UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: **UNIVERSITY OF PADUA**

Department of Biomedical Sciences

INTERNATIONAL PhD COURSE IN

ARTERIAL HYPERTENSION AND VASCULAR BIOLOGY

XXIV CYCLE

RESVERATROL, A NATURAL MOLECULE AGAINST HYPERTENSION AND CARDIOVASCULAR DISEASES: DEVELOPMENT OF PRODRUGS TO ENHANCE ITS BIOAVAILABILITY

Coordinator : Ch.mo Prof. Gian Paolo Rossi

Tutor : Ch.mo Prof. Garbisa Spiridione

Co-Supervisor : Dott. Mario Zoratti

PhD student : Alice Bradaschia

Contents

Riassunto dell'attività svolta

Lo scopo del mio progetto di dottorato è lo sviluppo di prodrugs di resveratrolo, il nostro polifenolo modello, resistenti al metabolismo durante l'assorbimento intestinale capaci di rigenerare il composto naturale grazie all'azione di enzimi ubiquitari. I polifenoli vegetali sono una vasta famiglia di composti naturali presenti in molti cibi e bevande. Molti di loro possiedono notevoli proprietà biologiche. Il resveratrolo per esempio, può aiutare a prevenire le malattie cardiovascolari. È in grado di abbassare la pressione sanguigna, ridurre la perossidazione lipidica l'aggregazione piastrinica. Inoltre, il resveratrolo ha proprietà antiossidanti, antiinfiammatorie e antiaterosclerotiche. Questi potenziali effetti terapeutici trovano però un ostacolo nella scarsa biodisponibilità di questo composto e dei polifenoli in generale. Come risultato di un basso livello di assorbimento e di rapido metabolismo nelle cellule intestinali ed epatiche, nel sangue vengono ritrovate solo piccole quantità di polifenoli, e principalmente sotto forma di metaboliti.

La più importante modificazione metabolica coinvolge gli ossidrili, e dato che enzimi con attività esterasica sono ubiquitari nel corpo umano, è sembrato logico cominciare il lavoro sviluppando derivati carbossiesterei. Il progetto è avvenuto in collaborazione con un gruppo di chimici organici che hanno sintetizzato i composti utilizzati in questa tesi.

Dato che i polifenoli generalmente sono poco solubili in acqua, e la solubilità è un fattore determinante per la biodisponibilità di un composto, un primo precursore di resveratrolo è stato ottenuto funzionalizzando gli ossidrili con gruppi glucosio, attraverso un linker succinico (RGS); studi di farmacocinetica con questo composto mostrano una cinetica di assorbimento ritardata rispetto al resveratrolo, un risultato potenzialmente utile. I livelli e la composizione dei metaboliti in circolo sono però del tutto analoghi a quelli ottenuti somministrando resveratrolo; questo suggerisce un'idrolisi del derivato a resveratrolo nel tratto intestinale, prima dell'assorbimento. Quindi, l'utilità del legame carbossiestereo *in vivo* è limitata. (Capitolo 1, Biasutto *et al.*,Bioorg Med Chem Lett. 2009 Dec 1;19(23):6721-4.).

Sono stati successivamente sintetizzati altri precursori del resveratrolo, con un legame acetalico che lega gruppi protettivi/solubilizzanti agli ossidrili. Il legame acetalico è caratterizato da una bassa polarità e da uno scarso ingombro sterico, e si prevede che favorisca il passaggio attraverso le bio-membrane. È suscettibile all'idrolisi in acido ma è comunque ancora troppo stabile per l'uso *in vivo.* È stata sintetizzata una prima

generazione di tre composti, funzionalizzati con semplici gruppi protettori. Il composto sostituito con un $-CH_2CH_2O-$ (monomero di glicole etilenico) si è dimostrato il più promettente della serie. Si è quindi deciso di incrementare la lunghezza della catena per verificare come e se questo possa influenzare l'assorbimento. I risultati di esperimenti *ex vivo,* utilizzando segmenti d'intestino, e di determinazioni farmacocinetiche hanno permesso di determinare che la lunghezza della catena del gruppo solubilizzante influenza positivamente l'assorbimento. (Capitolo 3)

Alla ricerca di funzionalità protettive con ideali caratteristiche di stabilità, sono stati successivamente sintetizzati derivati carbamolici. Dato che gli iniziali N,N-dimetil carbamati erano quasi insolubili in acqua e scarsamente assorbiti *in vivo*, sono stati prodotti analoghi derivati con un costrutto glucosilbutilico o con una corta catena di PEG. Il legame carbamolico si è dimostrato stabile *in vitro*. In esperimenti di assorbimento attraverso la parete intestinale, il derivato glucosilato è in grado di passare dal lato apicale a quello basolaterale senza perdere i gruppi protettivi, ed inoltre è assorbito anche *in vivo*. Invece il derivato PEGilato non viene assorbito. Questi derivati N,N-disostituiti vengono assorbiti senza metabolizzazione, ma sono troppo stabili per permettere la rigenerazione del composto parente (resveratrolo) entro alcune ore dalla somministrazione. L'assorbimento di derivati glucosilici non metabolizzati sia in esperimenti *ex vivo* che in *in vivo,* suggerisce il coinvolgimento dei trasportatori del glucosio. Esperimenti d'inibizione del trasporto glucosidico con intestino espiantato di ratto hanno dimostrato che esiste una grande variabilità di permeazione di questo tipo di composto, sia in presenza che in assenza di inibitori. Questo ha reso finora difficile giungere ad una definitiva conclusione sul sistema di trasporto. (Capitolo 4)

È stata quindi sviluppata una seconda generazione di carbamoil derivati del resveratrolo, i carbamati N-monosostituiti che sono noti per essere meno stabili degli N,N-disostituiti. Un primo set di derivati incorpora, come gruppo protettivo, la 6-deossi-6-galattosammina. La scelta di questo gruppo è stata suggerita sia dalle sue proprietà solubilizzanti sia dalla possibilità che i trasportatori della famiglia dei GLUT possano mediare il trasporto di questo tipo di derivati. Anche se promettenti per certi tipi di applicazioni, questi derivati non sono comunque ben assorbiti nell'intestino, probabilmente a causa della loro elevata idrofilicità. Risultati migliori sono stati ottenuti con un secondo set di composti che presentano il 3-ammino-1,2-propandiolo come gruppo protettore, il quale mentre assicura una sufficiente solubilità in acqua, garantisce anche una maggiore affinità per la fase meno polare e possiede un minor peso molecolare, fattori che sono importanti per un efficiente

assorbimento dopo somministrazione orale. Nel caso dei due isomeri monosostituiti, nel sangue si ritrovano livelli dell'ordine del μM, persistenti per ore. Tuttavia, i due idrossili liberi di questi composti sono substrati per le glucuronosiltrasferasi, e questa modificazione avviene prima che la rigenerazione di resveratrolo sia completa. (Capitolo 5).

Un nuovo approccio è ora in corso, combinando gli aspetti positivi che sono emersi dagli esperimenti con i derivati acetalici e cabamoilici.

In questi nuovi composti il gruppo carbamoilico è usato per legare corte catene di oligo-etileneglicole. Questi nuovi derivati dovrebbero essere ben assorbiti (in funzione della lunghezza della catena) e con una adatta cinetica di idrolisi. Al momento della pubblicazione di questa tesi sono disponibili solo risultati preliminari. (Capitolo 5).

Durante un periodo di sei mesi (Ottobre 2010-Aprile 2011) presso il gruppo di ricerca del Prof. Mervaala's all'Università di Helsinki, ho condotto uno studio sui possibili effetti positivi del resveratrolo e della restrizione calorica sulla cardiotossicità e il danno renale indotti dal sunitinib in ratti spontaneamente ipertesi, usando ratti normotesi come controllo. Il sunitinib è un inibitore delle tirosin-chinasi (TKI). I bersagli del sunitinib sono i recettori dei fattori di crescita, i più importanti dei quali sono i recettori del fattore di crescita endoteliale (VEGF) e i recettori del fattore di crescita derivato dalle piastrine (PDGF). Il sunitinib aumenta la sopravvivenza di pazienti con carcinoma renale e stromale gastrointestinale e ha dimostrato di possedere attività anche contro altri tipi tumori solidi. I risultati preliminari non hanno permesso di evidenziare differenze statisticamente significative fra i controlli e i ratti trattati in termini di disfunzione sistolica e diastolica e di funzionalità cardiaca. Per trarre conclusioni maggiormente significative, sarà necessario condurre ulteriori esperimenti.(Capitolo 6)

Per finire, in durante lo stadio iniziale del progetto ho partecipato ad uno studio sul metabolismo di polifenoli in cellule in cultura. Gli studi di assorbimento/metabolismo con monostrati di cellule intestinali Caco-2 hanno messo in luce l'esistenza di eterogeneità nell'espressione degli enzimi metabolici di Fase II (solfo- e glucuronosil-trasferasi) all'interno della stessa linea cellulare. Una semplice strategia che ci ha consentito di uniformare nuovamente popolazioni con diversa attività metabolica è stata quella di indurre l'espressione degli enzimi metabolici mediante coltivazione con concentrazioni minime di xenobiotico (nel nostro caso quercetina) (Biasutto, L., et al. (2009), Chembiochem. 2008 Nov 3;9(16):2633-42., Capitolo 2).

SUMMARY

The aim of my doctorate work is the development of "pro-drugs" of resveratrol, our polyphenol model, resistant to metabolism during absorption and capable of regenerating the natural compound thanks to the action of ubiquitous enzymes. Plant polyphenols are a vast family of natural compounds present in many foods and drinks. A large amount of data shows that many of them have noteworthy biological properties. Resveratrol, for example, can help prevent cardiovascular disease in humans, because it is able to lower blood pressure and lipid peroxidation, to induce vasodilatation and to reduce platelet aggregation. Moreover, resveratrol has antioxidant, anti-inflammatory, and anti-atherosclerotic properties, resulting in protection of the cardiovascular system, improvement of age-related cognitive decline and prevention and therapy of cancer. These potential positive effects are hampered by the low bioavailability of this compound and of polyphenols in general. As a result of a low level of absorption and a rapid metabolism in intestinal and liver cells, only small amounts of polyphenols are found in the bloodstream, and then mostly as metabolites.

The most relevant metabolic modifications involve the hydroxyls, and since enzymes with esterase activity are ubiquitous in the body, it seemed logical to begin work by developing carboxyester derivatives. The project is carried out in collaboration with a group of organic chemists who synthesise the compounds and also contribute analytical know-how.

Since polyphenols generally have a low solubility in water, and solubility is a key factor contributing to the bioavailability of a compound, my research group synthesized a first resveratrol derivative in which succinyl linkers connected resveratrol hydroxyls on one side and glucose residues on the other via carboxyester bonds. Pharmacokinetic studies with this compound showed that absorption kinetics were delayed in comparison to resveratrol, a potentially useful outcome. Pharmacokinetic studies with this compound showed that the levels and the composition of the metabolite mix in the bloodstream were the same as those obtained using resveratrol itself. This suggested that the compound was hydrolysed to resveratrol in the gastro-intestinal tract, before absorption. The carboxyester bond system thus turned out to be too labile in vivo, and therefore of limited usefulness (Chapter 1, Biasutto *et al.*,Bioorg Med Chem Lett. 2009 Dec 1;19(23):6721-4) Other bond systems, such as the ether and sulfonate linkages, proved on the contrary to be too stable.

We therefore turned to other functionalities. In one approach the acetal bond system was used to link protective/solubilising groups to resveratrol hydroxyls. The acetal linkage is

characterized by low polarity and steric hindrance, and it is expected to encourage passage through biomembranes. It is hydrolysed under acid conditions, but, as we discovered, it is rather too stable for use *in vivo*. A first generation of three derivatives were synthesized, which contained simple capping groups. The compound comprising a-CH_2CH_2O-(ethylene glycol monomer) unit proved to be the most promising. It was therefore decided to increase the length of the polyether chain to asses how this factor influenced absorption. *Ex vivo* and *in vivo* experiments with a series of acetal derivatives bearing short oligo-ethylene glycol chains thus allowed us to evaluate and appreciate the positive effect this type of substituents can have on the absorption process (Chapter 3).

In the search for a protective functionality with ideal stability characteristics, we then turned to carbamate derivatives. The prototype N,N-dimethyl carbamate derivative proved to be nearly insoluble in water. To confer this necessary property, N,N-disubstituted carbamate derivatives bearing a glucosylbutyl construct or a short PEG chain were synthesised and tested. The N,N-disubstituted carbamate linkage is stable under the conditions of our *in vitro* experiments. In *ex vivo* absorption experiments using intestine segments, the glucosylated derivative demonstrated its ability to translocate from the apical to the basolateral side, without loss of the protective groups. Some absorption was observed also *in vivo*. On the other hand no resveratrol derivatives could be detected on the basolateral side when the PEGylated molecule was used. These N,N-disubstituted carbamate derivatives can thus be absorbed without metabolization, but they also are too stable to allow the regeneration of the parent compound (resveratrol) within hours after absorption. Absorption of the intact glucosyl derivative both in *ex vivo* and *in vivo* studies suggested the involvement of glucose transporters in transepithelial transport. Inhibition experiments with explanted rat intestine demonstrated a great variability in the permeation of this compound, both in the presence and absence of would-be inhibitors, which has so far prevented a definite conclusion regarding the mechanism of transport. (Chapter 4).

We then developed a second generation of carbamoil derivatives of resveratrol, turning to N-monosubstituted carbamates which are known to be less stable than N,N-disubstituted ones. A first set of derivatives incorporated 6-deoxy-6-galactosamine. The choice was suggested by the solubilizing properties of the sugar group and by the possibility that intestinal transporters of the GLUT families might mediate the uptake of this kind of derivatives. The compounds of this first set, while very promising for certain types of application, were not however adsorbed well in pharmacokinetics experiments, presumably due to their too-strong hydrophilicity. Somewhat more satisfactory results were obtained

with a second set. This incorporated glycerol-like 3-amino-1,2-propanediol which, besides ensuring sufficient aqueous solubility, provided a more significant affinity for less polar phases (biomembranes) and a lower molecular weight than the galactosamine-based prodrugs. These parameters are important for an efficient adsorption in the bloodstream after oral administration. In the case of the two isomeric mono-substituted compounds, μM levels were measured in blood samples, persisting for hours. However, the two free hydroxyl groups in these derivatives are targets for glucuronosyltransferases, which modify them largely before the regeneration of resveratrol can be completed.

A new approach has now been adopted, combining the positive aspects which have emerged from the investigation of acetal and carbamoil derivatives. In these new compounds the carbamate group is used to link short oligo-ethyleneglycol chains. These constructs may be expected to be well absorbed (depending on chain length) and to hydrolyse with suitable kinetics. Only preliminary results with one compound are available at the time this thesis is submitted. (Chapter 5).

During my graduate studies I also spent six months (October 2010-April 2011) in the group of Prof. E. Mervaala's at the University of Helsinki, Finland. During this period I performed a preliminary study on the possible positive effects of resveratrol and caloric restriction against sunitinib-induced cardiotoxicity and renal damage in spontaneously hypertensive rats, using normotensive WKY rats as control. Sunitinib is a tyrosine kinase inhibitor (TKI). The targets of sunitinib are growth factor receptors, the most important of which bind vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF). The drug prolongs survival in patients with renal cell carcinoma and gastrointestinal stromal tumors and has demonstrated single agent activity against a number of other solid tumors. The results did not reveal statistically significative differences between control and treated rats. Further experiments will be needed before a definite conclusion can be reached. (Chapter 6)

Finally, it should be mentioned that in an early side project I participated in studies on the metabolism of polyphenols by cultured cells. Work with monolayers of colonic Caco-2 cells revealed a remarkable heterogeneity in the expression of Phase II metabolism enzymes (sulfo- and glucuronosyl-transferases) within the same cell line. A simple strategy which allowed us to regenerate a uniform activity in the different populations consisted in inducing the expression of metabolic activity by cultivating the cells with low concentrations of a xenobiotic compound (in our case quercetin) (Biasutto, L., et al. (2009), Chembiochem. 2008 Nov 3;9(16):2633-42., Chapter. 2).

Organization of the thesis

After a relatively brief general introduction about the effects of resveratrol on cardiovascular system, the bulk of this thesis is organized in 5 chapters corresponding each to a specific topic of research within the common subject of this thesis: development of new resveratrol derivatives for the full exploitation of its pharmacological possibilities. This organization has been favoured over a more traditional, monograph-style layout in part because my work actually developed as a series of closely related - but distinct - activities, and mainly with the intent of facilitating reading. Two chapters correspond to papers already published. A further chapter is about the experiments that I have carried out during the six month in the group of prof. Mervaala's. Thus, the chapters are not homogenous in length and relevance. I hope the benefits of such an organization outweigh this disadvantage.

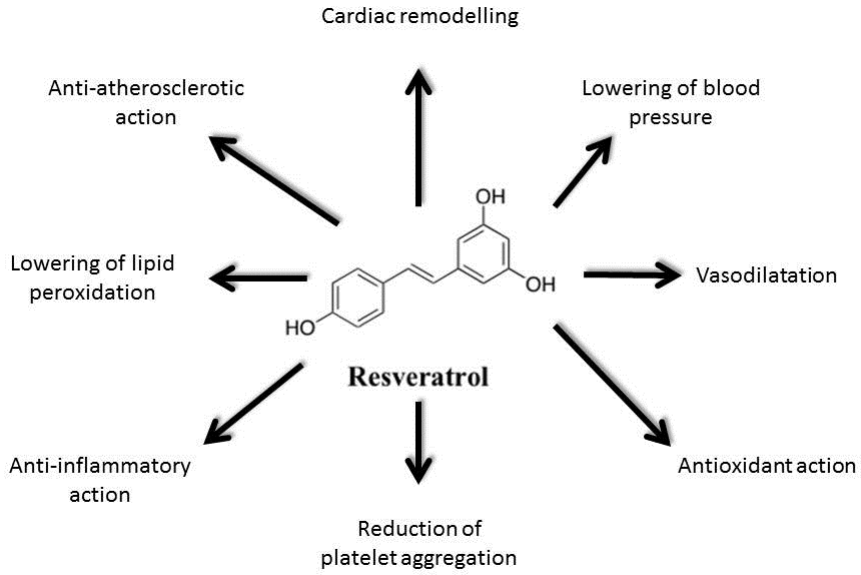
INTRODUCTION

Cardiovascular diseases (CVDs) are the major causes of morbidity and mortality in the developed Nations. CVDs are caused by disorders of the heart and blood vessels, and include:

- raised blood pressure - hypertension
- coronary heart disease - disease of the blood vessels supplying the heart muscle
- cerebrovascular disease - disease of the blood vessels supplying the brain
- peripheral arterial disease - disease of blood vessels supplying the arms and legs
- rheumatic heart disease - damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria
- congenital heart disease - malformations of heart structure existing at birth
- deep vein thrombosis and pulmonary embolism - blood clots in the leg veins, which can dislodge and move to the heart and lungs.

Some of the main factors that can expose to the risk to developing CVD are not modifiable, as for example, age, sex and genetic predisposition. However lifestyle, a modifiable factor, has great relevance in the development of CVD. The deep connection between dietary patterns and cardiovascular disease indicates the dual nature of the interaction: diet may cause cardiovascular disease and on the other hand changes to the diet can be an effective intervention in the prevention and treatment of damage to the cardiovascular system. Several epidemiological studies (1,2) have associated a lower incidence of cardiovascular diseases with a moderate red wine assumption, in particular in French people (3), a population generally consuming a fat-rich diet. Red wine contains a wide variety of bioactive compounds, which are predominantly phenolic in nature (4). These include flavonols such as quercetin, catechins and epicatechins, proanthocyanidins, anthocyanins, phenolic acids including gallic acid, caftaric acid, caffeic acid, p-coumaric acid, and the stilbene resveratrol. Among these phenolic compounds, resveratrol (3,5,4'-trihydroxystilbene) has drawn major attention. Resveratrol is present in the skin of red grapes and thus in red wine. Moreover, resveratrol is also present in a large number of fruits, such as cranberry, blueberry, mulberry, peanuts and in a wide variety of flowers. It is synthesized in response to environmental stressors including water deprivation, UV radiation and especially fungal infection (5). Several studies support the notion that

resveratrol can help prevent cardiovascular disease in humans. These benefits have been associated to a number of effects, including: (a) antioxidant action, (b) lowering of blood pressure, (c) vasodilatation, (d) anti-atherosclerotic action (lowering of lipid peroxidation, reduction of platelet aggregation and anti-inflammatory action), (e) cardiac remodeling.



Diverse cardioprotective role of resveratrol

The biological effects of resveratrol involve its capacity to target many intracellular molecules. The best known of these is Sirt1. Sirt1 is the founding member of sirtuin class of nicotinamide adenine di-nucleotide (NAD^+)-dependent histone deacetylases. In mammals there are seven members of the sirtuin family, Sirt1-Sirt7 (6). Sirt1 activates transcription factors and enzymes, regulating in this way a number of intracellular pathways, and it has been implicated in many vital processes, such as cell-cycle regulation, DNA repair, cell survival, gluconeogenesis, insulin sensitivity, muscle cell differentiation, lipid metabolism and fat mobilization (7).

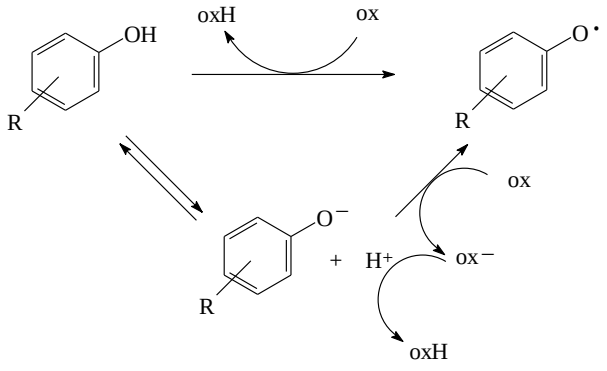
ANTIOXIDANT EFFECTS

It's now been accepted that risk factors other than increased plasma cholesterol and obesity play an important role in the development of CVD (8). Indeed the prominence of oxidative stress, vascular inflammation, and endothelial dysfunction for the development of cardiac

disease has been recently highlighted (9, 10). Several studies demonstrate that antioxidants, such as tempol (1-Oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine), significantly prevent organ damage induced by a variety of factors, including hypoxia, ischemia/reperfusion (I/R) injury, and angiotensin II/high salt loading. But tempol is toxic for humans, and this is a limitation for its clinical application. Numerous studies with antioxidative vitamin E supplement, an effective antioxidant in animals, have reached disappointing conclusions. The use of some antihypertensive drugs has been reported to decrease oxidative stress in many clinical treatments, but no antioxidant is currently used for the treatment of patients with cardiovascular disease because those available are not safe, specific and effective. On the other hand, a drug targeting multiple points may exhibit better therapeutic efficacy than one blocking or activating a single target in a complex context (11). A possible explanation is that common disorders such as cardiovascular diseases are the result of multiple molecular abnormalities. Using a drug for each relevant target would imply taking several types of medication, resulting in higher costs, more interactions between drugs and lower compliance.

Resveratrol is best known for its antioxidant properties *in vivo*, and several protective intracellular pathways of resveratrol are associated with oxidative stress.

The aromatic structure of resveratrol allows delocalization of charges or unpaired electrons, rendering him greatly more acidic than alcohols and good radical scavengers (and thus antioxidants). Radical scavenging may result from hydrogen donation or, more plausibly, electron transfer after deprotonation (Scheme 1).



ox = $O_2^{\bullet-}$, ROO•, Cu^{2+}, Fe^{3+}, ...

Radical scavenging by a generic phenolic compound.

However, the protective effects of resveratrol against oxidative injury both *in vivo* and *in vitro* are likely to be due to the activation of pathways that up-regulate the cells' natural

antioxidant defenses rather than the direct scavenging activity of reactive oxygen species (ROS).

Resveratrol represses pro-oxidative genes (such as nicotinamide adenine dinucleotide phosphate oxidase and myeloperoxidase) and induces antioxidative enzymes or substrates of these enzymes including superoxide dismutase (SOD), catalase, thioredoxin, and glutathione peroxidase (GSH-Px). Thus, resveratrol inhibits oxygen free radical formation. Superoxide dismutase and Glutathione peroxidase (GSH-Px) are mainly responsible for the inactivation of O_2^- and hydrogen peroxide, respectively. The major SOD form regulated by resveratrol is Manganese SOD (MnSOD); it locates mainly in mitochondria and counteracts ROS production by the mitochondrial electron transport chain (12).

Several studies have reported that resveratrol increases plasma nitric oxide (NO) levels, enhances the expression of endothelial nitric oxide synthase (eNOS), and modulates the deacetylation of eNOS (13). One of the mechanisms that have been proposed for increased NO production involves the SIRT1 protein deacetylase enzyme. SIRT1 has been shown to directly deacetylate eNOS, thereby stimulating NO production (14).

HYPERTENSION

Hypertension is a chronic medical condition in which blood pressure is elevated. Hypertension can be classified as either essential (primary) or secondary. Essential or primary hypertension is common: about 90-95% of hypertension is essential hypertension. It means that no medical cause can be found to explain the high blood pressure. Secondary hypertension indicates that the high blood pressure is caused by (i.e., secondary to) other conditions, such as kidney disease or tumors (adrenal adenoma or pheochromocytoma).

Resveratrol increases the levels of the vasodilator NO, which protects against increased systemic blood pressure (SBP) and subsequent cardiac hypertrophy, and decreases heart weight, ET-1 and angiotensin II (AngII) concentrations, which are associated with higher SBP.

In several animal models of hypertension, chronic resveratrol administration reduces systemic blood pressure: in DOCA-salt sensitive rat (15), two-kidney one-clip hypertensive rat (16), in a rat model of pulmonary hypertension (17) and in obese Zucker rats (18).

In a study on nephrectomy rats, the authors showed significantly higher systemic blood pressure, heart weight, serum endothelin-1 and AngII concentrations respect to the sham-

operated control group, while serum NO concentrations were lower. After 4 weeks of treatment with resveratrol, the serum NO concentration had increased, whereas SBP, heart weight and ET-1 and AngII concentrations had decreased. These results suggest that resveratrol may be able to counter the increase in systemic blood pressure and subsequent cardiac hypertrophy *in vivo* and that the mechanisms responsible for this may involve, at least in part, modulation of NO, AngII and ET-1 production (19).

Resveratrol has also been show to prevent remodeling of the mesenteric artery wall of spontaneously hypertensive rats (SHR), which is also typically observed in hypertensive humans, and to limit the increase in compliance of SHR arteries (20).

In a study on DOCA-salt hypertensive rats, resveratrol attenuated the increase of systolic blood pressure, left ventricular wet weight, left ventricular wall thickness, diastolic stiffness constant and the decrease of cardiac contractility. Moreover resveratrol treatment decreased inflammatory cell infiltration, decreased cardiac fibrosis (left ventricular interstitial and perivascular collagen content) and improved cardiac and vascular function (15). In obese Zucker rats, a metabolic syndrome experimental model, the increased systolic blood pressure and reduced aortic eNOS expression were significantly improved by long-term administration of resveratrol. In the visceral adipose tissue (VAT) of this rat type, resveratrol treatment lowered tumor necrosis factor-alpha (TNF-α) production and increased the concentration of adiponectin. Moreover, resveratrol improved the inflammatory status peculiar to this model (18).

The development of pulmonary hypertension is induced by proliferation of pulmonary arterial smooth muscle cells, endothelial dysfunction, oxidative stress, and inflammation. In monocrotaline-treated rats, resveratrol attenuated right ventricular systolic pressure, increased expression of endothelial NO synthase, decreased oxidative stress, and improved endothelial function in small pulmonary arteries. In addition, resveratrol was able to decrease expression of inflammatory cytokines, such as (TNF-α) and interleukin 6 (IL-6), and to limit leukocyte infiltration in the lung. Resveratrol also inhibited proliferation of pulmonary arterial smooth muscle cells (17).

The increased level of NO induced by resveratrol is due to the augmentation of eNOS expression and activity (21). It has been proposed that these effects involve SIRT1, that has been shown to directly deacetylate eNOS (22).

ENDOTHELIUM

Endothelial cells are able to balance vasodilator signals, for example NO, and vasoconstriction effectors, such as endothelin-1 (ET-1). Recent evidence showed that resveratrol has the ability to regulate the production of these vasodilators and vasoconstrictors.

Indeed, resveratrol is able to positively modulate NO activity and biosynthesis. In rat aortic smooth muscle, resveratrol opened large-conductance calcium-operated potassium channels to mediate an increased NO release from the endothelial cells and thus relaxation (23). Potassium channel-independent mechanisms, probably implicating calcium channels, were also involved in vasorelaxant effects on the mesenteric artery (24). Resveratrol reduced agonist- and potassium-induced increases in intracellular calcium in aortic vascular smooth muscle cells. Resveratrol achieved this effect through direct inhibition of calcium channels or depletion of intracellular Ca^{2+} stores, thus slowing increases in intracellular calcium concentration (25). Instead, in endothelial cells, resveratrol increased agonist- induced intracellular calcium release, which may trigger NO synthesis and release, and decreased calcium sensitivity in rat aortic smooth muscle (26).

Endothelial cells are responsible for the synthesis of ET-1, which is a strong vasoconstricting factor. Resveratrol potentially inhibits stress-induced ET-1 gene expression, ET-1 mRNA levels and ET-1 promotor activity by interfering with the ERK 1/2 pathway (27). Resveratrol improves endothelial function trough the decrease of ET-1 levels (28).

ATHEROSCLEROSIS

Atherosclerosis is now considered as an inflammatory disease of the arterial intima, which is characterized by the accumulation of lipids and fibrous elements in the arteries. It is caused by the retention of modified low-density lipoproteins (LDLs) and hemodynamic and reductive-oxidative (redox) stress.

Progression of atherosclerosis is characterized by weakening of the fibrous cap. This can lead to plaque rupture with subsequent local thrombosis. Resveratrol exerts several biological actions on atherosclerosis. As previously mentioned (23,27,28), resveratrol can stimulate the production of NO, and decreases the expression of endothelin-1, thus providing thrombo-resistance and preventing atherogenesis (29).

Inflammation occurs during atherogenesis, and the major events in inflammation include activation of the inflammatory cells with subsequent enhanced infiltration, amplified release of chemoattractants/inflammatory cytokines, and increased leukocyte-endothelial cell interactions. Prostaglandin E2 (PGE_2) plays a key role in inflammation. Its synthesis is catalyzed by COX-2. Resveratrol can inhibit cyclooxygenase (COX) 2 expression and this contributes to a reduction of inflammatory molecules such as prostaglandin E2 (30).

The circulating cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) are markers of inflammation in atherosclerotic plaques. Resveratrol, in cultured murine macrophages, was able to suppress IL-6 gene expression, protein synthesis and secretion (31) and also to inhibit the release of both IL-8 and granulocyte-macrophage colony-stimulating factor, which is important for the development of inflammation (32).

Resveratrol reduces the production of matrix metalloproteinases (MMPs)(33) Matrixmetalloproteinases not only accelerate formation and progression of plaque, but also facilitate weakening of the fibrous cap. Therefore, resveratrol is a hopeful medication for plaque stabilization.

In addition to these anti-inflammationtory effects, Resveratrol also appears to contribute to atheroprotection through the reduction of oxidative damage. Indeed, oxidative stress has been shown to induce endothelial dysfunction and the development of atherosclerosis (34). Importantly, Resveratrol significantly inhibits reactive oxygen species (ROS) production (35) and increases the expression of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase (36), thereby potentially reducing the formation of atherosclerotic lesions. Another mechanism by which resveratrol reduces ROS and contributes to atheroprotection is through the increase of SIRT1 levels, because SIRT1 overexpression attenuates mitochondrial oxidative damage in endothelial cells (37,38).

Low-density lipoprotein (LDL) oxidation represents a primary event of endothelial injury and also triggers atherosclerotic plaque formation. High plasma levels of LDLs are associated with increased risk of atherosclerosis and thus removal of LDLs is important for the treatment of inflammatory response (39).

Resveratrol reduces the oxidation of low-density lipoproteins (40) as well as their uptake into the vessel wall (41). Moreover, resveratrol enhances cholesterol efflux, thus affecting another crucial step of atherogenesis (42).

The thrombosis process plays a critical role in the development and progression of atherosclerosis. After injury to endothelium, platelets aggregate and adhere to the injured surface. The platelet-derived factor is released by deposited platelets. This growth factor

stimulates initiation and progression of atherosclerosis through the stimulation of the migration and proliferation of smooth muscle cells. Resveratrol inhibits platelet aggregation and activation by various mechanisms. In particular, resveratrol blocks the interaction of platelets with collagen and thrombin (43,44).

Moreover, the inhibition of the synthesis of thromboxane A2 (TxA_2), a potent inducer of platelet aggregation - due to COX-1 inactivation - is a mechanism suggested for the anti-platelet aggregation effect (45) of resveratrol. In addition, resveratrol reduces the intracellular calcium concentration ($[Ca^{2+}]i$) of platelets, which plays a key role in platelet aggregation, and thus it can prevent platelet activation (46).

CARDIAC REMODELLING

Chronic cardiovascular disease, such as hypertension, heart failure or myocardial infarction, induce remodeling of heart. The remodeling process is characterized by hypertrophy of myocytes, hyperplasia of fibroblasts and vascular smooth muscle cells, excessive collagen deposition and conduction abnormalities.

As described above, resveratrol is able to prevent increased blood pressure in animal models, thus it can protect the heart from structural remodeling (i.e. left ventricular hypertrophy, LVH) associated with pressure overload.

Another anti-hypertrophic mechanism of resveratrol is via AMPK and its upstream kinase LKB1. AMPK not only reduces the hypertrophic response, it also delays the transition from cardiac to heart failure (47). In hypertensive patients and rats, oxidative stress and lipid peroxidation products, such as 4-hydroxy-2-nonenal (4-HNE), are elevated (48). 4-HNE produces an inhibitory effect on the LKB1/AMPK signaling pathway, with consequent induction of mTOR/p70S6 kinase-mediated protein synthesis and cardiac myocyte cell growth. Resveratrol prevents the pro-hypertrophyc effect of 4-HNE by the activation of AMPK. Thus resveratrol inhibits unnecessary protein synthesis and prevents remodeling in the heart (49).

Resveratrol has also effects on cell proliferation. Block of cell proliferation could also improve cardiac function. In cultured rat cardiac fibroblasts, resveratrol inhibited their proliferation and differentiation to the hypersecretory myofibroblast phenotype; these are two critical steps in cardiac collagen deposition (50). Another probable mechanism through which resveratrol can inhibit the proliferation of cultured rat cardiac fibroblasts is the activation of the NO-cGMP signalling pathway (51).

Since inflammation is a key initiator of fibrosis, the anti-inflammatory properties of resveratrol could be another contributory mechanism to the changes in cardiac remodeling. In mouse cardiac fibroblasts, resveratrol inhibited the high expression of PI3K/Akt/ERK-dependent interleukin-17, a proinflammatory cytokine, induced by hight glucose; thus, resveratrol may decrease high glucose-mediated myocardial inflammation and remodeling (52).

ISCHEMIA/REPERFUSION INJURY AND PRECONDITIONING

Preconditioning (PC) is a protective and adaptive phenomenon that describes the development of resistance to damage to the heart. This effect is produced by short periods of ischemic stress, which make the heart more protected against another similar or greater stress. Not only short cyclic episodes of I/R but also a large number of stimuli such as pharmacological agents (agonists of opioid receptors, NO-donors), and various noxious stimuli (cytokines) have been found to produce a PC-like phenotype (53).

Existing evidence indicates that post-ischemic functional recovery induction by resveratrol requires NO production; this was demonstrated by the inhibitory effect of L-NAME (NG-nitro-L-arginine methyl ester, a non-selective nitric oxide synthase inhibitor) on the recovery (54).

Resveratrol likely activates both adenosine A1 and A3 receptors and this may protect cells from apoptosis. Adenosine A1 receptors are involved in preconditioning in rat hearts by transmitting a survival signal through the PI3-kinase-Akt-Bcl-2 signalling pathway (55). Activation of adenosine-A3 receptors may also precondition the heart by a survival signal through cAMP response element-binding protein (CREB) phosphorylation via PI3K/Akt and via MERK (mitogen-activated extracellular signal regulated protein kinase)/CREB pathways (56). Please note however that the group that published references 55 and 56 has recently been shown to have published falsified results in several occasions.

OTHER TERAPEUTIC EFFECTS

The therapeutic responses to resveratrol are not restricted to the cardiovascular system; there is growing evidence that this compound can block the carcinogenesis process by inhibiting targets such as kinases, ribonucleotide reductase and DNA polymerases (57). In

addition, resveratrol inhibits tumor-induced neovascularization (58,59) and induces cell cycle arrest and apoptosis (60).

Resveratrol also prevents or delays the onset of chronic age-associated diseases such as type II diabetes, improves insulin sensitivity, reduces blood glucose levels, and reduces high fat diet-induced obesity in rodents. Moreover, resveratrol showed neuroprotective effects in experimental animal models of injury or degeneration (61, 62). In addition to these properties, resveratrol can act as an analgesic (63) and has also been shown to reduce injuries to several organs, such as kidneys (64), lungs (65), intestine (66) and colon (67).

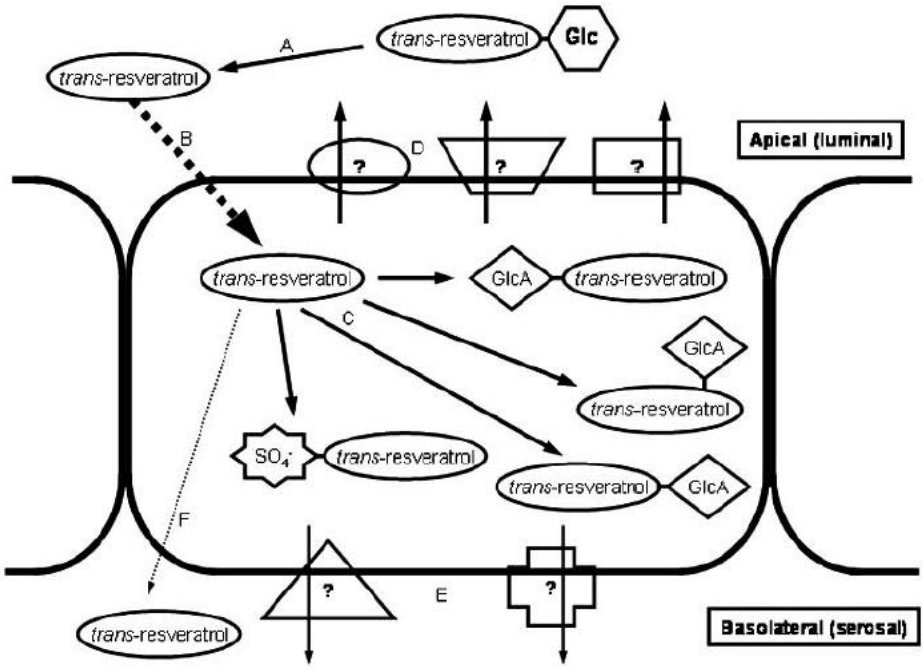
BIOAVAILABILITY AND *IN VIVO* METABOLISM OF RESVERATROL

All these potential positive effects are hampered by the low bioavailability of resveratrol. As a result of a poor solubility, low level of absorption and a rapid metabolism in intestinal and liver cells, only very low levels (in the nanomolar to micromolar range) of resveratrol are found in blood or plasma, even after a polyphenol-rich meal, and mostly as metabolites. This constitutes the major obstacle for a reliable assessment of its effects *in vivo* as well as, perhaps, for the full exploitation of its pharmacological possibilities.

Solubility has been identified as a critical parameter for the bioavailability of resveratrol. To overcome this obstacle many solutions have been adopted: the use of cyclodextrins (68, 69), of nanoemulsions (70), of micellar solutions of bile acids (71) and covalent modifications of the drug such as PEGylation (72). But, for example, enhancement of resveratrol solubility through complexation with cyclodextrins does not modify its pharmacokinetic profile (73).

In nature polyphenols occur mainly as glycosylated derivatives (piceid is a major one of resveratrol). While there are differences depending on the class of compounds considered and on the experimental model used, resveratrol is generally treated by intestinal enterocytes as a xenobiotic. According to the current model, the glycosylated derivative present in food is hydrolyzed to aglycone, because it is probably too hydrophilic to diffuse passively into the enterocytes. Once inside the intestinal cells, the aglycone is rapidly and efficiently converted into the corresponding hydrophilic phase-2 coniugates (glucuronide, sulfate) by detoxifying enzymes present in the cytosol. These metabolites are mostly re-exported by ABC/MDR-family translocators to the intestinal lumen, where they are eventually degraded by colonic microorganisms, and only a minor fraction is exported to

the basolateral side and enters the bloodstream. Analogous conjugation reactions also take place in the liver.



Intestinal uptake, metabolism and transport of resveratrol (Kroon et al, *Current Medicinal Chemistry*, 2010, *17*, 2442-2455)

Dosage has been proposed to be a key factor determining the outcome of polyphenol administration, and it has been repeatedly pointed out that many *in vitro* and *in vivo* studies have made use of unrealistically high concentrations of the compound, neglecting the limited bioavailability of dietary resveratrol. Administering higher doses to improve efficacy might not be possible as toxic effects have been observed at or above 1 g per kg (body weight) (74). Moreover, administering a daily dose to a human weighing 75 kg with 100 mg per kg (body weight) of resveratrol would require 2.7 kg of resveratrol a year, and production capacity would be insufficient for widespread use. For these reasons, blocking the metabolism of resveratrol and developing analogues with improved bioavailability may become increasingly important. Many drugs are afflicted by analogous bioavailability and metabolism problems, and one of the main strategies used to enhance effectiveness is based on protecting the reactive sites with removable groups, i.e. on the development of "prodrugs".

To achieve this goal, we are developing resveratrol derivatives protected at the sites of metabolic modification and/or with improved solubility characteristics, aiming for

compounds capable of crossing epithelia with higher absorption efficiency, and less prone to the action of detoxifying enzymes.

A more detailed overview of these various themes can be found in the introductory sections of the various chapters, where more specific information is presented to provide a context for each aspect of this work.

REFERENCES

1. de Lorimier, A.A. *et al,* 2000. Alcohol, wine, and health. Am. J. Surg. 180, 357–361

2. Gronbaek, M., *et al,* 2002. Alcohol, type of alcohol, and all-cause and coronary heart disease mortality. Ann. N. Y. Acad. Sci. 957, 16–20.

3. Renaud, S., de Lorgeril, M., 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339, 1523–1526

4. Soleas, G.J., *et al,* 1997. Wine as a biological fluid: history, production, and role in disease prevention. J. Clin. Lab. Anal. 11, 287–313

5. Roupe, K.A. *et al,* 2006. Pharmacometrics of stilbenes: seguing towards the clinic. Curr. Clin. Pharmacol. 1: 81–101.

6. Frye RA. *et al,* 2000. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem Biophys Res Commun; 273:793–8.

7. Finkel T, *et al,* 2009. Recent progress in the biology and physiology of sirtuins. Nature 91;460:587

8. Abeywardena, M.Y. *et al*, 2003. Dietary fats, carbohydrates and vascular disease: Sri Lankan perspectives. Atherosclerosis 171, 157–161.

9. Heitzer T. *et al,* 2001. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease Circulation. Nov 27;104(22):2673-8.

10. Zalba, G. *et al*, 2007. Oxidative stress, endothelial dysfunction and cerebrovascular disease. Cerebrovasc. Dis. 24 (Suppl. 1), 24–29

11. Hopkins AL. *et al*, 2008. Network pharmacology: the next paradigm in drug discovery. Nat Chem Biol 4:682–690

12. Tanno M. *et al,* 2010. Induction of manganese superoxide dismutase by nuclear translocation and activation of SIRT1 promotes cell survival in chronic heart failure J Biol Chem 285:8375–8382

13. Wallerath T. *et al,* 2002. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase Circulation 106:1652–1658

14. Mattagajasingh I. *et al,* 2007. SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. Proc Natl Acad Sci USA;104:14855-60.

15. Chan V.. *et al,* 2011. Resveratrol improves cardiovascular function in DOCA-salt hypertensive rats, Curr. Pharm. Biotechnol. 12 429–436

16. Toklu H.Z.. *et al,* 2010. Resveratrol improves cardiovascular function and reduces oxidative organ damage in the renal, cardiovascular and cerebral tissues of two-kidney, one-clip hypertensive rats, J. Pharm. Pharmacol. 62 1784–1793.

17. Csiszar A. *et al,* 2009. Resveratrol prevents monocrotaline-induced pulmonary hypertension in rats, Hypertension 54 668–675.

18. Rivera L.. *et al,* 2009. Long-term resveratrol administration reduces metabolic disturbances and lowers blood pressure in obese Zucker rats, Biochem. Pharmacol. 77 1053–1063.

19. Liu, Z. *et al,* 2005. Effects of trans-resveratrol on hypertension-induced cardiac hypertrophy using the partially nephrectomized rat model. Clin. Exp. Pharmacol. Physiol. 32, 1049–1054

20. Behbahani J. *et al,* 2010. Resveratrol and small artery compliance and remodeling in the spontaneously hypertensive rat, Am. J. Hypertens. 23 1273–1278.

21. Wallerath T. *et al,* 2002. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase, Circulation 106 1652–1658.

22. Arunachalam G. *et al,* 2010 SIRT1 regulates oxidant- and cigarette smoke-induced eNOS acetylation in endothelial cells: role of resveratrol, Biochem. Biophys. Res. Commun. 66–72.

23. Calderone V. *et al,* 2007. Functional contribution of the endothelial component to the vasorelaxing effect of resveratrol and NS 1619, activators of the large-conductance calcium-activated potassium channels. Naunyn. Schmiedebergs. Arch. Pharmacol. *375*, 73-80.

24. Gojkovic-Bukarica, L. *et al* 2008. A role of ion channels in the endothelium-independent relaxation of rat mesenteric artery induced by resveratrol. J. Pharmacol. Sci., 108, 124-130.

25. Campos-Toimil. *et al,* 2007. Effects of *trans*- and *cis*-resveratrol on Ca2+ handling in A7r5 vascular myocytes. Eur. J. Pharmacol., 577, 91-97.

26. Buluc M. *et al,* 2006. Resveratrol decreases calcium sensitivity of vascular smooth muscle and enhances cytosolic calcium increase in endothelium. Vasc. Pharmacol. 44, 231- 237.

27. Liu, J.C. *et al,* 2003. Inhibition of cyclic strain-induced endothelin-1 gene expression by resveratrol. Hypertension 42, 1198–1205.

28. Zou, J.G. *et al,* 2003. Effect of red wine and wine polyphenol resveratrol on endothelial function in hypercholesterolemic rabbits. Int. J. Mol. Med. 11, 317–320.

29. Davignon. *et al,* 2004. Role of endothelial dysfunction in atherosclerosis. *Circulation* 109**:** III27–III32.

30. O'Leary K.A. *et al,* 2004. Effect of flavonoids and vitamin E on cyclooxygenase-2 (COX-2) transcription, Mutat. Res. 551 245–254

31. Zhong, M. *et al,* 1999. Inhibitory effect of resveratrol on interleukin-6 release by stimulated peritoneal macrophages of mice. Phytomedicine 6, 79–84.

32. Culpitt, S.V. *et al,* 2003. Inhibition by red wine extract, resveratrol, of cytokine release by alveolar macrophages in COPD. Thorax 58, 942–946.

33. Dave M. *et al,* 2008. The antioxidant resveratrol protects against chondrocyte apoptosis via effects on mitochondrial polarization and ATP production. Arthritis Rheum 58:2786–2797

34. Mietus-Snyder. *et al,* 2000. Class A scavenger receptor up-regulation in smooth muscle cells by oxidized low density lipoprotein. J. Biol. Chem. 2755, 17661–17670

35. Soylemez S. *et al,* 2009. Resveratrol supplementation gender independently improves endothelial reactivity and suppresses superoxide production in healthy rats, Cardiovasc. Drugs Ther. 23 449–458.

36. Spanier G. *et al,* 2009. Resveratrol reduces endothelial oxidative stress bymodulating the gene expression of superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1) and NADPH oxidase subunit (Nox4), J. Physiol. Pharmacol. 60 (Suppl 4) 111–116.

37. Kao C.L. *et al,* 2010. Resveratrol protects human endothelium from H(2)O(2)- induced oxidative stress and senescence via SirT1 activation, J. Atheroscler. Thromb. 17 970–979.

38. Ungvari Z. *et al,* 2009. Resveratrol attenuates mitochondrial oxidative stress in coronary arterial endothelial cells, Am. J. Physiol. Heart Circ. Physiol. 297 H1876–H1881.

39. Dichtl, W. *et al,* 1999. Very low-density lipoprotein activated nuclear factor-jB in endothelial cells. Circ. Res. 84, 1085–1094

40. Frankel E.N. *et al,* 1993. Inhibition of human LDL oxidation by resveratrol, Lancet 341 1103–1104.

41. Auger C. *et al,* 2005. Dietary wine phenolics catechin, quercetin, and resveratrol efficiently protect hypercholesterolemic hamsters against aortic fatty streak accumulation, J. Agric. Food Chem. 53 2015–2021.

42. Berrougui H. *et al,* 2009. A new insight into resveratrol as an atheroprotective compound: inhibition of lipid peroxida-tion and enhancement of cholesterol efflux, Atherosclerosis 207
420–427.

43. Olas B. *et al,* 2002. Effect of resveratrol, a natural polyphenolic compound, on platelet activation induced by endotoxin or thrombin Thromb Res. Aug 15;107(3-4):141-5.

44. Wang Z. *et al,* 2002 Effects of red wine and wine polyphenol resveratrol on platelet aggregation in vivo and in vitro. Int J Mol Med. Jan;9(1):77-9

45. Fre´mont L. *et al,* 2000. Biological effects of resveratrol. Life Sci 66:663–673

46. Yang YM. *et al,* 2008. Resveratrol attenuates thromboxane A2 receptor agonistinduced platelet activation by reducing phospholipase C activity. Eur J Pharmacol 583:148–155

47. Beauloye C. *et al,* 2011. AMPK activation, a preventive therapeutic target in the transition from cardiac injury to heart failure Cardiovasc Res. May 1;90(2):224-33,

48. Dolinsky VW. *et al,* 2009. Resveratrol prevents the prohypertrophic effects of oxidative stress on LKB1 Circulation. Mar 31;119(12):1643-52.

49. Chan AY. *et al,* 2004. Activation of AMP-activated protein kinase inhibits protein synthesis associated with hypertrophy in the cardiac myocyte. J Biol Chem 279:32771–32779

50. Olson E.R. *et al,* 2005. Inhibition of cardiac fibroblast proliferation and myofibroblast differentiation by resveratrol. Am. J. Physiol.288, H1131- H1138.

51. Wang S.J. *et al,* 2007. Resveratrol inhibits proliferation of cultured rat cardiac fibroblasts: correlated with NO–cGMP signaling pathway. Eur. J. Pharmacol. 567, 26-35

52. Venkatachalam K. *et al,* 2008 Resveratrol inhibits high glucose induced PI3K/Akt/ERK-dependent interleukin-17 expression in primary mouse cardiac fibroblasts. Am. J. Physiol. Heart. Circ. Physiol. 294, H2078-H2087

53. Bolli R. *et al,* 2000. The late phase of preconditioning Circ Res. Nov 24;87(11):972-83

54. Bradamante S. *et al,* 2003. Resveratrol provides late-phase cardioprotection by means of a nitric oxide- and adenosine-mediated mechanism Eur J Pharmacol. Mar 28;465(1-2):115-23

55*. Das S. *et al,* 2005 Pharmacological preconditioning with resveratrol: role of CREB-dependent Bcl-2 signaling via adenosine A3 receptor activation, Am J Physiol Heart Circ Physiol. Jan;288(1):H328-35.

56*. Das S, *et al,* 2005 Resveratrol-mediated activation of cAMP response element-binding protein through adenosine A3 receptor by Akt-dependent and -independent pathways. J Pharmacol Exp Ther. Aug;314(2):762-9

57. Saiko P. *et al,* 2008**.** Resveratrol and its analogs: Defense against cancer, coronary disease and neurodegenerative maladies or just a fad? Mutat. Res. 658,68-94

58. Kimura, Y. & Okuda, H. 2001. Resveratrol isolated from *Polygonum cuspidatum* root prevents tumor growth and metastasis to lung and tumor-induced neovascularization in Lewis lung carcinoma-bearing mice. J. Nutr. 131, 1844–1849.

59. Tseng, S. H. *et al*. 2004. Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats. Clin. Cancer Res. 10, 2190–2202

60. Aggarwal, B. B. *et al*. 2004. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res. 24, 2783–2840

61. Wang, Q. *et al*. 2002. Resveratrol protects against global cerebral ischemic injury in gerbils. Brain Res. 958, 439–447

62. Sinha K. *et al,* 2002. Protective effect of resveratrol against oxidative stress in middle cerebral artery occlusion model of stroke in rats. Life Sci. 71, 655–665

63. Gentilli, M. *et al*. 2001. Resveratrol decreases hyperalgesia induced by carrageenan in the rat hind paw. Life Sci. 68, 1317–1321

64. Giovannini, L. *et al*. 2001. Resveratrol, a polyphenol found in wine, reduces ischemia reperfusion injury in rat kidneys. J. Cardiovasc. Pharmacol. 37, 262–270.

65. McClintock, S. D., Protection from half-mustard-gas-induced acute lung injury in the rat. J. Appl. Toxicol. 22, 257–262 (2002).

66. Korolkiewicz, R. P. *et al*. 2003. Differential salutary effects of nonselective and selective COX-2 inhibitors in postoperative ileus in rats. J. Surg. Res. 109, 161–169.

67. Martin A. R. *et al,* 2004. Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates apoptosis during early colonic inflammation in rats. Biochem. Pharmacol. 67, 1399–1410.

68. C. Lucas-Abellán, I. *et al,* 2007.Cyclodextrins as resveratrol carrier system, Food Chem. 104 39–44.

69 Lu Z. *et al,* 2009. Complexation of resveratrol with cyclodextrins: solubility and antioxidant activity, Food Chem. 113 17–20.

70. Li D.C. *et al,* 2009. Application of targeted drug delivery system in Chinese medicine, J. Control. Release 138 103–112

71. Atanacković M. *et al,* 2009. Solubilization of resveratrol in micellar solutions of different bile acids, Colloids Surf. B 72 148–154

72. Haag R. *et al,* 2006. Polymer therapeutics: concepts and applications. Angew Chem Int Ed Engl. 45:1198-1215

73. Das S. *et al,* 2008. The im pact of aqueous solubility and dose on the pharmacokinetic profiles of resveratrol. Pharm Res. Nov;25(11):2593-600.

74. Crowell J. A. *et al,* 2004 Resveratrol-associated renal toxicity. Toxicol. Sci. 82, 614–619

75. Baur JA, Sinclair DA. 2006. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov. Jun;5(6):493-506.

76. Wang H. *et al,* 2011. Resveratrol in cardiovascular disease: what is known from current research? Heart Fail Rev. Jun 19.

77. Kroon PA. *et al,* 2010. The cardiovascular nutrapharmacology of resveratrol: pharmacokinetics, molecular mechanisms and therapeutic potential. Curr Med Chem.;17(23):2442-55.

MATERIALS

Resveratrol was purchased from Waseta Int. Trading Co. (Shangai, P.R.China). Other starting materials and reagents were purchased from Aldrich, Fluka, Merck-Novabiochem, Riedel de Haen, J.T. Baker, Cambridge Isotope Laboratories Inc., Acros Organics, Carlo Erba and Prolabo, and were used as received.

STABILITY IN ACID AND NEAR-NEUTRAL SOLUTION

The chemical stability of the compounds was tested in aqueous media mimicking gastric (0.1 N HCl) and intestinal environment (PBS buffer 0.1 M, pH 6.8). A 50 μM solution of the compound was made diluting a 1000x stock solution in DMSO (the presence of 1% DMSO ensured solubility of all compounds and hydrolysis products eventually formed), and incubated at 37°C for 6 or 24 hours. Samples withdrawn at different reaction times were analyzed by HPLC-UV (see below). Hydrolysis products were identified by LC-MS analysis of selected samples.

STABILITY IN BLOOD

Rats were anesthetized and blood was withdrawn from the jugular vein, heparinized and transferred to tubes containing EDTA. Blood samples (1 ml) were spiked with 5 μM compound (dilution from 5 mM stock solutions in DMSO, 0.1% final DMSO) and incubated at 37°C for 1 or 4 hours. Samples withdrawn at different reaction times were collected and treated as described below. Cleared blood samples were finally subjected to HPLC-UV analysis.

PERMEATION OF THE RAT INTESTINAL WALL (EX VIVO)

Intestine was excised from a 18 h fasted rat and transferred into a saline solution (154 mM NaCl in water) at 37°C. The jejunum was cut into strips about 1 cm long, opened longitudinally, rinsed free of luminal content and mounted in Ussing-type chambers. Apical and basolateral compartments were filled with 1 ml of oxygenated Hepes buffer each (composition in mM units: NaCl 248, Glucose 55.3, $NaHCO_3$ 50, KCl 9.9, $MgSO_4$ 1.9, Hepes 40; pH: 6.8), and the assembly was incubated in a water bath at 37°C until all

the intestine strips (usually six) were mounted The buffer was then removed and substituted with 1 ml of a 20 μM solution of the compound to be tested in the same buffer on the apical side and with 1 ml of fresh Hepes buffer on the basolateral one. During the experiment, oxygen was bubbled through a tip in each compartment for about 50 seconds at approximately 10-min. intervals. An aliquot of each initial apical solution was incubated separately at 37°C for the period of the experiment, to check the stability of each compound under the experimental conditions. At the end of the experiment (2 h) 800μl of chamber contents on both apical and basolateral sides were collected and mixed with 8μl of 100mM ascorbic acid in water and 8μl of 6mM acetic acid. 2 hours represented the maximum incubation period, since the intestine can be considered vital until 3 h after excision, and preparation of the chambers requires about 1 hour. All samples were frozen within 5 min. after sampling, and later analyzed by HPLC-UV as described below.

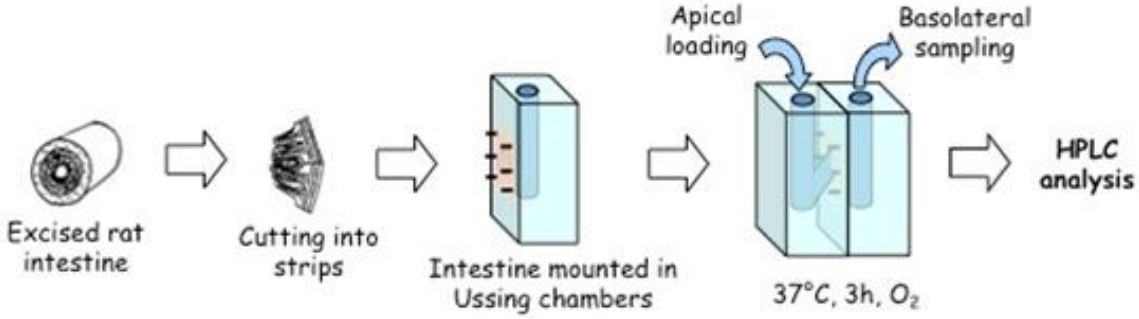


Fig.1 Scheme of Ussing chamber experiment.

In the experiments to assess the possible involvement of glucose transporters (Chapt. 4) we used the glucose transport inhibitors phloridzin (phloretin 2'β-D-glucoside, from apple wood, inhibitor of SGLT-1), phloretin (polyphenol found in apple; aglycone of phloridzin, inhibitor of GLUT2) and cytochalasin B (from *Drechslera dematioidea*, inhibitor of GLUT2) following the scheme reported in table 1.

Experiment 1	Experiments 2-6	Experiments 7-9	Experiment 10
Phloridzin 100 μM + cytochalasin B 10 μM	Phloretin 100 μM	Phloridzin 200 μM+ cytochalasin B 10 μM	Cytochalasin B 20 μM
Phloridzin 25 μM + cytochalasin B 20 μM	cytochalasin B 20 μM		Phloridzin 200 μM + cytochalasin B 10 μM
			Phloridzin 200 μM
			Phloretin 200 μM + cytochalasin B 20 μM
			Phloretin 200 μM

Table 1. Experiments with GLUT2 and SGLT1 inhibitors (see Chapt. 4, p. 74). The inhibitors were loaded only on the apical side

PHARMACOKINETIC STUDIES

The derivatives were administered to overnight-fasted rats as a single intragastric dose (0.088 mmol/Kg, dissolved in 250 μl DMSO). Blood samples were obtained by the tail bleeding technique: before drug administration, rats were anesthetized with isoflurane and the tip of the tail was cut off. A first blood sample was withdrawn, and served as blank. Other blood samples (60-100 μl each) were then taken from the tail tip at 10, 30, 60, 120, 240, 480 min. and 24 hours after drug administration. Blood was collected in heparinized tubes, kept in ice and treated within 20 min. The experiments were performed with the permission and supervision of the University of Padova Ethical Committee on Animal Experimentation (CEASA) which certifies compliance with Italian Law DL 116/92, embodying UE Directive 86/609. The area under the concentration-time curves (AUC) were determined by the linear trapezoidal method.

BLOOD SAMPLE TREATMENT AND ANALYSIS

Before starting the treatment, internal standard was added (4,4'-dihydroxybiphenyl, 25 μM final concentration). Blood was then stabilized with a freshly-prepared 10 mM solution of ascorbic acid (0.1 vol) and acidified with 0.6 M acetic acid (0.1 vol); after mixing, an excess of acetone (4 vol) was added, followed by sonication (2 min) and centrifugation (10,000 *g*, 8 min, 4°C). The supernatant was finally collected and stored at -20°C. Before analysis, acetone was allowed to evaporate at R.T. under N_2 flow, and up to 30 μl acetonitrile were added to precipitate residual proteins; after centrifugation, cleared samples were directly subjected to HPLC-UV analysis. Metabolites were identified by LC-MS analysis.

HPLC-UV ANALYSIS

Samples (20 μl) were analyzed using a reversed-phase column (Synergi-MAX, 4 μm, 150 x 4.6 mm i.d.; Phenomenex). Solvents A and B were H_2O containing 0.1% TFA and CH_3CN, respectively. The gradient for B was as follows: 10% for 2 min, from 10% to 35% in 20 min, then from 35% to 100% in 20 min; the flow rate was 1 mL/min. Alternatively, samples (2 μl) were analyzed using another reversed-phase column (Zorbax Eclipse Plus C18, 3 μm, 21 x 5.0 mm i.d.). We utilized two different gradients: in the first one the

gradient for B was as follows: 10% for 2 min, from 10% to 20% in 5 min, then from 20% to 30% in 1 min, then from 30% to 100% in 1 min; the flow rate was 0.6 mL/min. Alternatively, the gradient for B was as follows: 2%, from 2% to 10% in 0.3 min, then from 10% to 18% in 1.7 min, then from 18% to 28% in 0.5 min, 28% for 0.5 min then from 28% to100% in 1.8 min; the flow rate was 0.6 mL/min and the eluate was preferentially monitored at 286, 300 and 320 nm.

LC-ESI/MS ANALYSIS

LC-MS analyses and mass spectra were performed on selected sample (20 μl) with a 1100 Series Agilent Technologies system, equipped with MSD SL Trap mass spectrometer with ESI source operating in full-scan mode in both positive and negative ion mode.

Soluble polyphenols: synthesis and bioavailability of 3,4',5-tri(α-D-glucose-3-O-succinyl) resveratrol

Biasutto L., Marotta E., Bradaschia A., Fallica M., Mattarei A., Garbisa S., Zoratti M., Paradisi C.

Bioorganic & Medicinal Chemistry Letters, **2009**, 19, 6721-6724.

SUMMARY

We report the development of a chemical modification method of general applicability to polyphenols, which increases solubility to influence absorption. Glucosyl groups were added to the resveratrol kernel via a succinate linker, yielding 3,4',5-tri-(α-D-glucose-3-O-succinyl) resveratrol. The construct was only slowly hydrolysed in acid and at pH 6.8, but it was destroyed by blood esterases in less than one hour. In rats its administration resulted in a blood concentration vs. time curve shifted to longer times in comparison to resveratrol, a useful modulation of pharmacokinetics. The area-under-curve parameter and the metabolite mix were similar to those of resveratrol. The method may be advantageously employed to solubilise other polyphenols and to make them more palatable.

INTRODUCTION

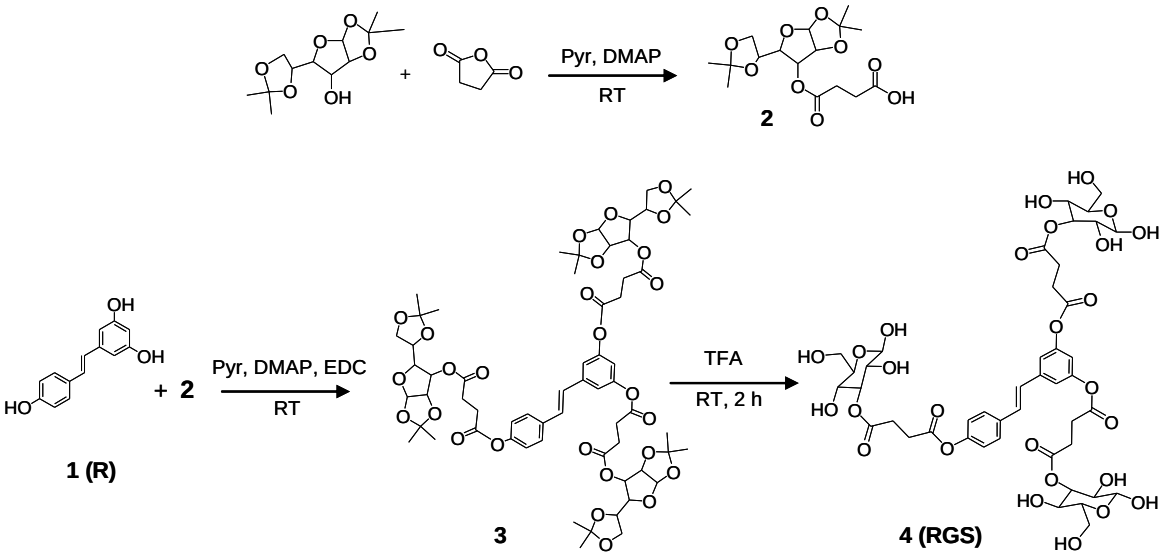
Optimization of bioavailability is fundamental for the full realization of the biomedical potential of nutraceuticals such as plant polyphenols. The research efforts of dozens of groups worldwide have proven that many polyphenols possess potentially very useful biochemical properties (1), offering promise for the fight against cardiovascular diseases, chronic inflammation and cancer. The promise however is only partially fulfilled: polyphenols are poorly absorbed and are present in the circulatory streams at very low levels, mostly as conjugates produced by phase II metabolism in the intestinal enterocytes and in the liver. Solubility is recognized as a key factor for bioaccessibility and thus bioavailability (2). The problems and approaches to the improvement of the bioavailability of antioxidants and polyphenols via formulations and modifications have been reviewed (3).

The generally low solubility of polyphenol aglycones can be attributed largely to a tendency to form aggregates via hydrophobic interaction of the aromatic systems and

hydrogen-bond formation by the hydroxyls. In nature polyphenols occur mainly as glycosylated derivatives. The current paradigm describing the absorption of these compounds in the intestine is that they are first deglycosylated at the intestinal wall, and then enter the cell by diffusion or via transport by carriers (4). The presence of glycosyl groups may nonetheless improve bioavailability by influencing phenomena taking place upstream of entry into enterocytes. A well-studied case is that of quercetin and its 3-O-glycosides (5). Kinetics and extent of absorption by rats depend strongly not only on the presence, but also on the identity of the sugar group, with uptake being most efficient for the most soluble derivatives. In this chapter is reported the development of a new resveratrol derivatives. Following nature's lead, glucosyl groups were added to the resveratrol kernel via a succinate linker, yielding 3,4',5-tri-(α-D-glucose-3-O-succinyl) resveratrol.

RESULTS

Synthetic procedures were optimized using 4,4'-dihydroxybiphenyl as a model compound. The final optimized protocol was then successfully applied to resveratrol (Scheme 1).



Scheme 1. Synthesis of 3, 4', 5-tri(α-D-glucose-3-O-succinyl)-resveratrol (RGS, **4**).

In the first step of the synthesis of a succinyl linker is attached to the 3-hydroxyl group of a glucose molecule used in a protected form (diacetone-D-glucose, DAG) to avoid reactions of the other hydroxyls, yielding diacetone-α-D-glucose-3-O-succinyl ester (**2**). Per-esterification of 4,4'-dihydroxybiphenyl or **1** to give 4, 4'-di(diacetone-α-D-glucose-3-O-succinyl)-biphenyl or 3,4',5-tri(diacetone-α-D-glucose-3-O-succinyl)-resveratrol (**3**),

respectively, was then performed by activating the free carboxylic group of **2** with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), using 4-N,N-dimethylamino pyridine (DMAP) as catalyst. The final step consisted in the deprotection of the glucose hydroxyl groups with trifluoroacetic acid (TFA), affording 4,4'-di(α-D-glucose-3-O-succinyl)-biphenyl or 3,4',5-tri(α-D-glucose-3-O-succinyl)-resveratrol (**4**; RGS) respectively. The solubility of **4** in water at room temperature is at least 90 mg/mL (89 mM). The major resveratrol phase II metabolites were also synthesised according to published procedures (7) as described in the Materials and Methods. They were used as standards to identify metabolites.

RGS underwent slow hydrolysis in 1N HCl (Fig. 1). After 6 h at 37°C, 80% of the starting material was still present as such, with the main product of hydrolysis being the di-substituted derivatives.

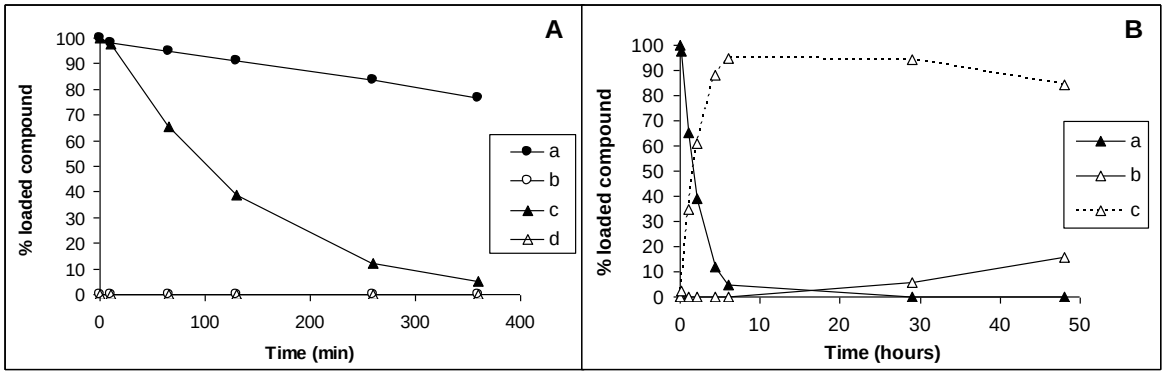


Figure 1. Stability of **4** in media mimicking gastric and intestinal environment (37°C). **A)** Over 6 hours: a) **4** and b) **1** in 1N HCl; c) **4** and d) **1** in PBS, pH 6.8. **B**) Over 48 hours: a) **4**, b) **1**, c) Mono- and di-(α-D-glucose-3-O-succinyl)-resveratrol in PBS, pH 6.8. Data are expressed as % of the initial molar amount used.

At pH 6.8 in phosphate-buffered saline (PBS) disappearance was faster. The process consisted in the loss of one entire glucose-O-succinyl moiety at a time. Loss of the first one was nearly complete after 6 hours, as showed by LC-MS analysis (8). Formation of resveratrol was observed after 28 h. At no time were detectable amounts of products arising from the loss of glucose molecules formed.

In pharmacokinetics experiments (see Supporting data for the experimental procedures) individual variability is generally high. We therefore performed our experiments utilizing the same set of rats for the administration of both resveratrol and the new derivative **4**. The identity of the circulating species was confirmed by LC-MS analysis using standards and by enzymatic treatment (Fig 2).

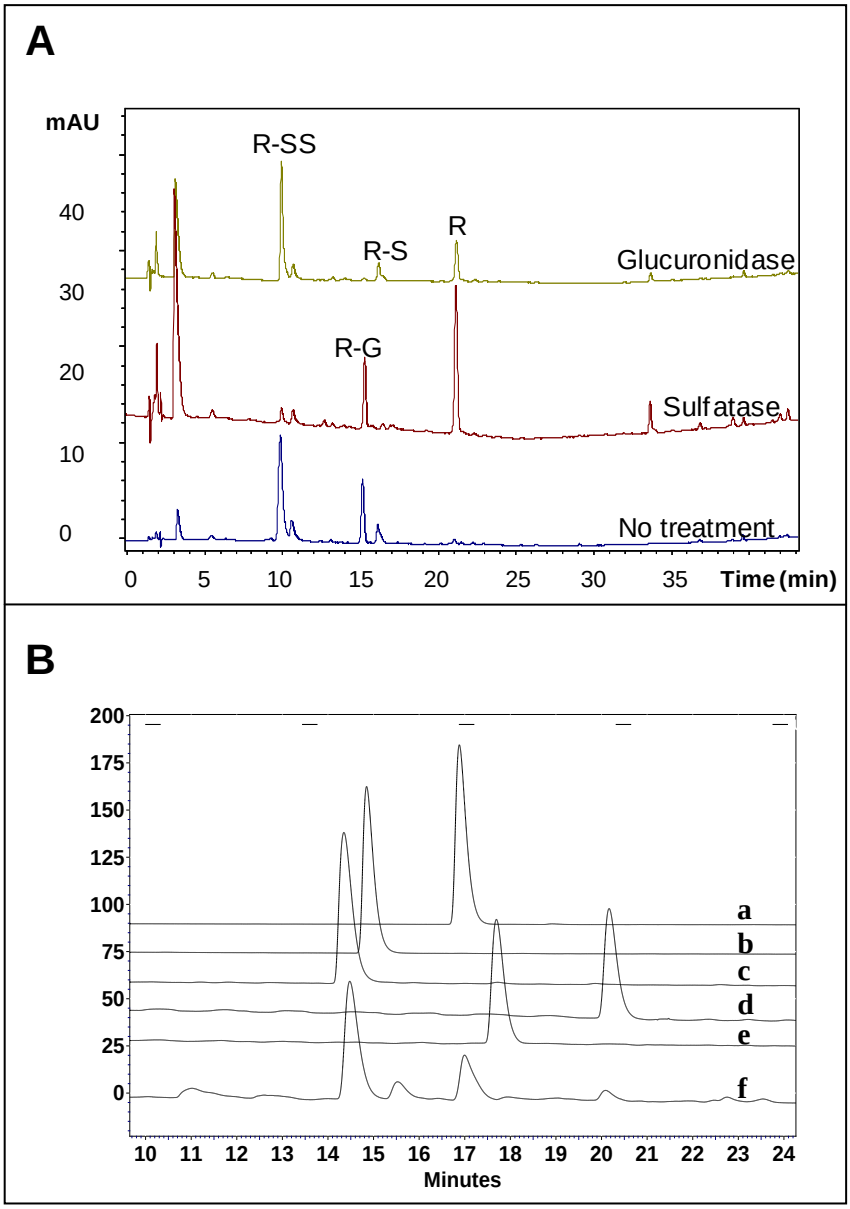


Fig. 2. Identification of resveratrol metabolites. **A)** HPLC-UV chromatograms (320 nm) of a blood sample withdrawn 4h after intragastric administration of **4**. The chromatograms were obtained from aliquots of the same sample: without any enzymatic treatment, and after treatment with sulfatase or with glucuronidase, as indicated. R-S: resveratrol-sulfate; R-G: resveratrol-glucuronide; R: resveratrol. **B)** Comparison between synthetic resveratrol conjugates and *in vivo* metabolites. HPLC chromatograms recorded at 320 nm of: a) R-3-glucuronide; b) R-4'-glucuronide; c) R-3,4'-disulfate; d) R-3-sulfate; e) R-4'-sulfate; f) rat blood sample collected 30 min after administration of 0.22 mmol/kg resveratrol.

In the case of resveratrol, blood analyses revealed relatively high amounts of resveratrol sulfate- and glucuronide-conjugates. In agreement with literature reports (9), C_{max} for the sum of all resveratrol-derived species (9.4 ± 8.5 µM) was reached at about 60 minutes after administration (Fig. 3A). The concentration vs. time curves of all metabolites followed the same pattern. Intact resveratrol appeared only at low levels (C_{max}: 1.2 ± 1.2 µM), and the corresponding peak earlier, at about 10 min. Administration of **4** resulted in a delayed

absorption, with the maximum concentration of total species in blood reached after approximately 4 hours (Fig. 3B).

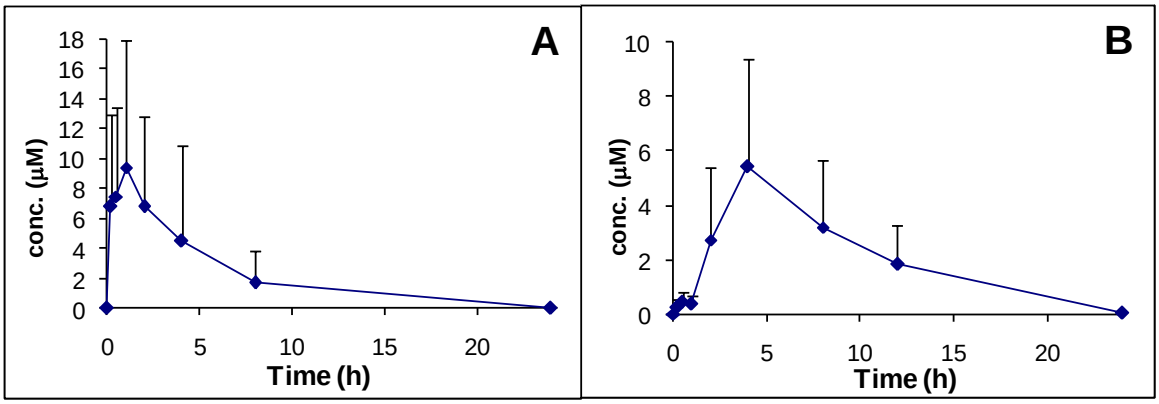


Figure 3. Pharmacokinetics of **A) 1** and **B) 4**. Mean values of total circulating species derived from the respective parent compounds (N = 3).

The overall C_{max} was 5.4 ± 4.0 µM. The species present in the bloodstream were also in this case resveratrol (at low levels) and mostly its metabolites, in proportions similar to those observed after administration of resveratrol itself (Fig. 4). When values of the area under concentration-time curves (AUC) are compared, no significant differences can be noted depending on whether resveratrol or **4** was administered. Similar results were obtained administering **4** as a water, rather than a DMSO, solution (not shown; N = 3).

Compound **4** is water-soluble and its stability vs. chemical hydrolysis is satisfactory. In particular, it is nearly stable in an acidic environment mimicking that of the stomach, so that it can be supposed to survive the gastric stage with only very limited destruction. Interestingly, hydrolysis of the succinate-resveratrol bond is much faster than that of the succinate-glucose bond, so that **4** is destroyed via successive losses of whole glucosylsuccinyl groups.

Administration of this solubilised form of resveratrol results in delayed absorption in comparison to the aglycone, without a significant difference in total absorption. Thus, administration of a proper mix of aglycone and derivative is expected to produce a prompt as well as long-lasting increase in circulating and body levels of the polyphenol and of its metabolites. The delay may be tentatively attributed to the time needed for complete hydrolysis of **4** to resveratrol in the intestinal tract, with the subsequent absorption of the resveratrol thus formed.

A

Circulating specie	AUC (μM * h)	
	Resveratrol	RGS
R	1,1 ± 1,1	0,2 ± 0,2
R-S	2,4 ± 1,7	3,5 ± 2,6
R-G	35,5 ± 36,9	21,2 ± 15,8
R-SS	14,4 ± 20,0	27,0 ± 18,7
Total	53,4 ± 57,7	51,8 ± 37,2

B

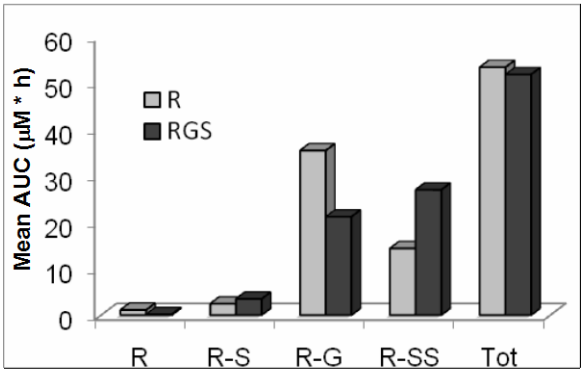


Figure 4. Mean AUC values for resveratrol and its main metabolites. The determinations were performed using three rats, each of which received both resveratrol and **4** with a 2-week interval. R: Resveratrol; R-S: Resveratrol sulfate; R-G: Resveratrol glucuronide; R-SS: Resveratrol disulfate. **A)** Error notations are standard deviations. **B)** Column plots of the data tabulated in A) (error notations omitted).

While polyphenols generally occur in nature as glycosylated derivatives, in many cases these products are not commercially available. Furthermore, monoglycosylation in some cases does not make the molecule soluble. For example rutin (quercetin-3-*O*-rutinoside, an abundant glycoside of quercetin) only dissolves at about 1 gram in 8 liters of water (10), i.e. about $2x10^{-4}$ M (2.3 grams/liter at pH 9 and decreasing with pH [11]). Quercitrin and genistin, common glucosides of quercetin and genistein respectively, have very low solubility in cold water (10). The procedures described above provide a straightforward approach to soluble polyphenol prodrugs. Linkage to more than one sugar residue is expected to lead to products with higher water solubility. Bioavailability may be improved by optimizing the choice of the glycosyl component or by linkage to a polymeric soluble molecule. New synthetic polyphenols are beginning to be explored as drugs and they are certain to be affected by the same (or worse) bioavailability problems as the natural compounds. Modulating solubility may have an impact and it would facilitate administration.

Modification of OHs may have to do not only with bioavailability, but also with complex formation with salivary proteins in the mouth. Sensations of bitterness and astringency are often associated with these compounds and in particular with their polymers (12), but they would not be expected to be induced by derivatives with "capped" hydroxyls. The other components forming RGS, succinic acid and glucose, are molecules already abundant and ubiquitous in the body, and thus certainly safe or beneficial as nutrients. Therefore, this type of molecules may find relevant technological applications in the food industry, such as, in the formulation of fortifying ingredients and supplements.

EXPERIMENTAL SECTION

MATERIALS AND METHODS

Materials and instrumentation.

Resveratrol was purchased from Waseta Int. Trading Co. (Shangai, P.R.China). Other starting materials and reagents were purchased from Aldrich, Fluka, Merck-Novabiochem, Riedel de Haen, J.T. Baker, Cambridge Isotope Laboratories Inc., Acros Organics, Carlo Erba and Prolabo, and were used as received. ^{1}H NMR spectra were recorded with a Bruker AC 250F spectrometer operating at 250 MHz. Chemical shifts (δ) are given in ppm relative to the signal of the solvent. HPLC-UV analyses were performed by a Thermo Separation Products Inc. system with a P2000 Spectra System pump and a UV6000LP diode array detector (190-500 nm). LC-ESI/MS analyses and mass spectra were performed with a 1100 Series Agilent Technologies system, equipped with binary pump (G1312A) and MSD SL Trap mass spectrometer (G2445D SL) with ESI source. TLCs were run on silica gel supported on plastic (Macherey-Nagel Polygram®SIL G/UV$_{254}$, silica thickness 0.2 mm), or on silica gel supported on glass (Fluka) (silica thickness 0.25 mm, granulometry 60Å, medium porosity) and visualized by UV detection. Flash chromatography was performed on silica gel (Macherey-Nagel 60, 230-400 mesh granulometry (0.063-0.040 mm)) under air pressure. The solvents were analytical or synthetic grade and were used without further purification.

Stability under gastric and intestinal-like conditions.

The chemical stability of **4** was tested in aqueous media mimicking gastric (0.1 N HCl) and intestinal (PBS buffer 0.1 M, pH 6.8) environment. A 50 μM solution of the compound was made diluting a 1000× stock solution in DMSO, and incubated at 37°C for 48 hours; samples withdrawn at different times were analyzed by HPLC-UV. Hydrolysis products were identified by LC-MS analysis of selected samples.

Stability in blood.

Rats were anesthetized and blood was withdrawn from the jugular vein, heparinized and transferred to tubes containing EDTA. Blood samples (1 mL) were spiked with 5 μM of compound (dilution from a 1000× stock solution in DMSO), incubated at 37°C for 1 hour and then treated as described below. Cleared blood samples were finally subjected to HPLC-UV analysis.

HPLC-UV analysis.

Samples (20 μl) were analyzed using a reversed phase column (Synergi-MAX, 4 μm, 150 x 4.6 mm i.d.; Phenomenex). Solvents A and B were H_2O containing 0.1% TFA and CH_3CN, respectively. The gradient for B was as follows: 10% for 2 min, from 10% to 35% in 20 min, then from 35% to 100% in 20 min; the flow rate was 1 mL/min. The eluate was preferentially monitored at 286, 300 and 320 nm. Alternatively, a Gemini C18, 3 μm, 150 x 4.6 mm i.d.; Phenomenex column was used. In this case the gradient for B was as follows: 10% for 2 min, from 10% to 30% in 15 min, from 30% to 60% in 15 min, from 60% to 100% in 3 min; the flow rate was 0.7 mL/min.

LC-ESI/MS analysis.

Samples (20 μl) were analyzed using the same column, solvents and gradient profile used for HPLC-UV analyses. MS analysis was performed with an ESI source operating in full-scan mode in both positive and negative ion mode. Before LC-MS analysis, samples were further concentrated under vacuum (about 10×).

Pharmacokinetics.

Resveratrol or **4** were administered to overnight-fasted rats as a single intragastric dose (0.09 mmol/Kg, dissolved in 250 μl DMSO). Blood samples were obtained by the tail bleeding technique: before drug administration, rats were anesthetized with isoflurane and the tip of the tail was cut off; blood samples (80-100 μl each) were then taken from the tail tip at different time points after drug administration. Blood was collected in heparinized tubes, kept in ice and treated as described below within 20 min. A two-week recovery interval was obligatorily allowed between experiments utilising the same rat. The experiments were performed with the permission and supervision of the University of Padova Central Veterinary Service, which acts as Institutional Animal Care and Use Committee and certifies compliance with Italian Law DL 116/92, embodying UE Directive 86/609. The area under the concentration-time curves (AUC) were determined by the linear trapezoidal method.

Blood sample treatment and analysis.

Before starting the treatment, 4,4'-dihydroxybiphenyl was added as internal standard (25 μM final concentration). Blood was then stabilized with a freshly-prepared 10 mM solution

of ascorbic acid (0.1 vol) and acidified with 0.6 M acetic acid (0.1 vol); after mixing, an excess of acetone (4 vol) was added, followed by sonication (2 min) and centrifugation (10,000 *g*, 8 min, 4°C). The supernatant was finally collected (measuring its volume) and stored at -20°C. Before analysis, acetone was allowed to evaporate at room temperature under N_2 flow, and 30 μL of CH_3CN were added to precipitate residual proteins; after centrifugation, cleared samples were directly subjected to HPLC-UV analysis. Metabolites were identified by LC-MS analysis, enzymatic treatment on selected samples, and/or direct comparison with chromatograms of synthetic purified metabolites (main text, Fig. 2).

The recovery yield for resveratrol and the major metabolites was determined in a preliminary set of experiments using spiked blood samples. Internal standard recovery was 68.7 ± 6.3% (N = 7). The method was found to ensure a constant recovery ratio of the analytes to the internal standard: 1.18 ± 0.14 (N = 7), 0.90 ± 0.01 (N = 3), 0.64 ± 0.04 (N = 6) and 0.90 ± 0.02 (N = 3), for resveratrol and its 3,4'-disulfate, 3-glucuronide and 3-sulfate, respectively. Knowledge of these ratios allowed us to determine the unknown amount of analyte in a blood sample by measuring the recovery of the internal standard.

Enzymatic treatment (Fig. 2A).

Selected samples (100 μl) were diluted with the same volume of PBS 0.1M, pH 7, and then incubated with sulfatase (*Aerobacter aerogenes*, 10-20 U/mL, Sigma-Aldrich, 25 μl) or glucuronidase (*E. Coli K12*, 140 U/mL, Roche, 25 μl) at 37°C for 30 min. Acetone (200 μl) was then added to stop the reaction; the mixture was centrifuged (12000*g*, 4°C, 5 min), and supernatants were collected and stored at -20°C until analysis. Acetone was evaporated under a flow of nitrogen, and the concentrated samples were injected into the HPLC-UV system. Comparison of the chromatographic profiles of treated and untreated samples allowed the attribution of peaks representing sulfated or glucuronated species.

Chromatographic comparison of synthetic conjugates with metabolites observed in vivo (Fig. 2B).

Synthesized resveratrol conjugates were dissolved in a water:CH_3CN 7:3 (5 μM final concentration) and then analyzed as described above. A straightforward comparison of the chromatograms recorded at 320 nm allowed isomer-specific identification.

Synthesis of resveratrol sulfates. Synthesis was performed by a slight modification of the procedure reported in literature (Kawai, N.; Fujibayashi, Y.; Kuwabara, K.-I.; Ijuin, Y.; Kobayashi, S. *Tetrahedron* **2000**, *56*, 6467. Wenzel, E.; Soldo, T.; Erbersdobler, H.;

Somoza, V. *Mol. Nutr. Food Res.* **2005**, *49*, 482). Briefly, **1** (1 g, 4.39 mmol) and sulfur trioxide pyridine complex (2.6 g, 16.35 mmol) were dissolved in dry pyridine (10 mL). After stirring under nitrogen for 2 h at 60°C, the mixture was purified by flash chromatography using first $CHCl_3$:MeOH 9:1 as eluent to eliminate pyridine. The trisulfate was not formed in detectable amounts. Mono- and di- sulfate isomers were separated by semi-preparative HPLC. Synthesis mixtures (about 20 mg/mL, dissolved in the starting mobile phase) were separated by injecting 100 μl samples in a semi-preparative HPLC column (Gemini C18, 5 μm, 250 x 10 i.d. mm). Solvents A and B were 50 mM Ammonium Acetate (pH 6.5) and CH_3CN, respectively. The gradient for B was as follows: 18% for 6 min, from 18% to 22% in 1 min, 22% for 10 min and then from 22% to 90% in 5 min. The two isomers were identified by ^{1}H-NMR.

Synthesis of resveratrol glucuronides.

The published procedure (Brandolini, V.; Maietti, A.; Tedeschi, P.; Durini, E.; Vertuani, S.; Manfredini, S. *J. Agric. Food Chem*. **2002**, *50*, 7407. Wenzel, E.; Soldo, T.; Erbersdobler, H.; Somoza, V. *Mol. Nutr. Food Res.* **2005**, *49*, 482) was followed with some modification. **1** (2.28 g, 10 mmol), acetobromo-α-D-glucuronic acid methyl ester (2 g, 6.3 mmol) and metallic Na (0.23 g, 1 mmol) were dissolved in methanol (MeOH; 30 mL). Sodium reacts immediately to produce resveratrol anion. After stirring for 2 h at room temperature, the solvent was evaporated and the mixture was diluted in ethylacetate (EtOAc) (20 mL) and then washed with 0.01 N HCl (5 x 50 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was finally evaporated under reduced pressure and the residue was purified by flash chromatography on silica gel using $CHCl_3$:MeOH 95:5 as eluent to afford a mixture of two mono-aceto-α-D-glucuronide-methyl-esters (44%). No di- or tri-substitution products were obtained. The corresponding glucuronides were obtained incubating the product (1.8 g, 4.44 mmol) with NaOH 0.1 M (20 mL) and MeOH (20 mL). The mixture was stirred at reflux for 2 hours, and then diluted in EtOAc (20 mL) and washed with 0.01 N HCl (5 x 50 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was finally evaporated under reduced pressure and the residue dissolved in MeOH (50 mL). 50 mL water were added, and the mixture was stirred with ion-exchange (H^+) Amberlist resin until pH 3 was reached. The resin was finally filtered off, and the solution dried under vacuum and purified by flash chromatography using EtOAc:MeOH 9:1 as eluent (78%). The two isomers were separated by semi-

preparative HPLC as above; the gradient for B was as follows: 18% for 10 min, from 18% to 90% in 5 min, then 90% for 2 min. The two isomers were identified by ^{1}H-NMR.

Synthetic procedures.

Diacetone-α-D-glucose-3-O-succinyl ester (**2**). Synthesis was performed modifying a published procedure.[13] Briefly, DMAP (120 mg, 0.98 mmol, 0.26 eq) and succinic anhydride (1.6 g, 16 mmol, 4.2 eq) were added to a solution of DAG (1 g, 3.8 mmol) in dry pyridine (15 mL). After stirring for 20 h at room temperature, the mixture was diluted in $CHCl_3$ (30 mL) and washed with 0.5 N HCl (6 x 50 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was finally evaporated under reduced pressure to afford 1.08 g of the desired product (78%). ^{1}H NMR (250 MHz, $CDCl_3$) δ (ppm) : 1.47,1.36,1.27 (s, 12H, CH_3); 2.64 (m, 4H, CH_2); 3.99 (m, 2H, CH-4, CH-5); 4.17 (m, 2H, CH-6); 4.45 (m, 1H, CH-2); 5.22 (d, 1H, CH-3); 5.82 (d, 1H, CH-1);10.7 (br, 1H, OH).

4, 4'-di(diacetone-α-D-glucose-3-O-succinyl)-biphenyl. DMAP (120 mg, 0.98 mmol, 1.3 eq), EDC (0.58 g, 3 mmol, 4 eq) and 4,4'-diidroxybiphenyl (**2**; 140 mg, 0.75 mmol) were added to a solution of **3** (1.08 g, 3 mmol, 4 eq) in dry pyridine (15 mL). After stirring for 24 h at room temperature, the mixture was diluted in $CHCl_3$ (30 mL) and washed with 0.5 N HCl (6 x 50 mL) and then with 5% $NaHCO_3$ (3 x 50 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was finally evaporated under reduced pressure, to afford 560 mg of the desired product (86%). ^{1}H-NMR (250 MHz, $CDCl_3$) δ (ppm) : 1.44,1.33,1.23 (s, 24H, CH_3); 2.72-2.84 (m, 8H, CH_2); 3.98 (m, 4H, CH-4, CH-5); 4.16 (m, 4H, CH-6); 4.45 (m, 2H, CH-2); 5.22 (m, 2H, CH-3); 5.79 (d, 2H, CH-1); 7.09 (m, 4H, H-3, H-5, H-3', H-5', J_{3-2}=8.1 Hz); 7.47 (m, 4H, H-2, H-6, H-2', H-6', J_{2-3}=8.1 Hz). MS-ESI (CH_3CN): m/z 893, $[M+Na]^+$.

4,4'-di(α-D-glucose-3-O-succinyl)-biphenyl. 4,4'-di(diacetone-α-D-glucose-3-O-succinyl)-biphenyl (150 mg, 0.17 mmol) was dissolved in 12 M TFA (3 mL). After stirring for 1.5 h at room temperature, the product was precipitated with diethyl ether (10 mL) 3 times, and the solvent decanted after each precipitation. The white solid was dried under vacuum and then dissolved in 3 mL water to hydrolyse trifluoroacetic esters at glucose hydroxyls. The solution was finally lyophilized to afford 98 mg of the desired product (81%). ^{1}H-NMR (250 MHz, DMSO-d_6) δ (ppm) : 2.73-2.8 (m, 8H, CH_2); 3.07-5.05 (m, CH_2 D-glucose); 7.21 (d, 4H, H-3, H-5, H-3', H-5', J_{3-2}=8.1 Hz); 7.69 (d, 4H, H-2, H-6, H-2', H-6', J_{2-3}=8.1 Hz). MS-ESI (CH_3CN): m/z 733, $[M+Na]^+$.

3,4',5-tri(diacetone-α-D-glucose-3-O-succinyl)-resveratrol (**3**). DMAP (120 mg, 0.98 mmol, 1.8 eq), EDC (0.58 g, 3 mmol, 5.7 eq) and **1** (120 mg, 0.53 mmol) were added to a solution of **3** (990 mg, 2.75 mmol, 5.2 eq) in dry pyridine (15 mL). After stirring for 24 h at room temperature, the mixture was diluted in $CHCl_3$ (30 mL) and washed with 0.5 N HCl (6 x 50 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was finally evaporated under reduced pressure and the residue was purified by flash chromatography using ethylacetate/hexane 5:3 as eluent to afford 475 mg of the desired product (74%). ^{1}H-NMR (250 MHz, $CDCl_3$) δ (ppm) : 1.49,1.39,1.28 (s, 36H, CH_3); 2.77-2.9 (m, 12H, CH_2); 4.04-4.08 (m, 6H, CH-4,CH-5); 4.21 (m, 6H, CH-6); 4.5 (m, 3H, CH-2); 5.29 (m, 3H, CH-3); 5.85 (d, 3H, CH-1); 6.85 (t, 1H, H-4, J=2 Hz); 6.92-6.99 (d, 1H, H-7, J=16 Hz); 7.07-7.11 (d, 2H, H-5', H-3', J=8.6 Hz); 7.10 (d, 2H, H-2, H-6, J=2 Hz);7.49 (s, 2H, H-2', H-6', J=8.6 Hz). ^{1}H-NMR (250 MHz, DMSO-d_6) δ (ppm) : 1.39,1.27,1.19,1.18 (s, 36H, CH_3); 2.7-2.85 (m, 12H, CH_2); 3.8-3.9 (m, 6H, CH-4, CH-5); 4.15 (m, 6H, CH-6); 4.49 (m, 3H, CH-2); 5.04 (m, 3H, CH-3); 5.86 (d, 3H, CH-1); 6.83 (t, 1H, H-4, J=2 Hz); 7.12 (d, 2H, H-3', H-5', J=8.6 Hz); 7.25 (d, 2H, H-2, H-6, J=2 Hz); 7.28 (d, 1H, H-7, J=16 Hz);7.59 (d, 2H, H-2', H-6', J=8.7 Hz). MS-ESI (CH_3CN): m/z 1277, $[M+Na]^+$.

3,4',5-tri(α-D-glucose-3-O-succinyl)-resveratrol (**4**). **3** (150mg, 0.12 mmol) was dissolved in 12 M TFA (3 mL). After stirring for 1.5 h at room temperature, the product was precipitated with diethyl ether (10 mL) 3 times, and the solvent decanted after each precipitation. The white solid was dried under nitrogen and then dissolved in 3 mL water to hydrolyse trifluoroacetic esters at glucose hydroxyls. The solution was finally lyophilised to afford 121 mg of the desired product (98%). ^{1}H-NMR (250 MHz, DMSO-d_6) δ (ppm): 2.74-2.83 (d, 12H, CH_2); 3.07-5.06 (m, CH_2 D-glucose); 6.88 (s, 1H, H-4); 7.16 (d, 2H, H-3', H-5', J=7.9 Hz); 7.28 (s, 2H, H-2, H-6); 7.31-7.36 (d, 1H, H-7, J=16 Hz);7.64-7.67 (d, 2H, H-2', H-6', J=8.1 Hz). MS-ESI (CH_3CN): m/z 1037, $[M+Na]^+$.

BIBLIOGRAPHY AND NOTES

(1) For recent reviews on resveratrol see, e.g.: (a) Baur, J.A.; Sinclair, D.A. Nat. Rev. Drug Discov. **2006**, 5, 493. (b) Shakibaei, M.; Harikumar, K.B.; Aggarwal, B.B. Mol. Nutr. Food Res. **2009**, 53, 115. (c) Athar, M.; Back, H.H.; Kopelovich, D.R.; Bickers, D.R.; Kim, A.L. Arch. Biochem. Biophys. **2009**, 486, 95.

(2) (a) Fahr, A.; Liu, X. Expert Opin. Drug Deliv. **2007**, 4, 403. (b) Hurst, S.; Loi, C.M.; Brodfuehrer, J.; El-Kattan, A. Expert. Opin. Drug Metab. Toxicol. **2007**, 3, 469. (c) Stegemann, S.; Levellier, F.; Franchi, D.; de Jong, H.; Lindèn, H. Eur. J. Pharm. Sci. **2007**, 31, 249.

(3) (a) Ratnam, D.V.; Ankola, D.D.; Bhardwaj, V.; Sahana, D.K.; Kumar, M.N. J. Control. Release **2006**, 113, 189. (b) Scholz, S.; Williamson, G. Int. J. Vitam. Nutr. Res. **2007**, 77, 224.

(4) Passamonti, S.; Terdoslavich, M.; Franca, R.; Vanzo, A.; Tramer, F.; Braidot, E.; E. Petrussa, E.; Vianello, A. Curr. Drug Metab. **2009**, 10, 369.

(5) (a) Manach, C. ; Morand, C. ; Demigné, C. ; Texier, O. ; Régérat, F. ; Rémésy, C. FEBS Lett. **1997**, 409, 12. (b) Graefe, E.U.; Wittig, J.; Mueller, S.; Riethling, A.K.; Uehleke, B.; Drewelow, B.; Pforte, H.; Jacobasch, G.; Derendorf, H.; Veit, M. J. Clin. Pharmacol. **2001**, 41, 492. (c) Shimoi, K.; Yoshizumi, K.; Kido, T.; Usui, Y.; Yumoto, T. J. Agric. Food Chem. **2003**, 51, 2785. (d) Arts, I.C.; Sesink, A.L.; Faassen-Peters, M.; Hollman, P.C. Br. J. Nutr. **2004**, 91, 841.

(7) (a) Kawai, N.; Fujibayashi, Y.; Kuwabara, K.-I.; Ijuin, Y.; Kobayashi, S. Tetrahedron **2000**, 56, 6467. (b) Wenzel, E.; Soldo, T.; Erbersdobler, H.; Somoza, V. Mol. Nutr. Food Res. **2005**, 49, 482. (c) Brandolini, V.; Maietti, A.; Tedeschi, P.; Durini, E.; Vertuani, S.; Manfredini, S. J. Agric. Food Chem. **2002**, 50, 7407.

(8) LC-ESI/MS analyses and mass spectra were performed with a 1100 Series Agilent Technologies system using a reversed phase column (Synergi-MAX, 4 μm, 150 x 4.6 mm i.d.; Phenomenex). Solvents A and B were H_2O containing 0.1% TFA and CH_3CN. See the Supplementary data for further information.

(9) (a) Walle, T. Free Radic. Biol. Med. **2004**, 36, 829. (b) Manach, C.; Donovan, J.L. Free Radic. Res. **2004**, 38, 771. (c) Yang, C.S.; Sang, S; Lambert, J.D.; Lee, M.J. Mol. Nutr. Food Res. **2008**, 52 Suppl 1, S139.

(10) The Merck Index, 13th edition (2001). Merck and Co., Inc., Whitehouse Station, NJ, USA.

(11) Barreto, M.; Buescher, R.W.: http://ift.confex.com/ift/2004/ techprogram/paper_ 23374.htm

(12) (a) Lesschaeve, I.; Noble, A.C. Am. J. Clin. Nutr. **2005**, 81 suppl. 1, 330S. (b) Bajec, M.R.; Pickering, G.J. Crit. Rev. Food Sci. Nutr. **2008**, 48, 858.

(13) Bonina, F.; Puglia, C.; Rimoli, M.G.; Avallone, E.; Abignente, E.; Boatto, G.; Nieddu, M.; Meli, R.; Amorena, M.: de Caprariis, P. Eur. J. Pharm. Sci. **2002**, 16, 167.

Heterogeneity and standardization of Phase II metabolism in cultured cells

Biasutto L, Marotta E, De Marchi U, Beltramello S, Bradaschia A, Garbisa S, Zoratti M, Paradisi C.
Cell Physiol Biochem. 2009;23(4-6):425-30

ABSTRACT

Caco-2 cells are widely used for transepithelial transport and metabolism studies. We analysed the metabolites produced from quercetin (Q) duringtransport of this flavonoid across Caco-2 monolayers and by plastic-adhering cells. We found that the pattern of Phase II metabolic activity varies markedly depending on the particular cell clone, age of the cell culture, and stressful treatment such as freezing/ thawing. Prolonged culturing and stress cause a decrease of "detoxifying" conjugating activity. This can be re-established by growing the cells with a low concentration of the transport/metabolism substrate for a few days. We suggest this metabolism-activating procedure be used to make studies with these cells more readily comparable.

INTRODUCTION

Caco-2 cells (1-4) are very popular as a convenient stand-in for intestinal epithelium in transport studies. They are a human epithelial colon cancer cell line originating from a colon adenocarcinoma having both enterocytic and colonocytic characteristics. When cultured as a monolayer, they differentiate forming tight junctions and mimicking the villous intestinal epithelium in several respects. If formed on a porous support, such as a Transwell® filter, the monolayer separates an apical from a basolaterale compartment, both accessible to the experimenter, and allows to study the kinetics, mechanism and pharmacology of the passage of any given substance from one side to the other. They are most often reductively considered as a permeability barrier, but in addition to adhesion molecules, transporters and efflux "pumps" they can express Phase II metabolism enzymes: UDP-glucuronosyltransferases UGT1 and UGT2 (5, 6), sulfotransferases SULT1 and SULT2 (7-9) and methyl transferases (10, 11). The expression of these enzymes makes Caco-2 cells

potentially useful to study biotransformations taking place during the intestinal permeation step of the absorption of a drug or nutrient. Although the liver plays an even greater role in

the “detoxification” of xenobiotics, the metabolic role of the intestine is being increasingly recognized as crucial (e.g. [12]), especially since metabolic transformations are linked to bioavailability via the interplay with transport by MDR-family efflux proteins (e.g. [13-15]). Polyphenols, with their multiple OH groups, are readymade substrates for Phase II enzymes, and the resulting metabolites are known to be exported from enterocytes via transporters of the MDR family (e.g. [15-22]). Several studies have established a correlation between Caco-2 monolayer permeability and performance in other models of intestinal absorption (e.g. (23) and refs. therein). Variations in results from laboratory to laboratory and from cell batch to cell batch have however led to the realization that “Caco-2” has actually come to denote a collection of subpopulations yielding monolayers with different transport properties, depending also on such factors as cell batch, age (number of cell cycles), growth conditions and so forth. This heterogeneity seems to reflect in part that of the originally established culture. A few Caco-2 clones with greater homogeneity have been

isolated and characterized (24). This variability in transport/ permeability characteristics has been described in detail by several authors and in a couple of excellent recent reviews (23, 25). Much less attention has been paid to the variability in metabolic ability. This aspect should however be of

just as much concern, since permeation and metabolism are related in absorbing epithelia. We report here our observations concerning this variability, and a simple procedure to re-establish latent metabolic ability in deficient cell populations. The latter was based on the notion that expression of detoxifying enzymes may be induced by prolonged exposure to polyphenols. For example in the case of resveratrol and HepG2 cells exposure to 10 μM resveratrol for 24 hours induced a considerable increase in mRNA and protein expression for two isoforms of UDP-glucuronosyltransferases and a sulfotransferase (26). A similar potentiation of Phase II detoxifying enzymes was observed in the liver of rats fed resveratrol(27).

MATERIALS AND METHODS

Materials

All chemicals for buffer preparations were of laboratory grade, obtained from J. T. Baker, Merck, or Sigma. The composition of HBSS was as follows (in mM units): NaCl 136.9, KCl 5.36, $CaCl_2$ 1.26, $MgSO_4$ 0.81, KH_2PO_4 0.44, Na_2HPO_4 0.34, Glucose 5.55, pH 7.4 (with NaOH). Corning-Costar Transwell® Clear plates (12- or 6-well, polyester

membrane, 12 or 24 mm insert diameter, 1.0 or 4.7 cm^2 septum surface, respectively, 0.4 μm pore size) were purchased from Cellbio (Milan). Caco-2 cell lines were kindly provided by the groups of M. De Bernard, S. Garbisa and E. Papini of the Dept. of Biomedical Sciences, University of Padova. Another Caco-2 clone, originally from ATCC, was purchased from the Istituto Zooprofilattico di Brescia (Italy).

Cell culture

Cells were seeded in culture flasks and passaged in Dulbecco's Modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (Biospa), 1% penicillin/streptomycin solution (10000 U/mL and 10 mg/mL, respectively, in PBS), 1% glutamine (200 mM in PBS), 1% nonessential amino acids (100× solution) and 1% Hepes (1 M in PBS). For freezing, cells grown to near confluence in a 75 cm^2 culture flask were centrifuged and resuspended in 750 μL fetal bovine serum. 250 μl of a DMEM:DMSO, 3:2 mixture were then added very slowly; the cell suspension was transferred into a vial, allowed to freeze at -80°C and then transferred into liquid N_2 for storage.

Monolayer formation

Procedures were as described previously [28]. The cells were seeded onto tissue culture treated Transwell Clear® inserts at a density of 3×10^5 cells × cm^{-2}. Monolayers were grown in a humidified atmosphere with 5% CO_2 at 37°C. Trans Epithelial Resistence (TER) was measured periodically using a Millipore Millicell-ERS epithelial voltohmmeter and experiments were performed when values reached approximately 1500-2000 Ω × cm^2 (10-20 days post-seeding).

Transport across cell monolayers

The initial stock solution of quercetin was freshly made in DMSO and was then diluted with HBSS to give formally 50 μM solutions (loading solution; final DMSO concentration 0.1%), and sonicated to obtain a finer dispersion of undissolved suspended material. The cells monolayers supported on Transwell® septa were washed twice with warm HBSS. The loading solution was added to the apical side (0.5 or 1.5 mL for 12-mm and 24-mm septa, respectively), while HBSS was added on the basolateral side (1.5 and 2.6 mL, respectively). At the desired time, apical and basolateral solutions were transferred into glass vials and frozen for analysis by HPLC-UV and LC-MS. Basolateral solutions were analyzed without any treatment; apical solutions were diluted with an equal volume of

CH_3CN and filtered through 0.45 μm PTFE filters (Chemtek Analytica) to eliminate cell residues. HPLC-UV and LC-MS analyses were carried out as described below.

Metabolism studies

Cells growing in flasks were seeded onto 6-well plates at a density of 6×10^5 cells per well, and were grown until they reached 100% confluence (5 days). To evaluate metabolic activity, they were then washed with warm HBSS, and incubated for 8 h with 1 mL/well of a 20 μM freshly prepared solution of quercetin (dilution of a 1000× stock solution in DMSO; 0.1% final DMSO) in DMEM without phenol red (to avoid interference in HPLC analyses). Medium and cells were collected together after 8 hours of incubation. 100 μL of 0.6 M acetic acid and 100 μL of 10 mM ascorbic acid (freshly prepared solution) were added, and the samples were immediately stored at –20°C until treatment and analysis. Treatment consisted in addition of acetone (1 mL), followed by sonication (2 min), filtration through 0.45 μm PTFE syringe filters and concentration under N_2.

Induction of conjugating enzymes

Cells were grown for 3 or 6 days in culture medium supplemented with 1 μM quercetin (dilution from a 1000× stock solution, 0.1% final DMSO). The medium was replaced with fresh one every day. 5 days before the planned experiment the cells were seeded onto 6-well plates as above. Metabolism experiments were performed at the end of the 5-day period (confluence), which coincided with the end of the 3- or 6-day quercetin treatment.

HPLC-UV and LC-ESI/MS

HPLC-UV analyses were performed by a Thermo Separation Products Inc. system with a P2000 Spectra System pump and a UV6000LP diode array detector (190-500 nm). The sample solution (20 μL) was injected into a reversed phase column (Synergi-Max, 4 μm, 150 × 4.6 mm i.d.; Phenomenex). Solvent A was H_2O:CF_3COOH (99.9:0.1, v:v) and B was CH_3CN. The flow rate was 1 mL/min, the gradient for B was as follows: 17% (2 min), 25% (in 5 min), 35% (in 8 min), 50% (in 5 min), 100% (in 15 min). The eluate was preferentially monitored at 270, 300 and 370 nm. Due to their spectral characteristics, quercetin and its derivatives were best observed at 370 nm. Quercetin metabolites have UV-Vis spectra very similar to that of quercetin (Biasutto, L. et al., unpublished), and the same value of $\varepsilon_{370\ nm}$ was used for quantification.

LC-MS was performed on selected samples with a 1100 Series Agilent Technologies system, equipped with binary pump (G1312A), diode array detector (G1315B) and MSD SL Trap mass spectrometer (G2445D SL) with ESI source operating in full-scan mode in both positive and negative ion mode. The column, the solvents and the gradient profile were the same as used for HPLC-UV analyses. Before LC-MS analysis, samples were further concentrated under vacuum (about 10×). Metabolites were identified by their UV-Vis and mass spectra. For the purposes of this paper sulfate and methylsulfate derivatives are considered together as "sulfate".

RESULTS

The observations reported here were collected using four Caco-2 cell lines obtained from different sources. When used as received in monolayer transport experiments, one of these lines ("clone A") exhibited strong phase II conjugation activity, resulting in the presence in the basolateral compartment mostly of quercetin sulfate. Three other lines (B-D) had only weak methyl- and sulfotransferase activities, with quercetin being the main compound both in the apical and basolateral side. The "clone A" line tended to lose conjugating activity as the number of passages increased, and needed to be periodically re-established from frozen stocks. However in several instances we observed a loss of conjugative activity apparently induced by freezing and storage of the cells in liquid N_2. Fig. 1 illustrates this variability by plotting the relative abundance of the major Phase II metabolites found after 6 hours on the basolateral side of Transwell®-supported monolayers exposed to (formally) 50 µM quercetin on the apical side (representative experiments with the 4 clones).

The different metabolic behaviour did not depend on the number of seeded cells (Fig. 2A) or tightness of the differentiated monolayer (Fig. 2A, B).

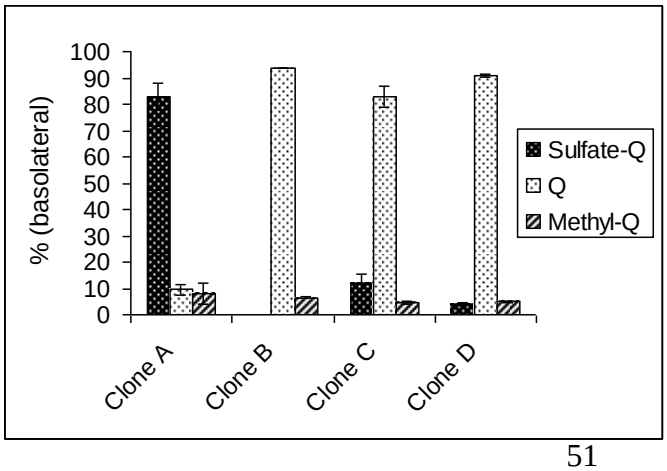


Fig. 1. A comparison of the conjugating activity of four Caco-2 clones. Relative abundance of quercetin and its major Phase II metabolites (sulfate- and methyl- quercetin) found after 6 hours in the basolateral compartment of Transwell®-supported monolayers exposed to (formally) 50 µM quercetin on the apical side

(representative experiment). The sum of the concentrations of all compounds is considered as 100%. Error bars indicate standard deviation (N = 4 for clone A; N = 3 for clones C and D; N = 1 for clone B).

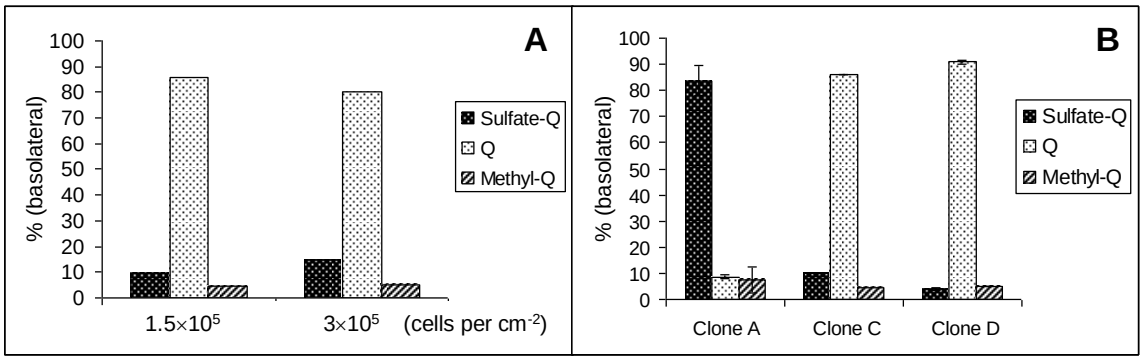


Fig. 2. Metabolic characteristics do not depend on cell density at seeding or on the value of Trans Epithelial Resistence (TER) of the differentiated monolayer. Shown is the relative abundance of quercetin and its major Phase II metabolites (sulfate- and methyl- quercetin) found after 6 hours on the basolateral side of Transwell®-supported monolayers exposed to (formally) 50 µM quercetin on the apical side (representative experiments). Shown are representative experiments exemplifying a general observation (N = 16). A) Data from two experiments with the same clone (clone D). Cells were seeded onto Transwell® inserts at two densities (1.5×10^5 and 3×10^5 cells per cm^{-2}), producing monolayers with TER values of 1800 and 3000 $\Omega \times cm^2$ at the time of the transport activity evaluation (13 days from seeding). B) The data shown are from: clone A: three experiments with average TER value of 1924 ± 203 $\Omega \times cm^2$; clone C: one experiment with TER = 1800 $\Omega \times cm^2$; clone D: three experiments with average TER value of 2150 ± 223 $\Omega \times cm^2$.

Data obtained with clone A in different time periods suggested a possible loss in expression of conjugating enzymes; the cells initially had considerable conjugating activity, but lost it after a undergoing many divisions or, in some cases, after freezing (Fig. 3).

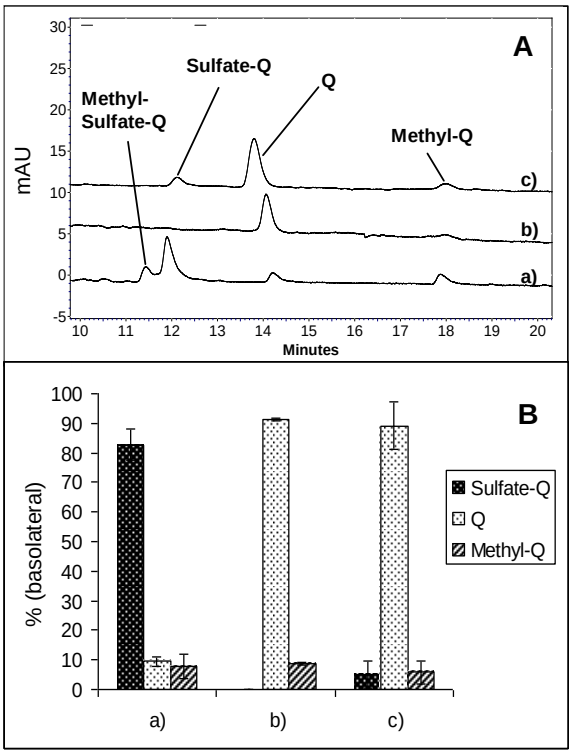


Fig. 3. Loss of metabolic activity. Relative abundance of quercetin and its major Phase II metabolites found after 6 hours on the basolateral side of Transwell®-supported monolayers exposed to (formally) 50 µM quercetin on the apical side. Experiments with clone A at different times: a) after only a few cell divisions after receipt of the cells; b) after culture for about 6 months; c) cells as in a) but after freezing and storage in liquid N_2. A) HPLC chromatograms (λ = 370 nm) of samples from representative experiments. B) Column plot of the averages obtained from 4, 3 and 5 experiments for a), b) and c) respectively.

It would be desirable to perform experiments with cells endowed with comparable metabolic activities. We therefore checked whether metabolism could be re-established by culture in the presence of low concentrations of the xenobiotic compound, i.e., quercetin. For practical reasons we performed these metabolism experiments on cells just reaching confluence after growing for different periods in 6-well plastic trays in the presence of 1 µM quercetin. Under these conditions the cells display a much more intense methyltransferase activity than when examined after reaching confluence and differentiation on Transwell® septa. This activity was not affected by quercetin (Fig. 4). On the other hand, sulfotransferase activity, nearly absent to start with, was progressively re-expressed within 6 days (Fig. 4). After 9 days metabolic activity was maintained at approximately the same level as after 6 days of growth, but signs of a cytotoxic effect of the polyphenol appeared (not shown).

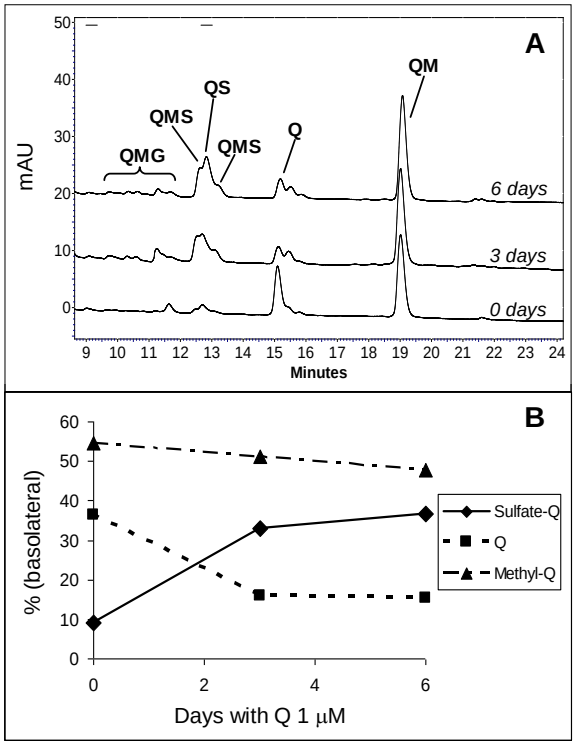


Fig. 4. Recovery of sulfotransferase activity. A) HPLC Chromatograms (370 nm) and B) relative abundance of quercetin and its major Phase II metabolites found in the medium and cells exposed to 20 μM Q for 8 hours. The data come from a representative experiment (out of 5) obtained with the same clone cultured for different time periods, as indicated, in the presence of 1 μM quercetin in the culture medium (see Materials and Methods). QMG: quercetin- methyl glucuronide; QMS: quercetin- methyl sulfate; QS: quercetin- sulfate; Q: quercetin; QM: Methyl- quercetin. The compounds were identified by LC-MS.

DISCUSSION

Caco-2 are a widespread model for transepithelial transport. If they are to provide reliable indications as to the absorption of a compound in the gastrointestinal tract they must correctly reflect metabolic as well as transport activity. Our experience with these cells was however that they can exhibit extremely variable phase II metabolic activity on a good substrate such as quercetin. Differences in metabolic capabilities can lead to discrepant results. For example in studying the transport of quercetin and its glycosides across Caco-2 monolayers a group (29) reported a rapid passage of unmodified quercetin, while others (28, 30) found conjugation products on both sides of the monolayer. Assessment of intestinal absorption *in vivo* is complicated by metabolic transformations taking place at

other sites, chiefly the liver, but experiments isolating the absorption step by using mounts of the intestinal wall or explanted intestine segments (31, 32) indicate that conjugative metabolism ought to be a feature of any reliable absorption model for polyphenols. If Caco-2 cells are to be used as such a model, they ought therefore to be metabolically active. Furthermore a standardization of the metabolic properties of the cells would be of great help in comparing results obtained from different laboratories.

Our results indicate that the expression of conjugating enzymes can be downregulated by prolonged culture under standard conditions or by "traumatic" events such as freezing followed by recovery and expansion of, perhaps, a selected population. Xenobiotic compounds can induce the expression of detoxifying enzymes in Caco-2 cells (e.g. [33, 34]). Quercetin is known to increase the expression of claudin-4, a protein of the tight junctions in Caco-2 cells lowering the permeability of a monolayer to markers of pericellular permeation (35). We found that culture of these cells with a low concentration of the intended transport/metabolism substrate, i.e., quercetin, succeeded in re-establishing expression of the relevant metabolising enzymes, i.e. sulfotransferases. We suggest therefore that Caco-2 cells to be employed in transport and/or metabolism studies be routinely "preactivated" by culture for a few days in the presence of low concentrations of the compounds of interest.

Heterogeneity of metabolic activity is probably a widespread characteristic of cultured cells, as suggested also by preliminary experiments with HepG2 cells (Biasutto, L. et al., unpublished). Inducibility of metabolic enzymes has been shown with different cells lines (26,36). "Preactivation" may represent a general strategy to restore metabolic homogeneity between different clones of the same cell line. Metabolic activation and standardization may be relevant also for Phase I metabolism, since various natural occurring flavonoids have been shown to modulate also specific cytocrome P450 isozymes (37-39).

REFERENCES

1 Meunier V, Bourrié M, Berger Y, Fabre G: The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications. Cell Biol Toxicol 1995;11:187-194.

2 Van Breemen RB, Li Y: Caco-2 cell permeability assays to measure drug absorption. Expert Opin Drug Metab Toxicol 2005;1:175-185.

3 Van de Kerkhof EG, de Graaf IA, Groothuis GM: In vitro methods to study intestinal drug metabolism. Curr Drug Metab 2007;8:658-675.

4 Sun H, Chow EC, Liu S, Du Y, Pang KS: The Caco-2 cell monolayer: usefulness and limitations. Expert Opin Drug Metab Toxicol 2008;4:395-411.

5 Gregory PA, Lewinsky RH, Gardner-Stephen DA, Mackenzie PI: Coordinate regulation of the human UDP-glucuronosyltransferase 1A8, 1A9, and 1A10 genes by hepatocyte nuclear factor 1α and the caudal-related homeodomain protein 2. Mol Pharmacol 2004;65:953-963.

6 Liu X, Tam VH, Hu M: Disposition of flavonoids via enteric recycling: determination of the UDP-glucuronosyltransferase isoforms responsible for the metabolism of flavonoids in intact Caco-2 TC7 cells using siRNA. Mol Pharm 2007;4:873-882.

7 Tamura HO, Taniguchi K, Hayashi E, Hiyoshi Y, Nagai F: Expression profiling of sulfotransferases in human cell lines derived from extra-hepatic tissues. Biol Pharm Bull 2001;24:1258-1262.

8 Chen Y, Huang C, Zhou T, Chen G: Genistein Induction of Human Sulfotransferases in HepG2 and Caco-2 Cells. Basic Clin Pharmacol Toxicol 2008;103:553-559.

9 Meinl W, Ebert B, Glatt H, Lampen A: Sulfotransferase forms expressed in human intestinal Caco-2 and TC7 cells at varying stages of differentiation and role in benzo[a]pyrene metabolism. Drug Metab Dispos 2008;36:276-283.

10 Zhang L, Zheng Y, Chow MS, Zuo Z: Investigation of intestinal absorption and disposition of green tea catechins by Caco-2 monolayer model. Int J Pharm 2004;287:1-12.

11 Poquet L, Clifford MN, Williamson G: Investigation of the metabolic fate of dihydrocaffeic acid. Biochem Pharmacol 2008;75:1218-1229.

12 Fisher MB, Labissiere G: The role of the intestine in drug metabolism and pharmacokinetics: an industry perspective. Curr Drug Metab 2007;8:694-699.

13 Benet LZ, Cummins CL: The drug efflux-metabolism alliance: biochemical aspects. Adv Drug Deliv Rev 2001;50 Suppl 1:S3-S11.

14 Benet LZ, Cummins CL, Wu CY: Unmasking the dynamic interplay between efflux transporters and metabolic enzymes. Int J Pharm 2004;277:3-9.

15 Silberberg M, Morand C, Mathevon T, Besson C, Manach C, Scalbert A, Remesy C: The bioavailability of polyphenols is highly governed by the capacity of the intestine and of the liver to secrete conjugated metabolites. Eur J Nutr 2006;45:88-96.

16 Crespy V, Morand C, Manach C, Besson C, Demigne C, Remesy C: Part of the quercetin absorbed in the small intestine is conjugated and further secreted in the intestinal lumen. Am J Physiol 1999;277:G120-G126.

17 O'Leary KA, Day AJ, Needs PW, Mellon FA, O'Brien NM, Williamson G: Metabolism of quercetin-7- and quercetin-3-glucuronides by an in vitro hepatic model: the role of human β-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. Biochem Pharmacol 2003;65:479-491.

18 Akao T, Sakashita Y, Hanada M, Goto H, Shimada Y, Terasawa K: Enteric excretion of baicalein, a flavone of Scutellariae Radix, via glucuronidation in rat: involvement of multidrug resistance-associated protein-2. Pharm Res 2004;21:2120-2126.

19 Akao T, Hanada M. Sakashita Y, Sato K, Morita M, Imanaka T: Efflux of baicalin, a flavone gluuronide of Scutellariae Radix, on Caco-2 cells through multidrug resistance-associated protein 2. J Pharm Pharmacol 2007;59:87-93.

20 Williamson G, Aeberli I, Miguet L, Zhang Z, Sanchez MB, Crespy V, Barron D, Needs P, Kroon PA, Glavinas H, Krajcsi P, Grigorov M: Interaction of positional isomers of quercetin glucuronides with the transporter ABCC2 (cMOAT, MRP2). Drug Metab Dispos 2007;35:1262-1268.

21 Lambert JD, Sang S, Lu AY, Yang CS: Metabolism of dietary polyphenols and possible interactions with drugs. Curr Drug Metab 2007;:499-507.

22 Maier-Salamon A, Hagenauer B, Reznicek G, Szekeres T, Thalhammer T, Jäger W: Metabolism and disposition of resveratrol in the isolated perfused rat liver: role of Mrp2 in the biliary excretion of glucuronides. J Pharm Sci 2008;97:1615-1628.

23 Volpe DA: Variability in Caco-2 and MDCK cell-based intestinal permeability assays. J Pharm Sci 2008;97:712-725.

24 Salvini S, Charbonnier M, Defoort C, Alquier C, Lairon D: Functional characterization of three clones of the human intestinal Caco-2 cell line for dietary lipid processing. Br J Nutr 2002;87:211-217.

25 Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F: The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol 2005;21:1-26.

26 Lançon A, Hanet N, Jannin B, Delmas D, Heydel JM, Lizard G, Chagnon MC, Artur Y, Latruffe N: Resveratrol in human hepatoma HepG2 cells: metabolism and inducibility of detoxifying enzymes. Drug Metab Dispos 2007;35:699-703.

27 Hebbar V, Shen G, Hu R, Kim BR, Chen C, Korytko PJ, Crowell JA, Levine BS, Kong AN: Toxicogenomics of resveratrol in rat liver. Life Sci 2005;76:2299-2314.

28 Biasutto L, Marotta E, De Marchi U, Zoratti M, Paradisi C: Ester-based precursors to increase the bioavailability of quercetin. J Med Chem 2007;50:241-253.

29 Walgren RA, Walle UK, Walle T: Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. Biochem Pharmacol 1998;55:1721-1727.

30 Murota K, Shimizu S, Chujo H, Moon JH, Terao J: Efficiency of absorption and metabolic conversion of quercetin and its glucosides in human intestinal cell line Caco-2. Arch Biochem Biophys 2000;384:391-397.

31 Crespy V, Morand C, Besson C, Manach C, Démigné C, Rémésy C: Comparison of the intestinal absorption of quercetin, phloretin and their glucosides in rats. J Nutr 2001;131:2109-2114.

32 Dragoni S, Gee J, Bennett R, Valoti M, Sgaragli G: red wine alcohol promotes quercetin absorption and directs its metabolism towards isorhamnetin and tamarixetin in rat intestine in vitro. Br J Pharmacol 2006;147:765-771.

33 Lampen A, Ebert B, Stumkat L, Jacob J, Seidel A: Induction of gene expression of xenobiotic metabolism enzymes and ABC-transport proteins by PAH and a reconstituted PAH mixture in human Caco-2 cells. Biochim Biophys Acta 2004;1681:38-46.

34 Xu C, Li CY, Kong AN: Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch Pharm Res 2005;28:249-268.

35 Amasheh M, Schlichter S, Amasheh S, Mankertz J, Zeitz M, Fromm M, Schulzke JD: Quercetin enhances epithelial barrier function and increases claudin-4 expression in caco-2 cells. J Nutr 2008;138:1067-1073.

36 Sun XY, Plouzek CA, Henry JP, Wang TT, Phang JM: Increased UDP-gluconosyltransferase activity and decreased prostate specific antigen production by biochanin A in prostate cancer cells. Cancer Res 1998;58:2379-2384.

37 Wood AW, Smith DS, Chang RL, Huang MT, Conney AH: Effects of flavonoids on the metabolism of xenobiotics. Progr Clin Biol Res 1986;213:195-210.

38 Ho PC, Saville DJ, Wanwimolruk S: Inhibition of human CYP3A4 activity by grapefruit flavonoids, furanocoumarins and related compounds. J Pharm Pharm Sci 2001;4:217-227.

39 Ciolino HP, Daschner PJ, Yeh GC: Dietary flavonols quercetin and kaemferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. Biochem J 1999; 715-722.

Synthesis and biological characterization of prodrugs of resveratrol based on the reversible protection of phenolic hydroxyl groups as acetals

INTRODUCTION

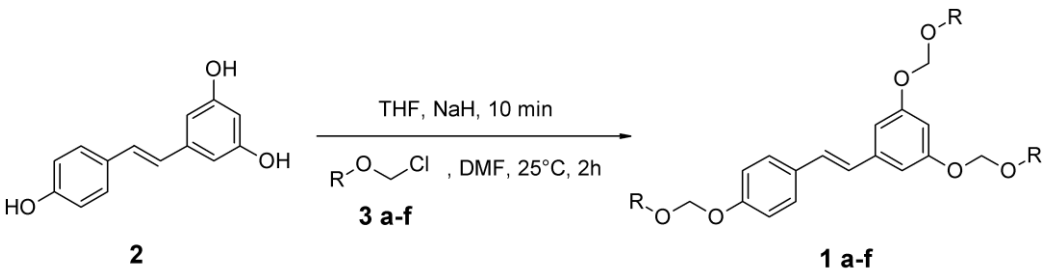
The efficacy of an ingested drug or nutraceutic compound depends on its ability to reach its target(s) in an active form, i.e., on its "bioavailability". This in turn depends on several factors, pertaining to the different stages of absorption and elimination: release from the "matrix" (e.g. food or a medicinal pill), crossing from the gastrointestinal tract into the body, metabolism, and excretion. Optimization of these aspects is fundamental for the success of a drug or the full realization of the biomedical potential of nutraceuticals such as resveratrol, the natural compound of interest here.

I report here the characterization and in vitro and in vivo pharmacokinetic evaluation in rat of prodrugs of resveratrol utilizing the acetal bond system to link protective/solubilising groups to resveratrol hydroxyls. The acetal linkage, often used for protection of hydroxyls in synthetic procedures, offers the advantage of low polarity and steric hindrance, expected to favour passage through biomembranes. It is furthermore acid-sensitive, a characteristic which may lead to a requirement for protection by a suitable formulation during gastric transit, but also offers in principle a tool for targeted release of the active principle in acidic environments. Its use in prodrugs has precedents (1), and recently a variant has been used to produce derivatives of quercetin, another remarkable polyphenol, with enhanced stability and membrane permeability (2, 3). A first generation of derivatives were synthesized (compounds a, b and c in scheme 1), which contain protective groups for the phenolic moiety commonly utilized in organic chemistry. Compound c proved to be the most promising of the series. It was therefore decided to increase the length of the polyether chain to asses how this factor can influence the adsorption.

Polyethyleneglycol (PEG) has been used in a number of formulations and prodrugs to increase solubility, improve absorption and limit immune response (4, 5). PEG is known to be non-toxic, non-antigenic and biocompatible, to be rapidly eliminated from the body and to have pronounced solubilising properties. This type of modification can increase resistance to hydrolases and stability in the gastrointestinal tract. PEG has been successfully used as a "carrier" of peptides and proteins as well as of small bioactive molecules. In this latter case however an intrinsic limitation is its low drug loading capability, i.e. the high MW of prodrugs incorporating polymeric chains. We have

produced a resveratrol prodrug bearing PEG chains linked via carboxyester moieties (6). In experiments assessing transport across the wall of rat intestine ex vivo, this construct succeeded in determining the translocation of unmodified resveratrol. When using resveratrol as such, only conjugation metabolites appeared on the basolateral side. However, the same facilitating and shielding effect was obtained using an equivalent mixture of PEG and resveratrol, suggesting that the carboxyester linkage had been lysed during incubation and the effect was due to the liberated PEG chains. To overcome this fragility we have thus turned to the acetal linkage. To remedy the low loading capacity we have used as capping groups a few short polyether chains of defined-size and performed a structure-function study to determine the optimal size for absorption in pharmacokinetics.

RESULTS



R = Me **(a),** Et **(b),** EtOMe **(c),** $(EtO)_2Me$ **(d),** $(EtO)_3Me$ **(e),** $(EtO)_4Me$ **(f),** $(EtO)_6Me$ **(g)**

Scheme 1: synthesis of acetal derivatives from resveratrol and alkoxymethylene chlorides.

STABILITY IN ACID AND NEAR-NEUTRAL SOLUTION AND IN BLOOD

The derivatives **1 a-g** proved to be stable under neutral conditions for at least 24 hours, in simulated intestinal fluid (PBS, pH 6.8) as well as in blood, as expected for acetals. In 0.1 M HCl the various compounds hydrolysed at similar rates, except for derivative **1b**, which decomposed at a considerably slower rate. Two types of kinetic profiles are observed: hydrolysis appears to follow zero-order kinetics for derivatives **1 a-b** and (pseudo) first order kinetics for derivatives **1 c-g**.

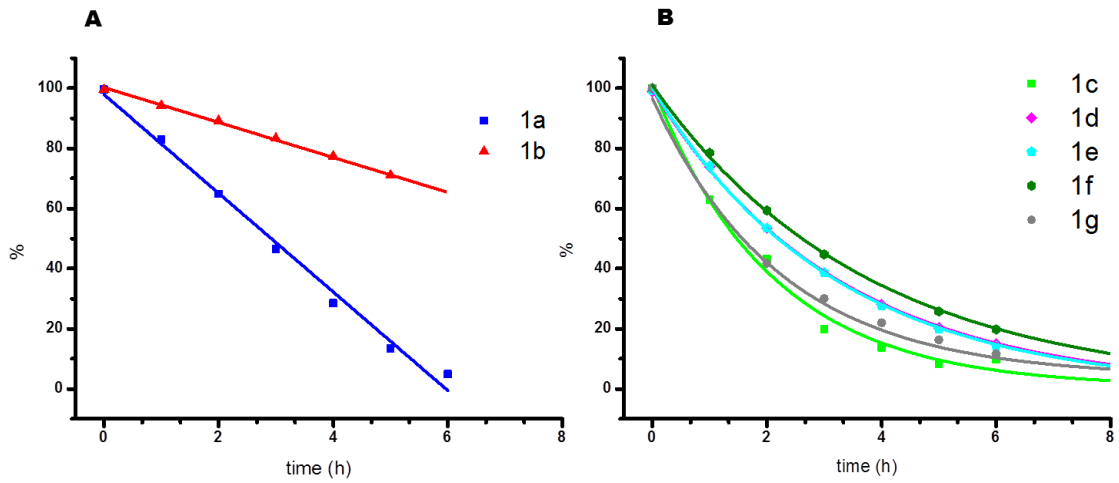


Fig. 1: concentration vs time profiles for hydrolysis of resveratrol acetal derivatives in acidic solution. Experimental points for compounds **1 a-b** fit a linear decay (A), while those for compounds **1 c-g** fit an exponential decay (B).

This duality may be only apparent, originating from the presence of undissolved material in the case of compounds 1a and 1b, the two least soluble ones. The point is currently being investigated. The available results of kinetic determinations are summarized in Table 1.

		Half-life time (h)						
		1a	**1b**	**1c**	**1d**	**1e**	**1f**	**1g**
media	PBS pH 6.8	> 24	> 24	> 24	> 24	> 24	> 24	> 24
	HCl 0.1 N	2.9	8.6	1.4	2.2	2.2	2.6	1.6
	blood	> 4	> 4	> 4	> 4	> 4	> 4	> 4

Table 1: half-lifetimes of trisubstituted resveratrol derivatives in different media.

PHARMACOKINETICS AND ABSORPTION

Compounds **1 a**-g were tested for permeation of the explanted rat intestinal wall in Ussing chamber experiments. No detectable amounts of compounds **1 a-b** appeared in the basolateral chamber. In reaching a maximum level with compound **1 f,** no detectable amounts of resveratrol or other hydrolysis products were found in basolateral side.

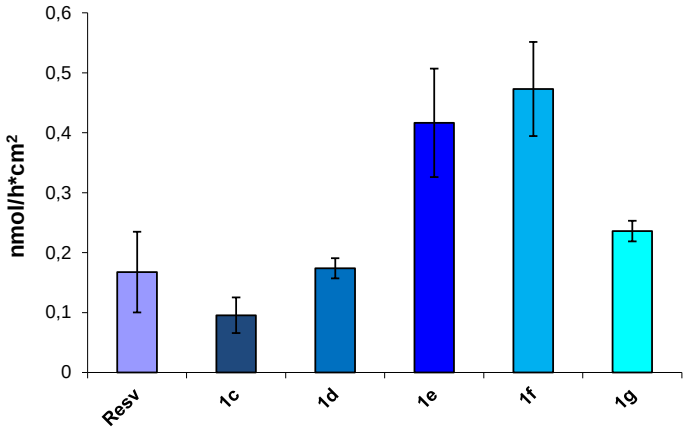


Fig 2: Flow rate of resveratrol-comprising molecules through the intestinal wall of resveratrol and compounds 1 c-g in Ussing chamber experiments (N=3)**.** Basolateral species are the intact prodrugs if acetalic derivatives are loaded in the apical side. If resveratrol is loaded, basolateral species are almost only Phase II metabolites. See the experimental section for details.

All derivatives were subjected to pharmacokinetic tests in rats. The administration of 0.088 mmol/Kg of compounds **1 a-b** did not result in the appearance of resveratrol, derivatives or metabolites in blood samples. After administration of compound **1c**, it was not possible to observe it as such, but only some other peaks probably due to its hydrolysis process. After administration of compounds **1 d-g**, it was possible to observe them in blood samples as such within 10 minutes of administration, with a concentration peak between 30 and 60 minutes (Fig. 3). In pharmacokinetics experiments with compounds **1d** and **1e**, a peak was also present with a retention time corresponding to the di-substituted derivatives (Fig. 3).

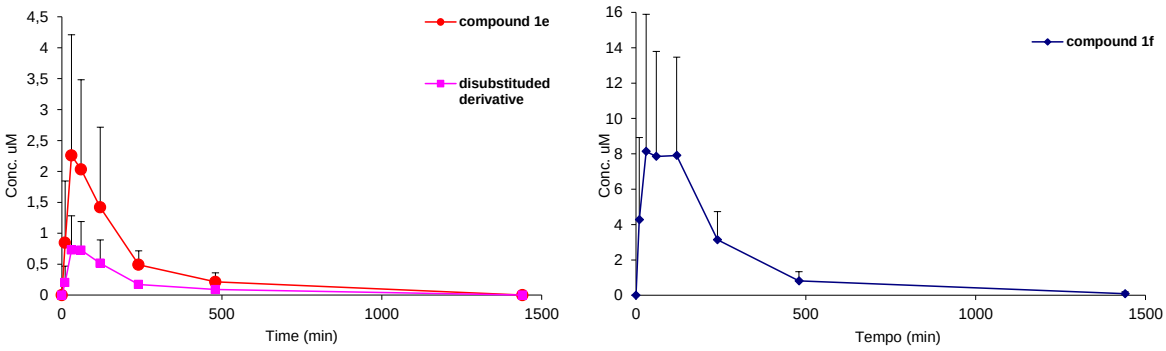


Fig. 3: Pharmacokinetic profiles for compound **1 e** and **1f**. The square represent the concentration of a metabolite deriving from hydrolysis of one acetal bond.

LC-MS analysis allowed the identification of these minor chromatographic peaks as due to demethylation products, presumably deriving from the decomposition of the "MethylPEG" moieties at the terminal ether bond, probably due to the action of the intestinal bacterial flora.

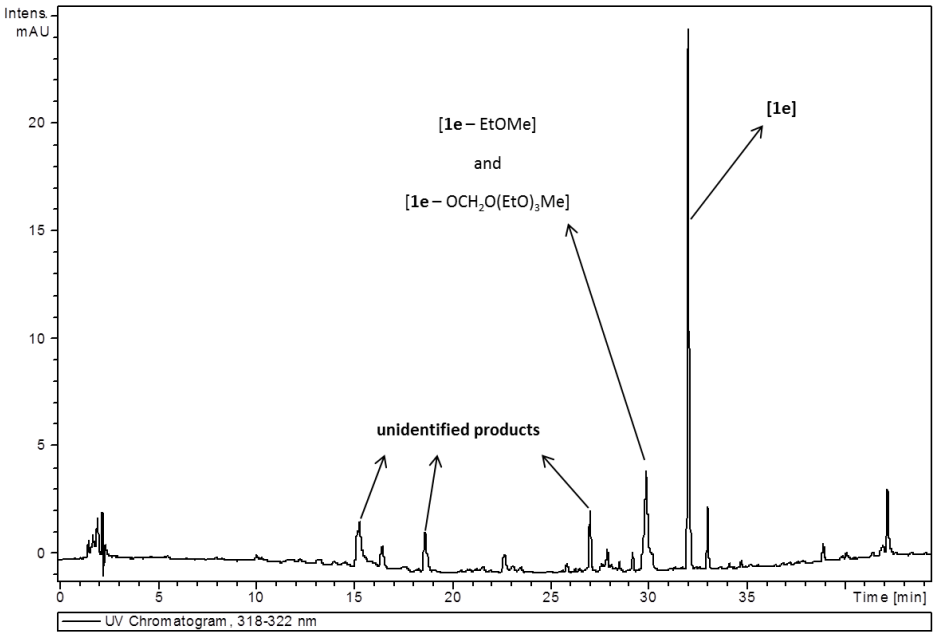


Fig. 4: LC-MS chromatogram of a blood sample taken 1 h after administration of compound 1e, showing the presence of compound 1e and partial hydrolysis products.

The Area Under the Curve (AUC) parameter increased as the length of the oligo-ethyleneglycol chain increased, and reached values considerably higher than those of resveratrol upon administration of resveratrol itself with, again, a maximum with compound **1f**.

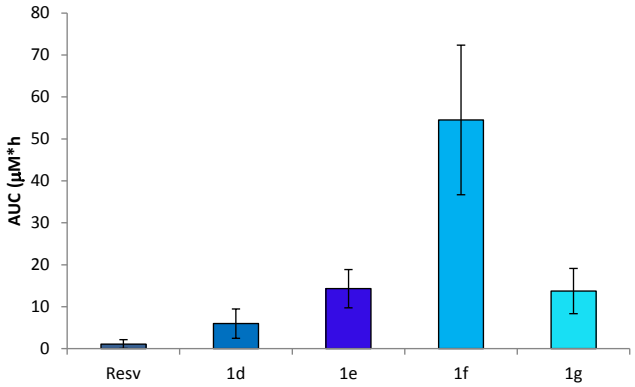


Fig. 5. AUC values for resveratrol and compounds**1 d-g**.

DISCUSSION

Resveratrol is rapidly absorbed by oral administration but it undergoes extensive metabolization and excretion. One promising method to prevent resveratrol metabolism is the protection of hydroxyl groups by a labile bond in a prodrug approach. The desirable bond should be chosen for optimal stability in vivo. The acetal bond is suitable for the protection of hydroxyl functions and it is particularly stable at the near-neutral pH prevailing in most animal tissues. It is however slowly labile at acidic pH, and this opens the possibility to restore resveratrol in specific biological compartments characterized by low pH values. We have synthesized a set of resveratrol derivatives with an acetal bond on each of the three hydroxyls, verifying the predicted stability of acetals at the pH of the intestine and in blood and confirming their sensitivity to an acid environment. Introduction of the substituent groups modified absorption: the functionalization of resveratrol with MOM (1a) or EOM (1b) blocked completely the enteric absorption of the resulting derivatives together with a substantial lowering of water solubility. The introduction of one to six $–CH_2CH_2O-$ units in the chain advantageously conferred instead water solubility and increased uptake by rat intestine, as a function of the oligomer chain length. These results, in keeping with the remarkable properties of PEG, offer the enticing possibility of achieving high-level systemic or targeted delivery of resveratrol-generating molecules, bypassing, at least for a while, the xenobiotics-inactivating defence systems of the gut and liver.

REFERENCES

1. Nomura M. *et al,* 2003. Synthesis of the cyclic and acyclic acetal derivatives of 1-(3-C-ethynyl-beta-D-ribo-pentofuranosyl)cytosine, a potent antitumor nucleoside. Design of prodrugs to be selectively activated in tumor tissues via the bio-reduction-hydrolysis mechanism. Bioorg Med Chem. May 29;11(11):2453-61.

2. Kim MK. *et al,* 2010. Enhanced stability and intracellular accumulation of quercetin by protection of the chemically or metabolically susceptible hydroxyl groups with a pivaloxymethyl (POM) promoiety. J Med Chem. Dec 23;53(24):8597-607.

3. Kim MK. *et al,* 2011. Remarkable Stability and Cytostatic Effect of a Quercetin Conjugate, 3,7-Bis-O-Pivaloxymethyl (POM) Quercetin. ChemMedChem. Nov 18. doi: 10.1002/cmdc.201100478. [Epub ahead of print]

4. Harris JM, Chess RB. 2003. Effect of pegylation on pharmaceuticals. Nat Rev Drug Discov. Mar;2(3):214-21.

5. Pasut G, Veronese FM, 2011. State of the art in PEGylation: The great versatility achieved after forty years of research. J Control Release. Nov 7. [Epub ahead of print]

6. Biasutto L. *et al,* 2009. Absorption and metabolism of resveratrol carboxyesters and methanesulfonate by explanted rat intestinal segments. Cell Physiol Biochem.;24(5-6):557-66. Epub 2009 Nov

N,N-DISUBSTITUTED CARBAMOIL PRODRUGS OF RESVERATROL

INTRODUCTION

Plant polyphenols exhibit, at least *in vitro*, a variety of potentially useful biochemical activities. Indeed, a vast literature documents effects of potential relevance for major health-care endeavours. For example resveratrol, the model polyphenol used in the present work, can prevent or slow the progression of a wide variety of illnesses, including cardiovascular disease, cancer, and ischemic injuries, as well as enhance stress resistance and extend the lifespans of various organisms from yeast to vertebrates. Studies dealing with the mechanisms underlying the bioactivity of resveratrol have been summarized in recent reviews (1-4). The biological processes involved are by no means limited to the general chemical reactivity of polyphenols as anti- or pro-oxidants. Various members of the family are known to modulate signal-transducing proteins ranging from channels (5) to cyclooxygenases (6). Effects on gene expression are important (1, 7).

Resveratrol has been shown to improve glucose homeostasis, reduce obesity, and alleviate hypertension. These are the group of symptoms that define the metabolic syndrome. Swine fed with a hypercholesterolemic diet developed glucose intolerance in addition to dyslipidemia. In resveratrol-treated animals researchers have observed not only a reduction of the adiposity and cholesterol levels, but also an increased membrane-bound fraction of the glucose transporter Glut-4 in cardiomyocytes (8). Thus, in addition to its lipid-lowering effect, resveratrol may improve hyperglycemia by increasing cellular glucose uptake. Resveratrol has also been found to decrease excess weight gain in multiple animal models of overfeeding or hyperlipidemia. For example, Dal-Pan et al. (9) showed that 4 weeks of resveratrol supplementation decreased weight gain, increased basal metabolic rate, and promoted early satiety in lemurs. These beneficial effects of resveratrol are thought to be due to its ability to activate SIRT-1 and AMP-activated protein kinase (AMPK), two important regulators of mitochondrial metabolism and glucose metabolism (10).

Bioavailability is fundamental for the full realization of the biomedical potential of nutraceuticals; many polyphenols have pharmacokinetic/pharmacodynamic and physico-chemical properties that limit their chance of being developed into pharmaceutical products. The efficacy of orally administrated resveratrol depends on its absorption,

metabolism, and tissue distribution. Only trace amounts (below 5 ng/mL) of unchanged resveratrol could be detected in human blood after a 25 mg oral dose (11). The dose escalation approach has been investigated, using a total dose range of 25–5,000 mg (12) but even in this way it was not possible to achieve the concentration necessary for resveratrol to exert its bioactivity. Indeed in enterocytes resveratrol is rapidly converted by conjugating enzymes to metabolites that are re-exported, largely to the intestinal lumen, by ABC transporters such as P-glycoprotein. Liver sulfotransferases (SULTs) and glucuronosyltransferases **(**UGTs) then intervene on the molecules that have entered the circulation. Studies on *in vivo* bioavailability and metabolism of resveratrol indicate that resveratrol-3-*O*-glucuronide and resveratrol-3-sulfate are both significant metabolites in rat urine, mouse serum, and are formed by rat hepatocytes. It is important to note that no phase I metabolites of resveratrol such as oxidation, reduction or hydrolysis products have been detected.

One of the main strategies used to prevent drug metabolism and enhance bioavailability and effectiveness is based on the development of "prodrugs" by protecting the phase II metabolism reactive sites (hydroxyl moieties) with removable groups. The purpose of these protections is to slow down or eliminate phase II metabolic processes during the absorption, thus resulting in higher systemic levels of polyphenol following the removal of protective groups by hydrolysis promoted by ubiquitous enzymes.

An ideal prodrug has both good absorption and bioconversion characteristics. Carbamate esters are one of the most popular types of prodrugs, with examples reported for duocarmycin (13), camptothecin (14), entacapone (15) and 3-PPP (16). We report here on the performance of two groups of N,N-disubstituted resveratrol carbamates. Tri (N,N-dimetilcarbamoil) Resveratrol (RTC) and Tri(N,N-dimetiltiocarbamoil) Resveratrol (RTTC) (Fig. 1) bear two methyl groups on the nitrogen atom and are poorly soluble, which poses practical problems.

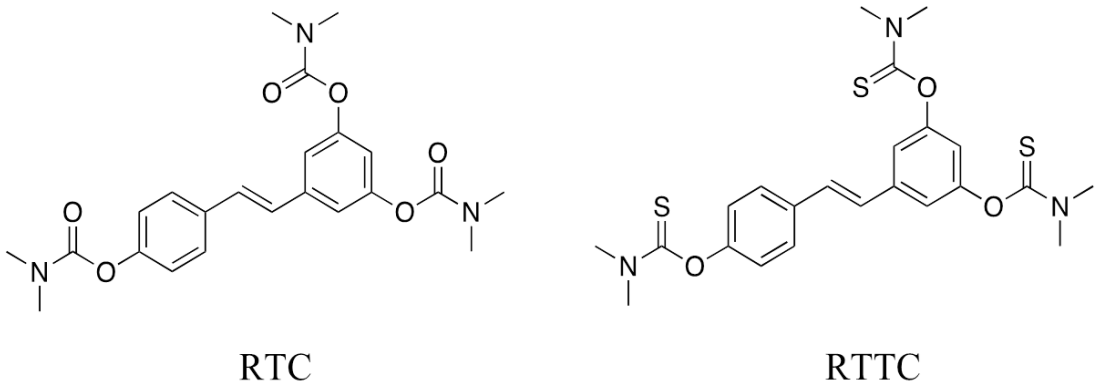


Fig.1. Chemical structure of RTC and RTTC

To improve solubility we then synthesised 3,5,4'-tri-[N-(3-glucosil) 4(butil-N-metilcarbamoil)]resveratrol (RTCG), 4'-[N-(3-glucosil) butil-N-metilcarbamoil] resveratrol (RMCG) and 3,5,4'-tri-[N-mPEG-N-metil-carbamoil] resveratrol (RTC(PEG350)) (Fig. 2).

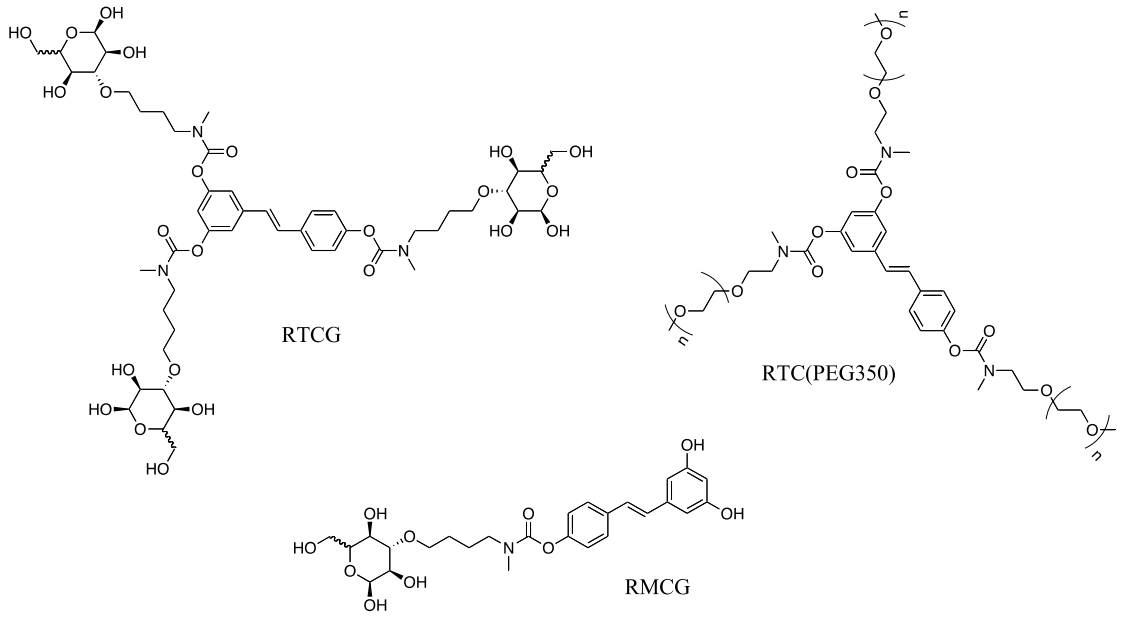


Fig.2. Chemical structure of RTCG, RMCG and RTC(PEG350)

Many prodrugs designed to increase water solubility involve the addition of an ionizable promoiety to the parent molecule. Because charged molecules have greater difficulty crossing biological membranes, one must balance increased water solubility with the potential for decreased permeability. Based on this consideration we chose non-ionizable solubilizing agents. Conversion of a compound into a more hydrophilic one is not

necessarily going to help absorption, since the energetic barrier opposing crossing the lipid core of biological membranes is expected to become more important. However both groups we used, glucose and PEG, have features that may lead both to increased solubility and to increased absorption.

It might be possible, by using the derivatization with glucose, to take advantage of the presence of glucose transporters in the absorbing epithelium. While flavonoid or resveratrol intestinal uptake is often thought to involve simple diffusion through the enterocyte membrane, studies have implicated intestinal transporters of the SGLT and GLUT families in the uptake of glycosylated derivatives (17, 18). The presence of a sugar moiety might a priori enhance the absorption and the targeting of the polyphenol, because this family of transporters is widely distributed in tissues, cells and muscles.

Chemical attachment of poly(ethylene glycol) [PEG] moieties to therapeutic compounds (a process known as 'pegylation') represents an approach that may enhance important drug properties. The FDA has approved PEG for use as a vehicle or base in foods, cosmetics and pharmaceuticals, including injectable, topical, rectal and nasal formulations. PEGylation often leads to the improvement of drug absorption and pharmacokinetics (19-23). PEG is known to be non-toxic, non-antigenic and biocompatible, to be rapidly eliminated from the body, to be soluble both in water and many organic solvents and to have pronounced solubilizing properties. In this chapter, PEG was chosen as a carrier to modify resveratrol because PEG conjugation can increase both water and lipid solubility of a drug. Improved water solubility may enhance the oral bioavailability of resveratrol as the concentration in the intestine can be increased, and improved lipid solubility may facilitate the penetration of resveratrol through biological membranes. In addition, PEG as a carrier may extend the half-life in circulation of resveratrol by protection from enzymatic activities through a "cloud effect".

In vitro stability studies, pharmacokinetics assays and investigations of absorption by explanted rat intestinal segments were thus performed on the synthesized derivatives in order to evaluate prodrug approaches to increased bioavailability after oral administration of resveratrol.

RESULTS

STABILITY IN ACID, NEAR-NEUTRAL SOLUTION AND IN BLOOD

As mentioned above, RTTC and RTC are poorly soluble in water. 1% DMSO was therefore routinely present in their solutions. Nonetheless, in the case of RTTC a fine precipitate is visible after 24 hours. This had prevented the determination of the stability. All the other compounds proved to be sufficiently stable over a 24 h period, both in gastric and intestinal environment-mimicking solutions (0.1 N HCl and 0.1 M PBS buffer, pH 6.8 respectively). Fig. 1 shows an example. In blood all the compounds were stable over a 1h period of incubation at 37°C.

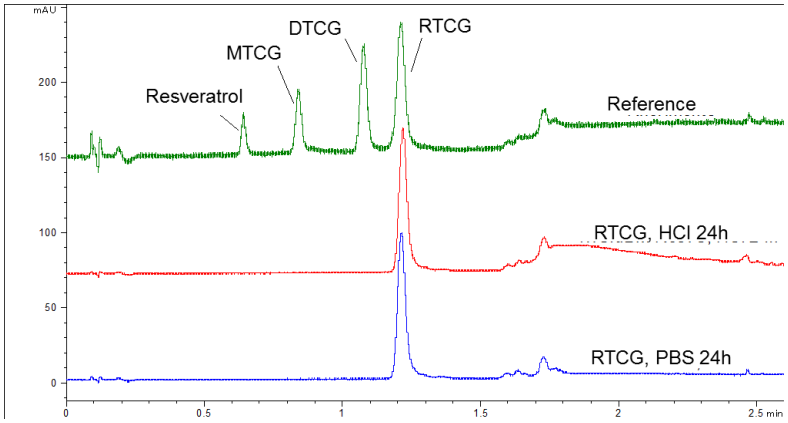


Fig.2. HPLC chromatogram of RTCG for acid and near-neutral samples after 24h of incubation. On the X-axis is reported the time of the chromatographic analysis.

PERMEABILITY STUDIES

The Ussing-type chamber system allows the study of transepithelial transport separately from other processes taking place in the intestinal lumen, in blood, or in the liver.

No RTTC, RTC(PEG350) and RMCG or derivatives/metabolites thereof could be detected in the basolateral-side chamber after incubating portions of rat intestinal wall with 20 µM of compounds on the basolateral side (10% DMSO; see chapt. Materials and Methods). On the contrary, RTC and RTCG were found in the basolateral side solution. The amount translocated after a 2,5-hour incubation corresponded to 2,5 ± 2,7 and 1.66 ± 0,72 % (N = 3), respectively, of the amount initially present in the apical chamber (0,50 ± 0,54 and 0,33 ± 0,14 nmoles, respectively). It should be emphasized that only the prodrugs themselves

were detected in the basolateral chamber. Resveratrol and its metabolites were below the detection threshold, and no products deriving from partial deprotection of the hydroxyl groups (hydrolysis of one or two of the three carbamate groups) could be detected either.

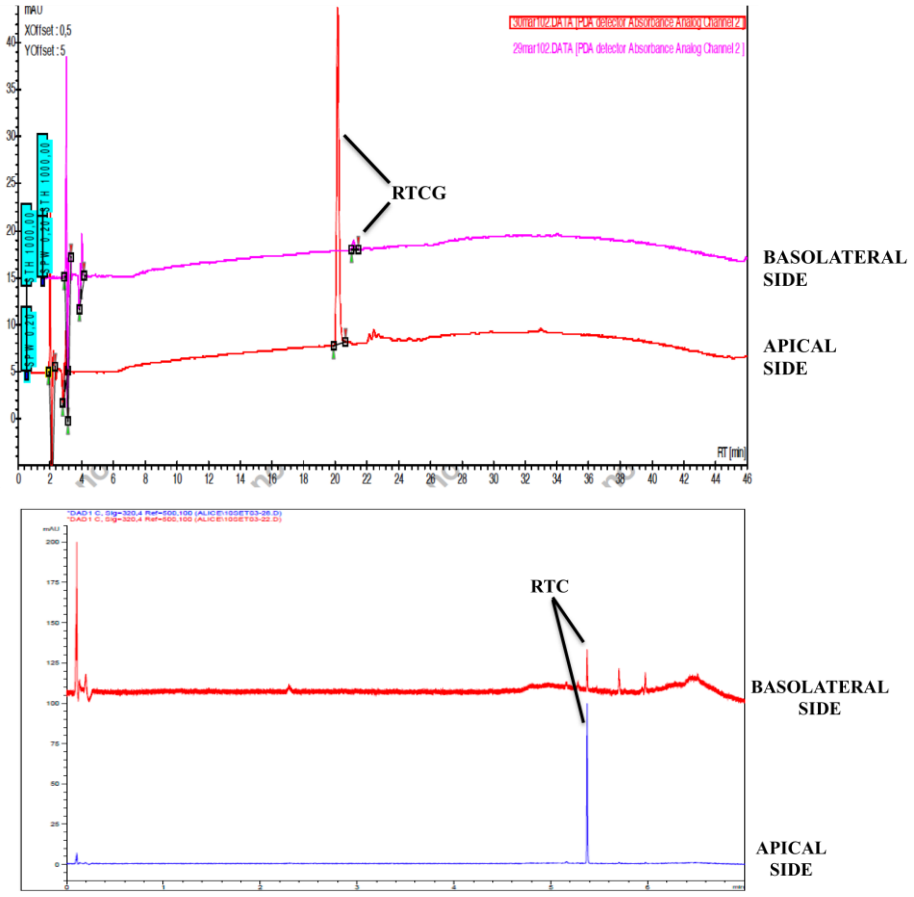


Fig.3. Exemplary HPLC chromatograms from RTCG and RTC permeation studies. The samples analysed were taken after two hours of incubation. The two rightmost small peaks in the chromatogram of the basolateral side sample in the lower panel are unrelated background signals.

PHARMACOKINETIC STUDIES

After oral administration of RTC or RTTC to rats, pharmacokinetic determinations in blood samples showed only limited absorption, with the presence of some resveratrol derivatives which could not be identified by HPLC/ESI MS because the achievable signal-to-noise ratio was insufficient.

RTC(PEG350) and RMCG were found to be present in the bloodstream, but only in traces. On the other hand, RTCG was adsorbed to a quantifiable extent; its concentration reached

a maximum about 60 minutes after administration. It was not possible to detect resveratrol or metabolites.

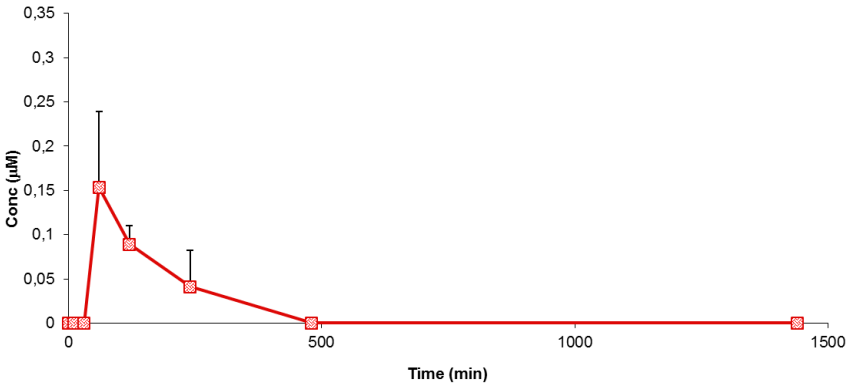


Fig.4. Pharmacokinetics profile of RTCG. The error barsindicate the deviation from the mean.

Dietary glucose crosses the apical membrane of the enterocyte by the Na^+/glucose cotransporter (SGLT1) and exits across the basolateral membrane through the facilitative transporter GLUT2. Before a meal, the concentration of glucose in the lumen is very low. Any glucose is rapidly captured by SGLT1, which is ideal for this purpose, being a low-capacity, high-affinity transporter and the only transporter capable of moving glucose against a concentration gradient. GLUT2 is a high-capacity, low-affinity facilitative transporter that equilibrates glucose between plasma and enterocyte. After a meal, there is a high effective glucose concentration at the surface of the apical membrane. The initial glucose transport across the apical membrane results in rapid insertion of GLUT2 into the apical membrane from intracellular vesicles underlying the membrane. Apical GLUT2 is now the major pathway of absorption. Then, when the glucose concentration in the lumen falls, the whole signaling system is reversed so that GLUT2 is inactivated and traffics away from the apical membrane to restore the situation before a meal (24, 25). To determine if the administration of glucose could enhance the adsorption of RTCG, thus providing evidence for a role of GLUT2 in its transport, in a set of experiments we supplied rats with glucose in their drinking water (40 g/l) for three days before carrying out the pharmacokinetic determinations.

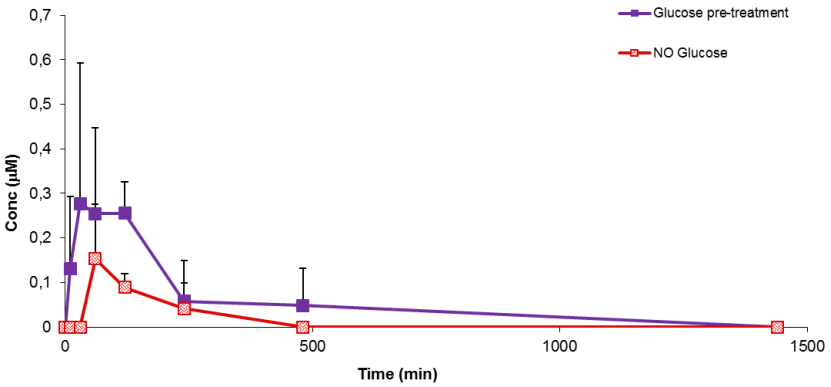


Fig.5. Comparision of pharmacokinetics profiles of RTCG with or without glucose pre-treatment.

Indeed, the administration of glucose improved absorption (Fig. 5), with the AUC going from approx. 22,36 ± 3.47 µmol/l*min (N=2) to 82,55 ± 8,04 µmol/l*min (N = 3). The kinetic profile did not change dramatically. The result however provides a preliminary evidence of an involvement of glucose transporters in the uptake of derivatives comprising a glucose (or galactose) molecule, a result of possible relevance for the possibility of a permeation of these compounds through the BBB. Resveratrol or its metabolites could not be detected in these experiments either.

To gain a better understanding of this process, we investigated the absorption process of RTCG using segment of excised rat jejunum (Ussing chambers). We performed ten experiments in the presence of different concentrations and combinations of inhibitors of SGLT-1 (phlorizin) and GLUT-2 (phloretin and cythocalasin B) (see chapt. Materials and Methods). Unfortunately we observed a great variability of the permeation of this compound, both in the presence and absence of would-be inhibitors. This made it difficult to determine if any observed difference in the translocation of the compound in a given experiment was due to the presence of the inhibitors or not.

CONCLUSIONS

The resveratrol conjugates investigated in this study constitute a new set of derivatives intended to explore the feasibility of the pro-drug approach to improve polyphenol absorption and efficacy. The chemical modifications reported here can indeed protect resveratrol hydroxyls from first pass metabolism during the absorption phase. Moreover, the second group of compounds, with glucose and PEG moieties, is water-soluble (≥ 50mM for RTCG and peg ≥ 2mM for RTC(PEG350)). However these N,N-disubstituted

derivatives proved too stable towards hydrolysis, both in gastric and intestinal environment-mimicking solutions, and they were rather poorly absorbed after oral administration. Available data point to an excessive stability *in vivo* as well. Bond stability represents a key parameter for the preparation of effective prodrugs. The ideal bond has a considerable, but not indefinite, resistance to enzymatic hydrolysis *in vivo.*

Due to its chemical and physical characteristics, the stability of the carbamate ester group can be fine-tuned by acting on the substitution pattern of the N atom. N-monosubstituted carbamates are known to be less stable than N,N-disubstituted ones (26). Furthermore, these substituents can be chosen so as to impart the desired solubility properties to the whole molecule. We thus developed a second generation of carbamoil derivatives of resveratrol, which are presented in the next chapter.

REFERENCES

1. Harikumar KB. *et al,* 2008. Resveratrol: a multitargeted agent for age-associated chronic diseases. Cell Cycle;7:1020-1035.
2. Baur JA, Sinclair DA. 2006. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov;5:493-506.
3. Pervaiz S, Holme AL. 2009. Resveratrol: its biologic targets and functional activity. Antioxid Redox Signal. Nov;11(11):2851-97.
4. Shakibaei M. *et al,* 2009. Resveratrol addiction: to die or not to die. Mol Nutr Food Res;53:115-128
5. Illek B. *et al,* 1998. Flavonoids stimulate Cl conductance of human airway epithelium in vitro and in vivo. Am J Physiol;275:L902-L910.
6. Szewczuk LM. *et al,* 2004. Resveratrol is a peroxidase-mediated inactivator of COX-1 but not COX-2: a mechanistic approach to the design of COX-1 selective agents. J Biol Chem;279:22727-22737
7. Narayanan BA. *et al,* 2006. Chemopreventive agents alters global gene expression pattern: predicting their mode of action and targets. Curr Cancer Drug Targets;6:711-727
8. Robich MP. *et al,* 2011. Resveratrol modifies risk factors for coronary artery disease in swine with metabolic syndrome and myocardial ischemia. Eur J Pharmacol. Aug 16;664(1-3):45-53.

9. Dal-Pan A. *et al,* 2010. Resveratrol suppresses body mass gain in a seasonal non-human primate model of obesity. BMC PhysiolJun 22;10:11.
10. Fullerton MD. *et al,* 2010. SIRT1 takes a backseat to AMPK in the regulation of insulin sensitivity by resveratrol. Diabetes. Mar;59(3):551-3.
11. Walle T. *et al,* 2004. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos. Dec;32(12):1377-82
12. Walle T. 2011. Bioavailability of resveratrol. Ann N Y Acad Sci. Jan;1215:9-15
13. Nagamura S. *et al,* 1995. Synthesis and antitumor activity of duocarmycin derivatives. Chem Pharm Bull (Tokyo). Sep;43(9):1530-5.
14. Senter PD. *et al,* 2001. Identification and activities of human carboxylesterases for the activation of CPT-11, a clinically approved anticancer drug. Bioconjug Chem. Nov-Dec;12(6):1074-80
15. Savolainen J. *et al,* 2000. Synthesis and in vitro/in vivo evaluation of novel oral N-alkyl- and N,N-dialkyl-carbamate esters of entacapone. Life Sci;67(2):205-16.
16. Thorberg SO. *et al,* Carbamate ester derivatives as potential prodrugs of the presynaptic dopamine autoreceptor agonist (-)-3-(3-hydroxyphenyl)-N-propylpiperidine. J Med Chem. 1987 Nov;30(11):2008-12.
17. Gee JM. *et al,* 2000. Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. J Nutr. Nov;130(11):2765-71.
18. Cermak R. *et al,* 2004. Quercetin glucosides inhibit glucose uptake into brush-border-membrane vesicles of porcine jejunum. Br J Nutr. Jun;91(6):849-55.
19. Harris JM. *et al,* 2003. Effect of pegylation on pharmaceuticals. Nat Rev Drug Discov. 2:214-221.
20. Veronese FM, Pasut G. 2005. PEGylation, successful approach to drug delivery. Drug Discov Today. 10:1451-1458.
21. Veronese FM, Mero A. 2008. The impact of PEGylation on biological therapies. BioDrugs. 22:315-329.
22. Ryan SM. *et al,* 2008. Advances in PEGylation of important biotech molecules: delivery aspects. Expert Opin Drug Deliv. 5:371-383.
23. Hamidi M. *et al,* 2006. Pharmacokinetic consequences of pegylation. Drug Deliv. 13:399-409.

24. Gouyon F. *et al,* 2003. Simple-sugar meals target GLUT2 at enterocyte apical membranes to improve sugar absorption: a study in GLUT2-null mice. J Physiol. Nov 1;552(Pt 3):823-32

25. Kellett GL. Brot-Laroche E. 2005. Apical GLUT2: a major pathway of intestinal sugar absorption Diabetes. Oct;54(10):3056-62

26. Vacondio F. *et al,* 2010. Qualitative structure-metabolism relationships in the hydrolysis of carbamates. Drug Metab Rev. Nov;42(4):551-89. Review

N-MONOSUBSTITUTED CARBAMOIL PRODRUGS OF RESVERATROL

INTRODUCTION

Plants produce several types of low-molecular-mass natural products. The majority of these compounds (called 'secondary metabolites'), plays a role in the defense of plants against infections or stressful conditions. Most of them are under scrutiny for their therapeutic potential, both in terms of disease prevention and treatment.

It is often postulated that red wine is an important factor in the French Paradox, a term referring to the fact that people in France suffer from a relatively low incidence of coronary heart disease, despite their diet being rich in saturated fats (1). As resveratrol is particularly abundant in red wine and exhibits cardiovascular-beneficial properties, it has been hypothesized that the polyphenol is responsible for the putative advantageous effects of wine. Resveratrol interacts with a large number of receptors, kinases, and other enzymes that contribute to its biological effects. For example, oral administration of resveratrol protects against the development of diet-induced insulin resistance in rodents fed a high calorie diet (2) as well as in type 2 diabetic patients (3) and this is reportedly dependent upon activation of SIRT1 (4) as well as AMPK (5).

A few polyphenols bind proteins with high affinity. For example, epigallocatechin gallate (EGCG) binds the laminin receptor with nM affinity (6). In most cases, however, potentially useful interactions are considerably weaker. For example the IC_{50} for the inhibition of cyclooxygenases 1 and 2 by quercetin is around 5 μM (7). Resveratrol and other polyphenols inhibit IkB kinase (IKK), thus reducing phosphorylation and degradation of IkB and downregulating NF-kB. IKK is half-inhibited at a concentration of resveratrol around 1 μM (8). Relatively weak affinities constitute an impediment to the biomedical utilization of these compounds, since their bioavailability is notoriously low (9-11).

Resveratrol, like most flavonoids and isoflavones, diffuses into enterocytes as the aglycone, as shown by experiments with cultured cells. Piceid, the natural glucoside of resveratrol, is perhaps too hydrophilic to diffuse passively into the enterocytes and it is likely that it is deglycosylated by small intestinal lactase in humans, as has been shown for quercetin and other flavonoids (12). Once inside the intestinal cells, there is rapid and efficient metabolization to produce more hydrophilic glucuronide and sulfate conjugates.

These metabolites are mostly re-exported to the intestinal lumen. Thus, only low concentrations (nM-μM) of polyphenols, mostly in the form of conjugates, are found in plasma and lymph even after a polyphenol-rich meal. "Detoxification" by conversion into sulfates and glucuronides entails faster elimination by the renal and biliary routes. Even so, the *in vivo* efficacy of resveratrol, despite its low bioavailability, has led to speculation that its metabolites could retain some activity. In support of this idea, several metabolites retain the ability to activate SIRT1 and inhibit cyclooxygenase *in vitro* (13). However, resveratrol-3-sulfate fails to inhibit CYPs (14) and there is currently no evidence that any metabolite is able to cross the plasma membrane. While these aspects remain to be investigated, the development of analogues with improved bioavailability and reduced metabolism will become increasingly important in the exploitation of the potential positive effects of the natural polyphenols. New and synthetic polyphenols are beginning to be explored as drugs (for resveratrol-inspired ones see, e.g., [15-25]). They are certain to be affected by the same (or worse) bioavailability problems as the natural compounds.

To avoid drug metabolism and enhance bioavailability and effectiveness, one of the main strategies used is based on the development of "prodrugs" by protecting the reactive sites with removable groups. Metabolism and solubility have been identified as the critical parameters for the bioavailability of resveratrol and other polyphenols, amenable to manipulation via a prodrug strategy Several prodrugs have been developed to improve oral bioavailability of phenolic drugs. An ideal prodrug has both good absorption and bioconversion characteristics.

In this chapter I present the work I have carried out with the second generation of carbamoil-derivatives of resveratrol. As mentioned in the previous chapter, first-generation N,N-disubstituted compounds displayed excessive stability. Since N-monosubstituted carbamates are in general more prone to hydrolysis (26), we moved to this class of compounds, which indeed showed a satisfactory reactivity. The flexibility of the carbamoyl bond system furthermore allows one to "decorate" the prodrug with groups conferring desirable properties. One property we wished to modify was, again, solubility. Thus, a set of derivatives incorporates 6-deoxy-6-galactosamine. The choice was also suggested by the possibility that intestinal transporters of the GLUT families might mediate the uptake of this kind of derivatives. This set of compounds, while very promising for certain types of application, was not however adsorbed well from the intestine, presumably due to its too-strong hydrophilicity. Better results were obtained with the prodrug incorporating glycerol-like 3-amino-1,2-propanediol, which provides sufficient

aqueous solubility as well as a more significant affinity for less polar phases and a lower molecular weight, important for an efficient adsorption in the bloodstream after oral administration (Lipinsky's rule). As shown in chapter 3, the length of PEG chain can influence the adsorption. We therefore decided to join this positive characteristic with the promising rate of hydrolysis of monosubstituted carbamaboil bond. Thus, in still another approach, the adopted N-substituent was a very short PEG chain. This was done so as to hopefully confer to the prodrug some of the favourable properties of PEG (enhanced absorption, screening from enzymatic activities, thermodinamically favourable interaction with both water and apolar phases), while maintaining a relatively low MW and facilitating synthetic procedures thanks to the defined size of the oligomers (commercial long PEG chains are provided as a mixture of species of various lengths). This work is still in progress.

RESULTS

GALACTOSAMINE DERIVATIVES

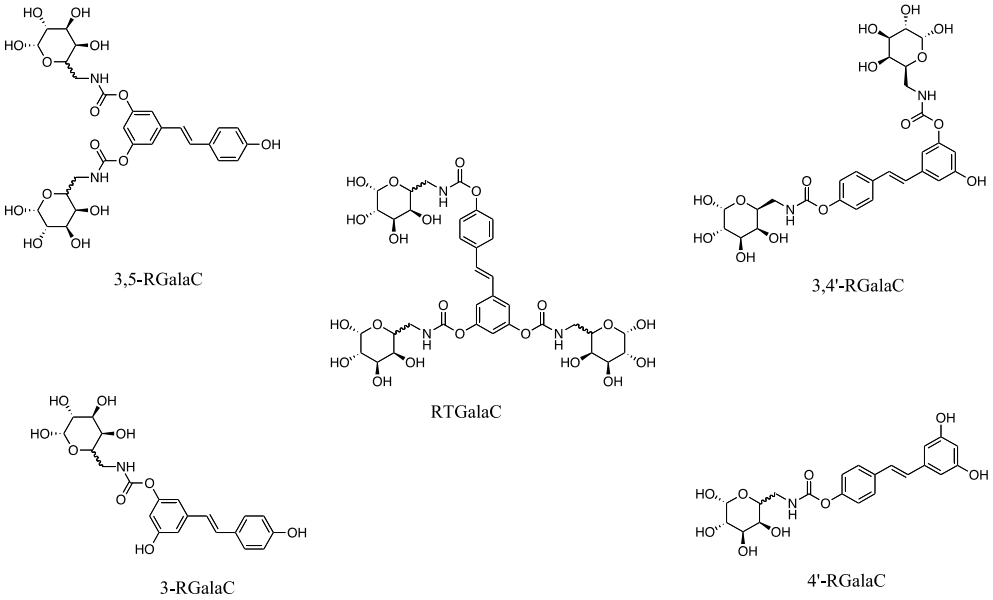


Fig.1. Chemical structure of Resveratrol galactosamine derivatives

STABILITY IN ACID AND NEAR-NEUTRAL SOLUTION

All the compounds proved to be stable over a 24 h period in acid solutions (0.1 N HCl). In near-neutral solutions (0.1 M PBS buffer, pH 6.8), the derivatives underwent hydrolysis of the carbamoyl bond with up to about 20% loss of the starting compound in the first hour for the 3,4',5-derivative and for the 3,5-derivatives, and 10% for the 3,4'-derivative. The hydrolysis of the fully substituted compounds generated intermediate products (di- and mono- derivatives). As was to be expected, since they have only one reactive group rather than three, the mono-substituted isomeric derivatives turned out be more stable than the trisubstituted one. At pH 6.8, 80% of the starting material was still present as such after 6 hours. Table 1 show the hydrolysis reaction rate costants (k) of the starting compounds. The reactions k were calculated through interpolation of data (performed by the Microcal Origin 8.0 program) with the following equation of pseudo-first order: $[C] = [C_0]*e^{-kt} + y0$ where:

[C] : concentration of the compound

$[C_0]$: concentration of the compound at the initial time t_0

t: time

RTGalaC	3,4'-RGalaC	3,5-RGalaC	4'-RGalaC	3-RGalaC
0,329±19,496	0,092±2,355	0,246±4,928	0,049±1,803	0,027±0,270

Table1. Hydrolysis reaction rate costants (k) in near-neutral solution. The data are expressed as h^{-1}.

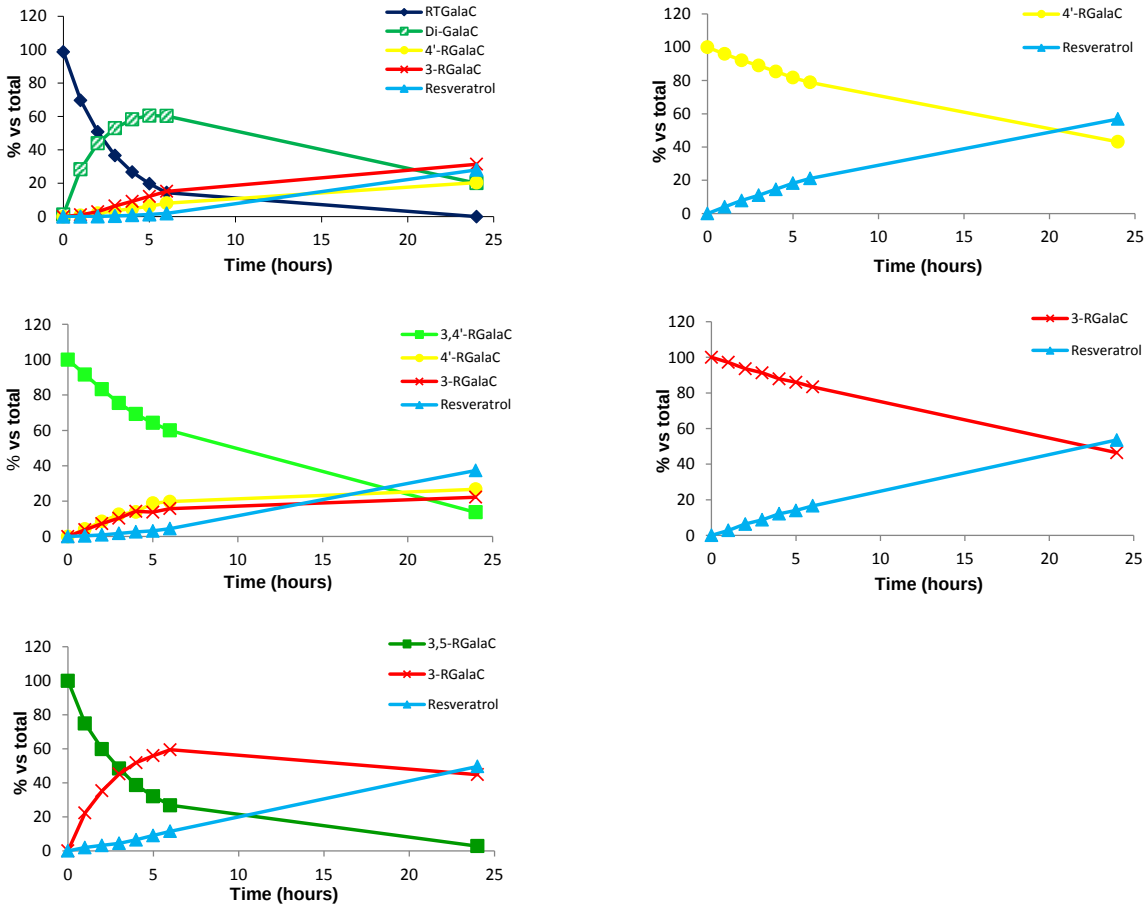


Fig.2. Stability of Resveratrol galactosamine derivatives in near-neutral solution. **A**) RTGalaC, **B**)3,4'-RGalaC, **C**) 3,5-RGalaC, **D**) 4'-RGalaC, **E**) 3-RGalaC. Data are expressed as % of the initially loaded compound.

Formation of resveratrol, i.e. the end-product of hydrolysis, was observed in all cases, with kinetics reflecting the nature of the starting material. After 24 hours the hydrolysis of mono-derivatives to resveratrol had proceded by about 57% (4'-GalaC) and 54% (3-RGalaC) respectively, while the complete hydrolysis of the other three compounds tested was in the 30-50% range.

STABILITY IN BLOOD

Hydrolysis in blood (Fig. 3) was faster than in PBS pH 6.8, presumably due to enzymatic action. A point worth noting is the difference in the rate of hydrolysis between the two isomeric mono-substituted compounds: for the 4'-derivative after 4h only 20% of starting material was present as such. At the same time of incubation, the 3-derivative was still present at 70% as such. This behavior is also illustrated by the hydrolysis reaction rate costants of the two compounds (Table 2).

RTGalaC	3,4'-RGalaC	3,5-RGalaC	4'-RGalaC	3-RGalaC
4,387±4,387	0,351±0,979	2,117±6,977	0.980±3.113	0.272±0.783

Table2. Hydrolysis reaction rate costants (k) in blood. The data are expressed as h^{-1}.

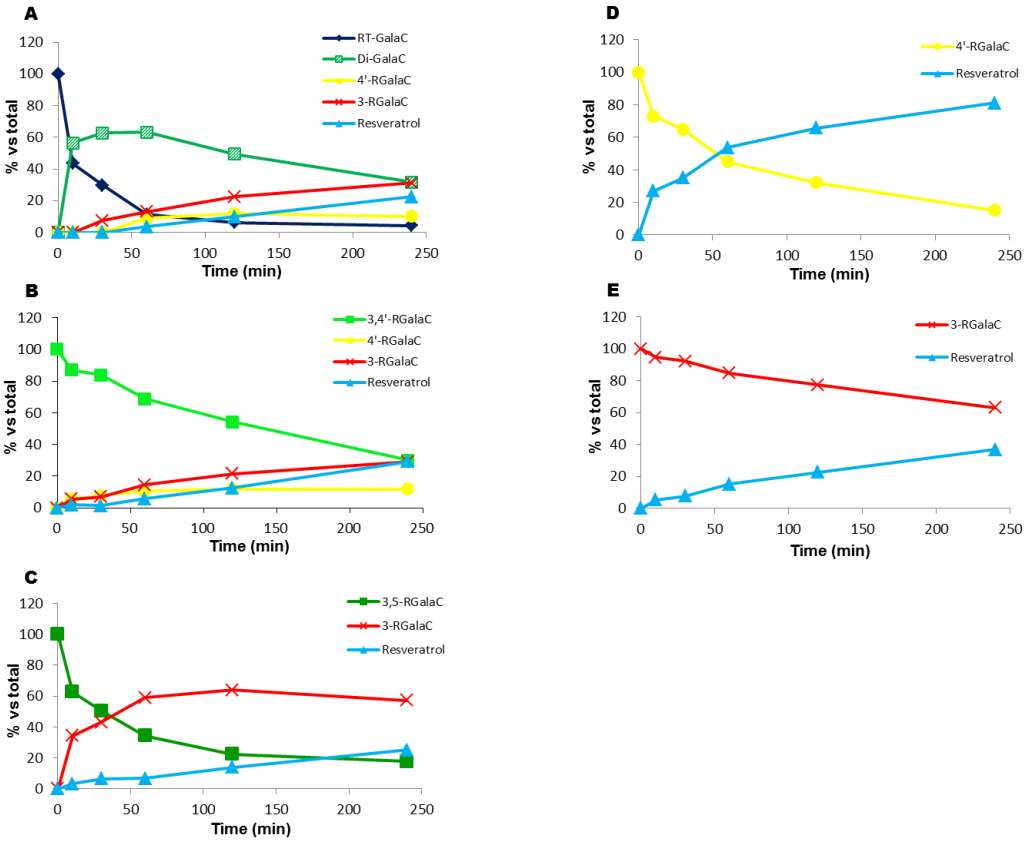


Fig.3. Stability of Resveratrol galactosamine derivatives in blood. **A**) RTGalaC, **B**) 3,4'-RGalaC, **C**) 3,5-RGalaC, **D**) 4'-RGalaC, **E**) 3-RGalaC. Data are expressed as % of the initially loaded compound

These *in vitro* stability studies confirmed that the rate of hydrolysis of the carbamoyl bond is of the correct order of magnitude for our intended applications. We therefore proceeded to directly investigate the behaviour of our compounds *in vivo* in pharmacokinetics studies.

PHARMACOKINETIC STUDIES

After oral administration of the compounds, pharmacokinetic determinations provided no evidence of absorption into the blood for 3,4',5- and 3,5-derivatives. Fig.4 displays the pharmacokinetic profile of 3,4'-, 3- and 4'- substituted derivatives. The 3,4'-disubstituted resveratrol derivative was poorly absorbed in comparison with the two mono-substituted ones (Fig. 4A). Absorption was rapid, reaching a low peak blood concentration 10 minutes

after administration, which then remained approximately constant for up to about 120 minutes. The only other species that could be detected was an unidentified resveratrol-comprising molecule. The molecule capped in position 3 reached an early peak in blood (Fig. 4B), with the concentration then decaying in an approximately exponential manner, down to undetectable levels after about 4 hours. Detection at 320 nm showed the presence of at least another resveratrol derivative, which remains unidentified. The 4'-substituted derivative again differed significantly from its isomer: it showed a delayed absorption with the maximum concentration of total species in blood reached at approximately 4 hours after administration. Again, a different unidentified derivative accounted for most of this material (Fig. 4C), and resveratrol as such could not be detected. On the basis of the retention times and in comparison with the glyceramine derivatives discussed below, we speculate that the unidentified resveratrol derivatives may well be glucuronides.

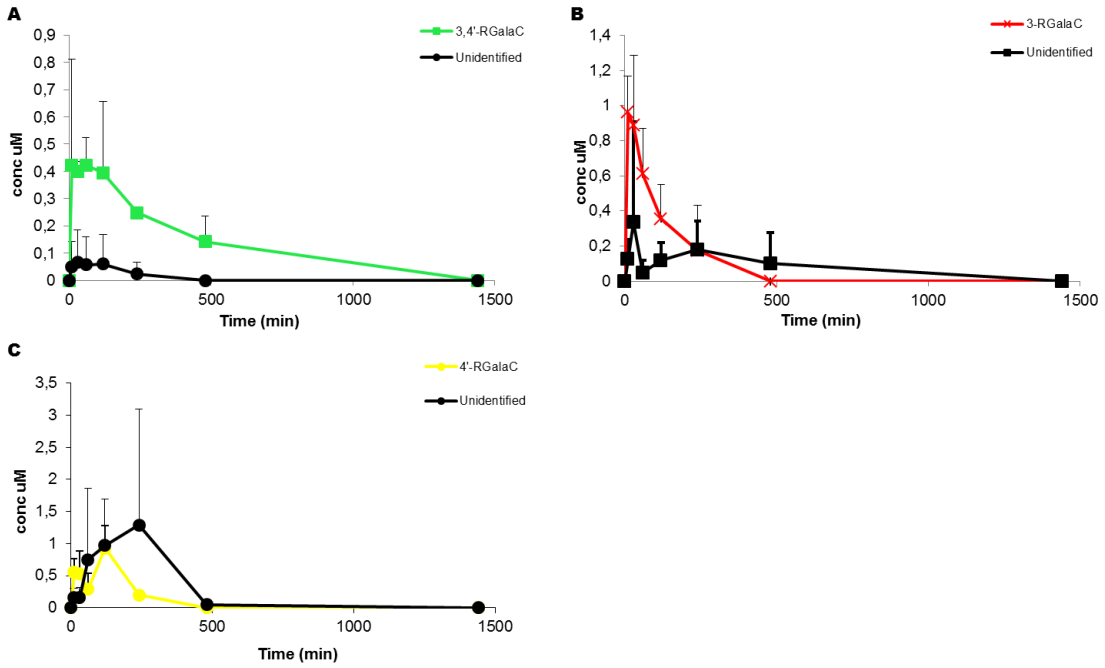


Fig.4. Pharmacokinetics of Resveratrol galactosamine derivatives. **A**) 3,4'-RGalaC, **B**) 3-RGalaC, **C**) 4'-RGalaC. Data represent average values obtained with different rats (N=3).

GLYCERAMINE DERIVATIVES

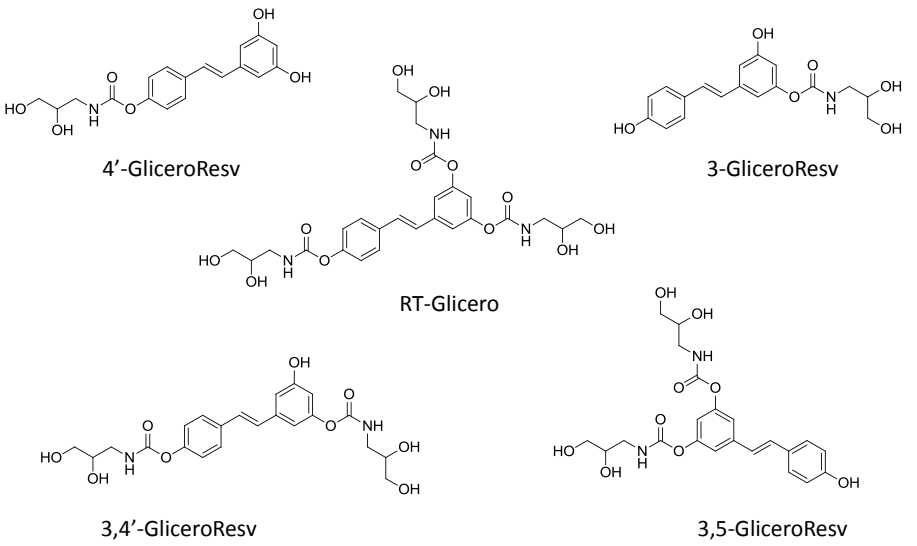


Fig.5. Chemical structure of Resveratrol glyceramine derivatives

STABILITY IN ACID AND NEAR-NEUTRAL SOLUTION

All the compounds shown in Fig. 5 proved stable over a 24 h period in 0.1 N HCl. In 0.1 M PBS buffer, pH 6.8, the derivatives underwent hydrolysis of the bond with about 30% loss of the starting compound in the first hour for the 3,4',5-derivative, 20% for the 3,5-derivatives and only 10% for the 3,4'-derivative. As expected, the 3- and 4'-derivatives reacted more slowly. Formation of resveratrol, i.e. the end-product of hydrolysis, was observed in all cases, with different kinetics. After 24 hours the mono-substituted derivatives were converted to resveratrol for about 57% (4'-GliceroResv) 58% (3-GliceroResv) respectively, while the other compounds produced 20-40% of resveratrol.

RT-GliceroResv	3,4'-GliceroResv	3,5-GliceroResv	4'-GliceroResv	3-GliceroResv
0,307±48,662	0,093±14,994	0,208±23,046	0,050±1,030	0,040±1,512

Table3. Hydrolysis reaction rate costants (k) in near-neutral solution. The data are expressed as h^{-1} .

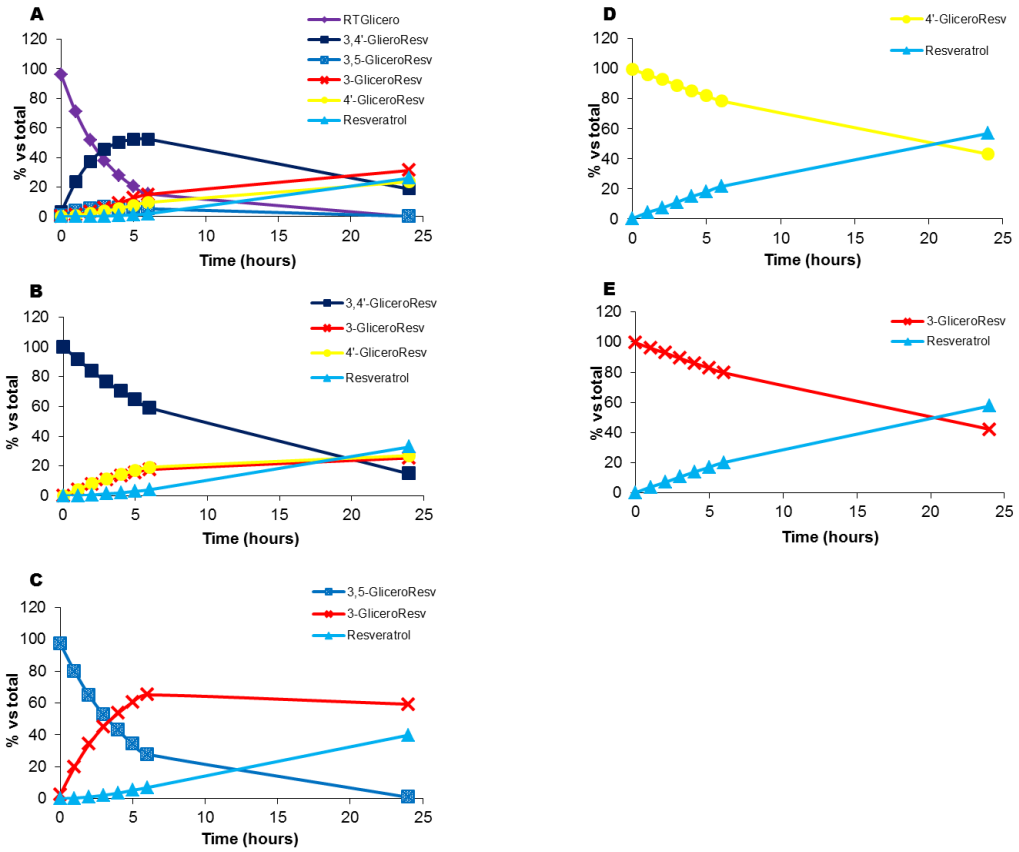


Fig.6. Stability of Resveratrol glyceramine derivatives in near-neutral solution. **A**) RTGlicero, **B**) 3,4'-GliceroResv, **C**) 3,5-GliceroResv, **D**) 4'-GliceroResv, **E**) 3-GliceroResv. Data are expressed as % of the initially loaded compound.

STABILITY IN BLOOD

Fig. 7 below presents the kinetics of reaction upon incubation of 5 µM compound in fresh rat blood. As expected, the rates of reaction were similar to those of the other carbamoyl derivatives. Again, as with the galactosamine derivatives (fig. 3), the rate of reaction proved to depend on the substitution position, with the compound substituted at position 4' reacting clearly more rapidly than the 3-substituted isomer (see the hydrolysis reaction rate costants of the two compound reported in table 4). This confirms the involvement of a regioselective (enzymatic) hydrolysis mechanism, since in PBS the kinetics are similar in the two cases.

RT-GliceroResv	3,4'-GliceroResv	3,5-GliceroResv	4'-GliceroResv	3-GliceroResv
1,479±11,539	0,459±3,380	1,398±32,467	0,607±33,489	0,119±0.187

Table4. Hydrolysis reaction rate costants (k) in blood. The data are expressed as h^{-1}.

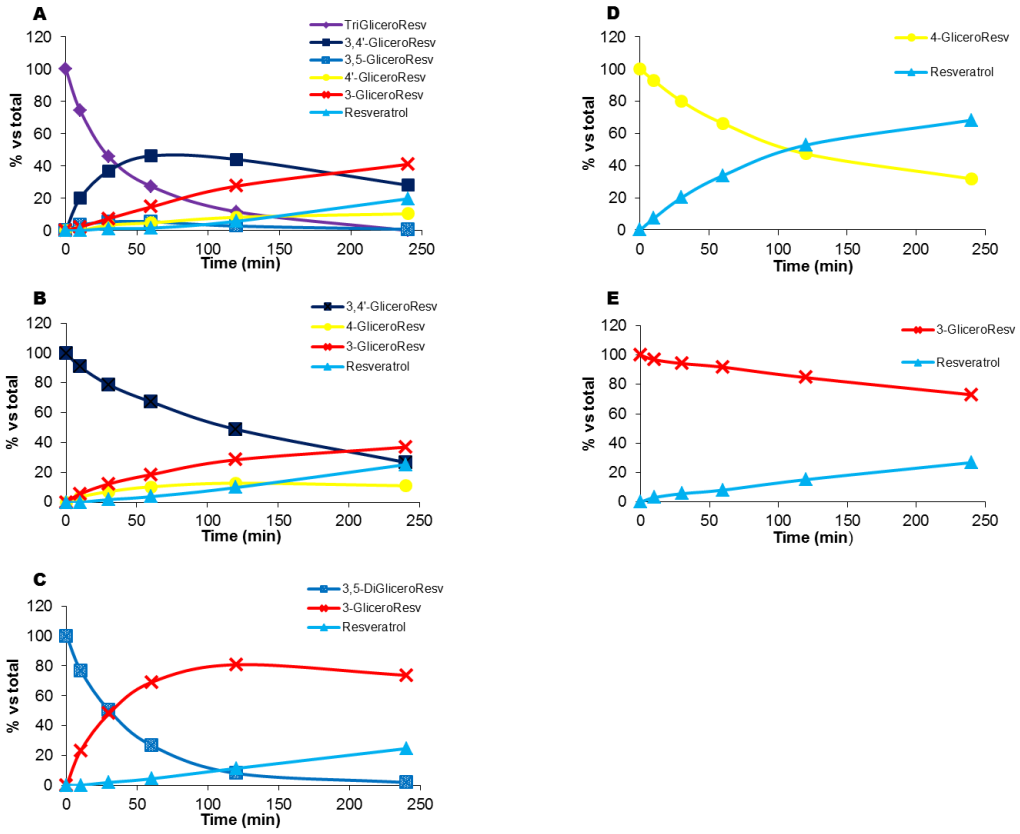


Fig.7. Stability of Resveratrol glyceramine derivatives in blood. **A**) RTGlicero, **B**) 3,4'-GliceroResv, **C**) 3,5-GliceroResv, **D**) 4'-GliceroResv, **E**) 3-GliceroResv. Data are expressed as % of the initially loaded compound

PHARMACOKINETIC STUDIES

After oral administration of the monosubstituted compounds, pharmacokinetic determinations showed that absorption had taken place, and the presence of altogether three different metabolites. The derivative capped in 4' produced only one metabolite, because the 3 and 5 positions are equivalent.

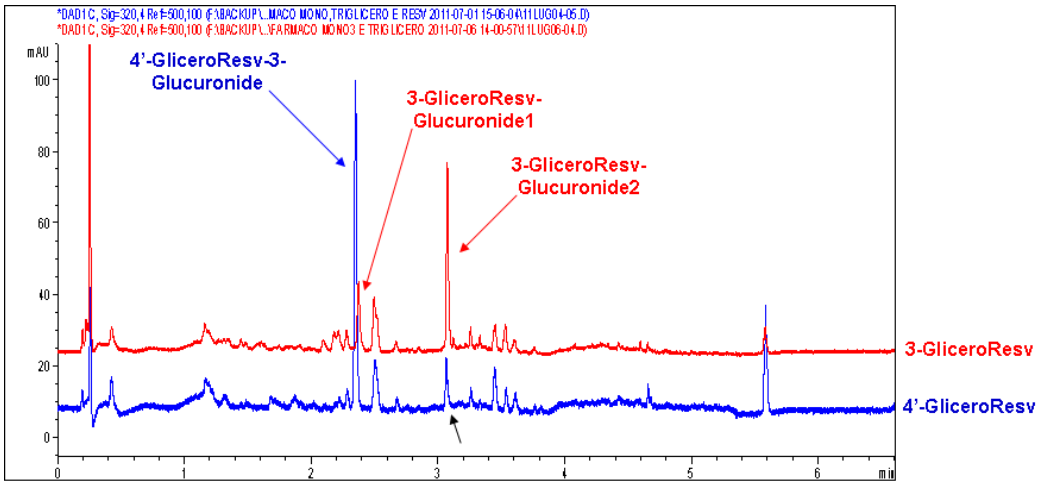


Fig.8. Pharmacokinetic profiles of 4'- and 3- derivatives. Blood samples taken 2 h and 1 h after administration of compound, respectively. The black arrow indicates the 4'-derivative present as such.

HPLC-MS analysis led to the conclusion that they were the products of glucuronidation of the mono-derivatives still bearing protective carbamoyl group(s) (Fig.9 shows an example). It was not possible to determine on which of the two free hydroxyls of the 3-derivatives the glucuronidation took place. Some unmodified 4'-GliceroResv was also detected in blood, which was not the case for its isomer.

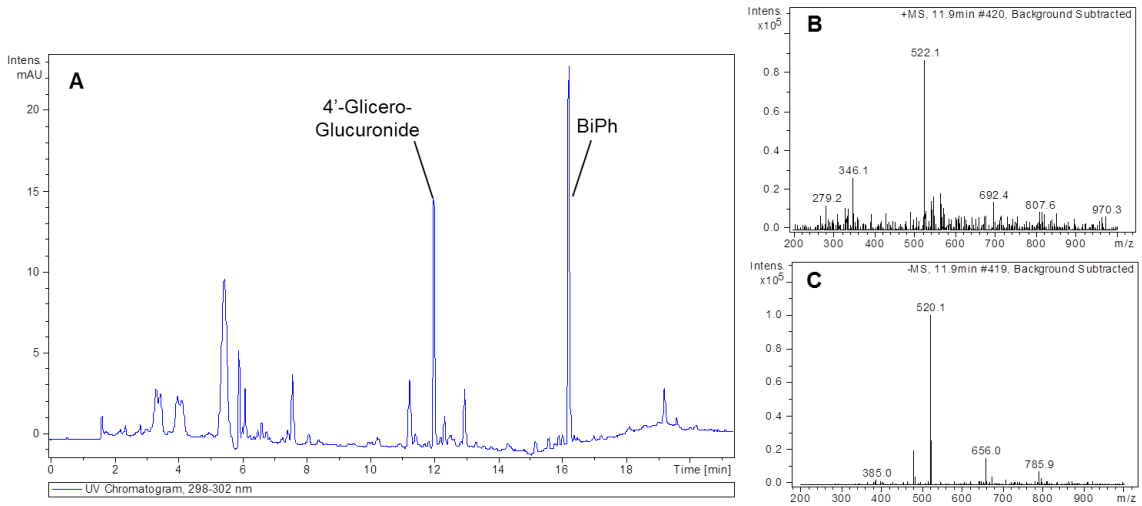


Fig.9. LC-MS chromatogram for a pharmacokinetic sample of 4'-GliceroResv (taken 2h after administration). Panel A presents the chromatogram ("BiPh" denotes the internal standard). In panels B and C the peaks represent the mass of the metabolite measured in the positive and negative ion current modes, respectively.

The 3- and 4'-derivatives were well taken up, but also extensively metabolized; the glucuronides reached a maximum concentration 60 minutes after administration. 4-GliceroResv glucuronide reached a concentration of 4,5 μM, whereas the two glucuronides

of the derivative capped in position 3 reached peak levels of 1,27 and 2,55 μM respectively.

The 3,4',5-, 3,5 and 3,4'-derivatives were poorly adsorbed. In the first case, the species detected in the bloodstream were the 3,4'-derivative, 4'-GliceroResv-3-Glucuronide (identified by comparison of the retention time with the glucuronidation product obtained from 4'-GliceroResv) and 3-GliceroResv-Glucuronide-2 (the position of glucuronidation is undetermined). After oral administration of the 3,5-derivative, absorption was rapid, reaching a maximum concentration of the prodrug as such 10 minutes after administration. The other species present were the 3-derivative and 3-GliceroResv-Glucuronide-1 (position of glucuronidation undetermined, but different from that of 3-GliceroResv-Glucuronide-2, based on chromatographic retention time). The pharmacokinetics of the 3,4'-derivative showed the presence of only the glucuronides of 4'- and 3-GliceroResv (the same observed in the 3,5-derivative pharmacokinetic) and no other intermediate products were detected.

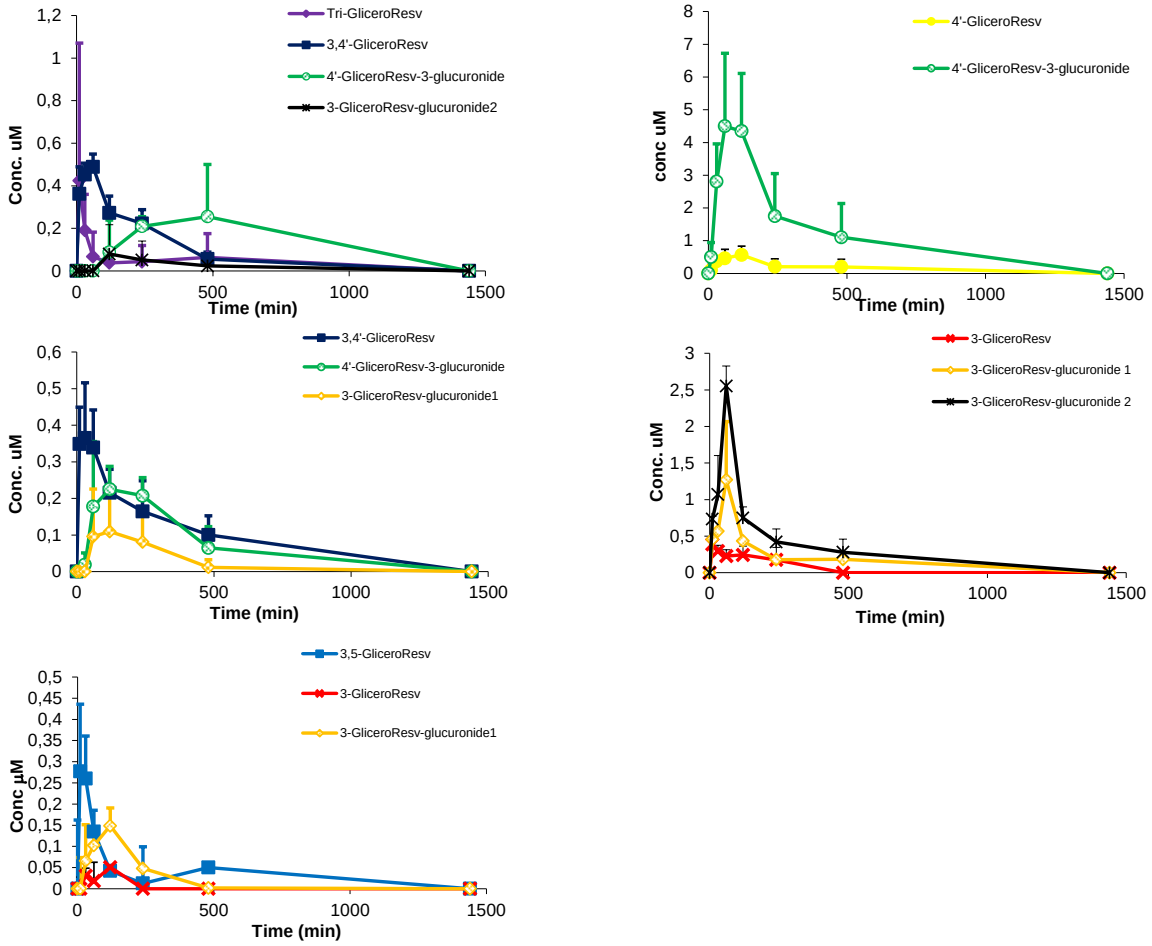


Fig.10. Pharmacokinetics of Resveratrol galactosamine derivatives. **A**) 3,4'-RGalaC, **B**) 3-RGalaC, **C**) 4'-RGalaC. Data represent average values obtained with different rats (N=3).

3,5,4'-TRI-[N-(METHOXYTRI-(ETHYLENGLICOL))-CARBAMOYL] RESVERATROL

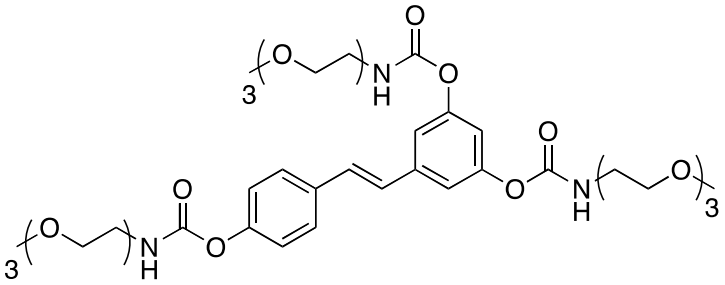


Fig.1. Chemical structure of **Resv(TGMECP)$_3$**

STABILITY IN ACID AND NEAR-NEUTRAL SOLUTION

Like the other carbamates, the compound proved to be stable over a 24 h period in the gastric environment-mimicking solution (0.1 N HCl). In intestinal environment-mimicking solutions (0.1 M PBS buffer, pH 6.8), it underwent hydrolysis of the carbamoyl bond with 10% loss of the starting compound in the first hour. The hydrolysis generated the expected partially substituted products (di- and mono- derivatives). No resveratrol, i.e. the end-product of complete hydrolysis, was detected until 24 h.

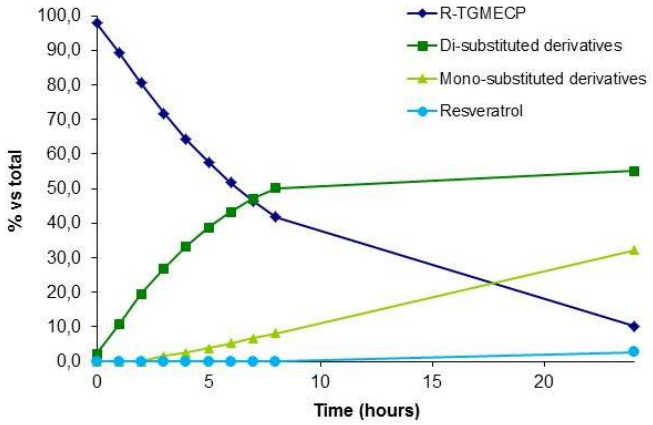


Fig.2. Stability of Resv(TGMECP)$_3$ in near-neutral solution. Data are expressed as % of the initially loaded compound

STABILITY IN BLOOD

Once absorbed, an orally administered prodrug should be able to re-generate the parent compound. RESV(TGMECP)$_3$ was thus incubated in blood at 37°C for 4 hour as a way to assess its stability in the bloodstream. The compound underwent hydrolysis, more rapidly than in PBS (pH 6.8) probably due to the presence of enzymes.

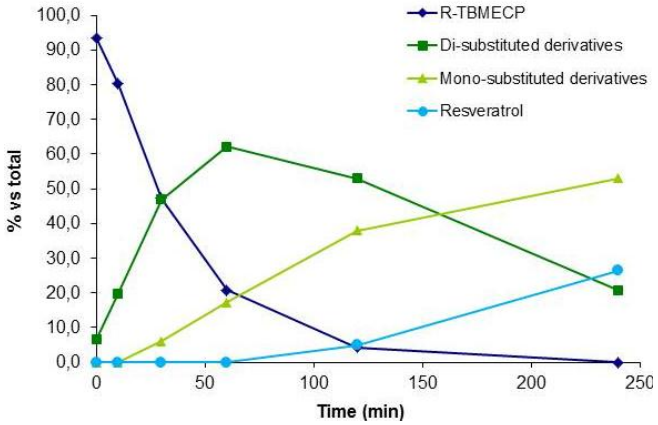


Fig.3. Stability of Resv(TGMECP)$_3$ in blood. Data are expressed as % of the initially loaded compound

PHARMACOKINETIC STUDIES

We performed so far five pharmacokinetics experiments with this compound but the results are unfortunately inconclusive, with a marked variability in the nature and concentration of analytes found in blood. Therefore I do not present here these data. Work is in progress to understand the origin of these problems.

CONCLUSIONS

The new resveratrol derivatives presented here proved to have a satisfactory stability vs. chemical hydrolysis. In particular, they were stable in acid solution, so they can be supposed to survive the gastric stage. In near-neutral solution and blood the rate of hydrolysis was relatively slow, allowing the absorption of the protected derivatives as such. In blood, the presence of prodrugs implies a progressive slow release of resveratrol. *In vivo* experiments they displayed satisfactory adsorption (in particular glyceramine

derivatives). However, it appears that as soon as a hydroxyl group is deprotected, it becomes a substrate for glucuronosyltransferases and this process takes place before the regeneration of resveratrol can be completed.

While the results with the first PEG carbamoil-derivative are not clear yet, given the encouraging results with PEG-acetal and monosubstituted carbamates, we are producing the other member of the "family", with a longer chain.

A further step of our research work will be the development of compounds with chemical characteristics (high lipophylicity) allowing absorption via the lymphatic system, to avoid hepatic metabolism.

REFERENCES

1. Renaud S. *et al,* 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet. Jun 20;339(8808):1523-6.
2. J.A. Baur. *et al,* 2006. Resveratrol improves health and survival of mice on a high-calorie diet, Nature 444 337–342.
3. Brasnyó P *et a,* 2011. Resveratrol improves insulin sensitivity, reduces oxidative stress and activates the Akt pathway in type 2 diabetic patients Br J Nutr. Aug;106(3):383-9
4. M. Lagouge *et al,* 2006. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha, Cell 127 1109–1122.
5. J.H. Um. *et al,* 2010. AMP-activated protein kinase-deficient mice are resistant to the metabolic effects of resveratrol, Diabetes 59 554–563.
6. Cai D. *et al,* 2002. Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro. Neuron 35:711–719.
7. Baptiste DC. *et al,* 2007. Update on the treatment of spinal cord injury. Prog Brain Res 161:217-233.
8. Savitz SI. *et al,* 2007. Future of neuroprotection for acute stroke: in the aftermath of the SAINT trials. Ann Neurol 61:396-402.
9. Ratan RR, Noble M. 2009. Novel multi-modal strategies to promote brain and spinal cord injury recovery. Stroke 40:S130-2.
10. Cafferty WB, Strittmatter SM. 2006. The Nogo-Nogo receptor pathway limits a spectrum of adult CNS axonal growth. J Neurosci 26:12242-12250.
11. Hannila SS, Filbin MT. 2008. The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. Exp Neurol 209:321-332.

12. Nemeth K. *et al,* 2003. Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans; Eur. J. Nutr. *42*, 29-42.

13. Calamini B. *et al,* 2010. Pleiotropic mechanisms facilitated by resveratrol and its metabolites Biochem J. Jul 15;429(2):273-82.

14. Yu, C. *et al,* 2003. Liquid chromatography/tandem mass spectrometric determination of inhibition of human cytochrome P450 isozymes by resveratrol and resveratrol-3-sulfate. Rapid Commun. Mass Spectrom. 17, 307–313

15. Murias M. *et al,* 2004. Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure-activity relationship. Bioorg Med Chem;12:5571-5578.

16. Murias M. *et al,* 2005. Antioxidant, prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure-activity relationship. Biochem Pharmacol;69:903-912.

17. Minutolo F. *et al,* 2005. Synthesis of a resveratrol analogue with high ceramide-mediated proapoptotic activity on human breast cancer cells. J Med Chem;48:6783-6786.

18. Murias M *et al,* 2008. Cytotoxic activity of 3,3',4,4',5,5'-hexahydroxystilbene against breast cancer cells is mediated by induction of p53 and downregulation of mitochondrial superoxide dismutase. Toxicol In Vitro;22:1361-1370.

19. Lee KW. *et al,* 2008. The resveratrol analogue 3,5,3',4',5'-pentahydroxy-trans-stilbene inhibits cell transformation via MEK. Int J Cancer;123:2487-2496.

20. Meng XL. *et al,* 2008. RV09, a novel resveratrol analogue, inhibits NO and TNF-alpha production by LPS-activated microglia. Int Immunopharmacol;8:1074-1082.

21. Gosslau A. *et al,* 2008. Trans- and cis-stilbene polyphenols induced rapid perinuclear mitochondrial clustering and p53-independent apoptosis in cancer cells but not normal cells. Eur J Pharmacol;587:25-34.

22. Bernhaus A. *et al,* 2009. Antitumor effects of KITC, a new resveratrol derivative, in AsPC-1 and BxPC-3 human pancreatic carcinoma cells. Invest New Drugs; in press (DOI: 10.1007/s10637-008-9183-7).

23. Jung JC. *et al,* 2009. Synthesis of novel trans-stilbene derivatives and evaluation of their potent antioxidant and neuroprotective effects. Eur J Med Chem;44:3166-3174.

24. Fan GJ. *et al,* 2009. 4,4’-Dihydroxy-trans-stilbene, a resveratrol analogue, exhibited enhanced antioxidant activity and cytotoxicity. Bioorg Med Chem;17:2360-2365.

25. Guha P. *et al,* 2009. Improved antiulcer and anticancer properties of a trans-resveratrol analog in mice. J Pharmacol Exp Ther;328:829-838.

26. Vacondio F. *et al*, 2010. Qualitative structure-metabolism relationships in the hydrolysis of carbamates. Drug Metab Rev. Nov;42(4):551-89.

A preliminary study about the effects of resveratrol and caloric restriction regimen against sunitinib-induced cardiotoxicity and renal damage

INTRODUCTION

The sirtuin family of NAD-dependent histone deacetylases (HDACs) in mammals consists of seven proteins, SIRT 1–7. SIRT-1, the best-studied among them reportedly improves the organism's health and survival (1). It is a ubiquitous, nuclear and cytoplasmic protein deacetylase, whose activity is controlled by cellular NAD+ availability (2). SIRT-1 regulates critical metabolic and physiological processes (3-6). SIRT-1 could be a target for the management of some pathologies: it can play a role thwarting atherosclerosis because it regulates tissue metalloproteinase-3 (TIMP3), an endogenous enzyme involved in the regulation of inflammatory processes and thus in the prevention of atherosclerosis (7). Furthermore, SIRT-1 has a direct role in controlling endothelium-dependent vasodilation. In endothelial cells, it increases nitric oxide production by stimulating endothelial nitric oxide synthase (eNOS) activity (8). In rat aortic smooth muscle cells, SIRT1 downregulates angiotensin II (Ang II) type 1 receptor expression, which is responsible for mediating Ang II-dependent vasoconstriction and sodium retention (9). This activity, combined with its ability to activate eNOS and thus to enhance endothelium-dependent vasodilation, suggests that SIRT1 may have a significant, direct function in controlling hypertension at the level of the vessel wall.

Resveratrol is a known natural activator of Sirt1 (10, 11). It is therefore often said to mimick calorie restriction- widely considered to be one of the best environmental intervention that provide health benefits to mammals- which also increases Sirt-1 activity (10, 12, 13).

There is evidence that resveratrol does not act directly on SIRT-1. However, it remains likely that SIRT-1 is activated in cells exposed to resveratrol by an indirect mechanism (14, 15) and that the effects of resveratrol and calorie restriction on the cardiovascular system may involve signaling through SIRT1 and the AMP-activated protein kinase (AMPK) pathways. AMP-activated protein kinase (AMPK) is a kinase that plays key roles in maintaining metabolic homeostasis in the heart, especially in the setting of energy stress, and it has vasculoprotective effects, both on vascular endothelial and smooth muscle cells

(16, 17). Moreover, dysfunction of the AMPK signalling pathway is involved in the genesis and development of various cardiovascular diseases, including atherosclerosis, hypertension and stroke (18). AMPK is activated by several natural compounds, including resveratrol, as clearly supported by data from several labs (e.g. 19-23).

The aim of the project I carried out during my stay at prof. Mervaala's laboratory was to investigate the possible positive effects of resveratrol and caloric restriction regimen against tyrosine kinase inhibitor (TKI)-induced cardiotoxicity and renal damage. For this purpose, we used sunitinib as TKI. It prolongs survival in patients with renal cell carcinoma and gastrointestinal stromal tumors and has demonstrated single agent activity against a number of other solid tumors. The targets of sunitinib are growth factor receptors, the most important of which are the vascular endothelial growth factor (VEGF) receptors, and platelet-derived growth factor (PDGF) receptors (24).

Although sunitinib is well-tolerated in many patients, significant toxicities are associated with its use. Indeed, a considerable proportion of patients treated with sunitinib develop hypertension, left ventricular dysfunction, other adverse cardiac events (congestive heart failure and myocardial infarction) and renal dysfunction (25-27). AMPK inhibition can play a central role in sunitinib cardiotoxicity (28, 29).

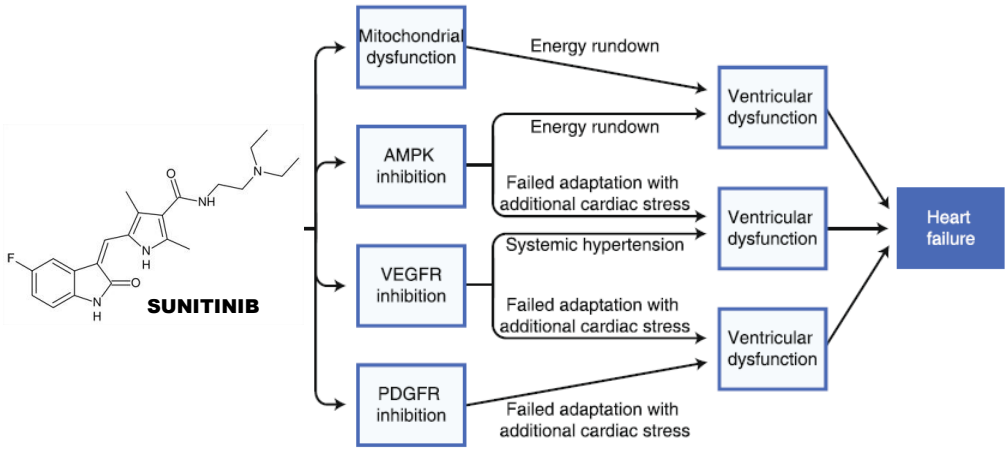


Fig. 1 Hypothesized mechanisms of sunitinib-induced cardiac dysfunction and downstream effects. AMPK: adenosine monophosphate (AMP)–activated protein kinase; PDGFR: platelet-derived growth factor receptor; VEGFR: vascular endothelial growth factor receptor. Modified from: Greineder C.F., Curr Hypertens Rep (2011) 13:436–441

It is reported that patients with a previous history of hypertension and coronary heart disease undergoing sunitinib therapy are exposed to an increased risk to develop cardiovascular events (26, 30). To investigate the effects of sunitinib and resveratrol, we thus utilized spontaneously hypertensive rats (SHR), which are considered a good animal

model of human essential or primary hypertension, and have been extensively used to study cardiovascular disease. In this strain of rats, like in human beings, hypertension starts with advancing age and the cause of the increased blood pressure remains unknown.

EXPERIMENTAL

STATISTICAL ANALYSIS

Data are presented as mean±SEM. Statistically significant differences in mean values were tested by ANOVA and Dunnett's multiple comparison test. A value of $P<0.05$ was considered statistically significant.

EXPERIMENTAL ANIMALS

We have utilized normotensive Wistar Kyoto male rats (WKY) and spontaneously hypertensive male rats (SHR). For the rats under caloric restriction (CR), the daily energy intake was restricted to 70% of the daily energy consumed by the control group The control groups had free access to chow. The body mass was recorded every day to assess the dose of drugs. Blood pressure- and body weight-matched SHR and normotensive WKY control rats were divided into seven groups, as described afterwards.

STUDY DESIGN

The study protocol was designed as showed in table1.

Experimental group	**Rat strain**	**Treatment**	**Dose**	**Follow-up period (weeks)**
WKY Control	*WKY*	*Vehicle*		*8*
SHR Control	*SHR*	*Vehicle*		*8*
SHR +CR 70%	*SHR*	*Vehicle + CR 70%*		*8*
SHR +SUN	*SHR*	*Sunitinib*	*3mg/kg*	*8*
SHR +CR 70% +SUN	*SHR*	*Sunitinib + CR 70%*	*3mg/kg*	*8*

SHR + RESV	*SHR*	*Resveratrol*	*40 mg/kg*	*8*
SHR +RESV+SUN	*SHR*	*Resveratrol + Sunitinib*	*40 mg/kg (Resv) + 3mg/kg (SUN)*	*8*

Table1. Design for in vivo study.

COMPOUNDS USED

Sunitinib

Sunitinib (N-(2-diethylaminoethyl)-5-[(Z)-(5-fluoro-2-oxo-1H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide) was administered at the dose of 3 mg/kg/day by gavage after diluting in hydroxymethylcellulose 0.5% in saline solution.

Resveratrol

Resveratrol (trans-3,5,4'-trihydroxystilbene) was given as dissolved in hydroxymethylcellulose 0.5% in aqueous solution, at the dose of 40 mg/kg/day by gavage.

BLOOD PRESSURE MEASUREMENTS

The blood pressure of the rats was measured with the CODA Non-Invasive Blood Pressure System for Mice and Rats (Kent Scientific Corporation, Torrington, Connecticut, USA) on non-anesthetized animals restrained in a rodent holder heated at 37°C. Measurements were performed in three sets of six cycles with five-second breaks between cycles and thirty-second breaks between sets. Blood pressure was measured weekly.

SAMPLE PREPARATION

At the end of the study (8 weeks), rats were anesthetized with CO_2/O_2 (95% / 5%) and decapitated. The hearts and kidneys were excised, washed with ice-cold saline, blotted dry,

weighted, and snap-frozen in liquid nitrogen or isopentane (-35°C). All the samples were stored at -80°C until analysis. Also the mesenteric arteries were excised.

ASSESSMENT OF ENDOTHELIAL FUNCTION

The mesenteric arteries were cleaned of adherent connective tissue and cut into 3 mm long sections, beginning 5 mm distally from the mesenteric artery-aorta junction. The mesenteric artery rings were carefully stretched between stainless steel hooks, connected to an isometric force transducer (EMKA Technologies, Paris, France) and suspended in an organ bath chamber in Krebs-Ringer buffer (pH 7.4) of the following composition (mM): NaCl 119.0, $NaHCO_3$ 25.0, glucose 11.1, and $CaCl_2$ 1.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, and aerated with 96% O_2 and 4 %CO_2. The rings were equilibrated for 60 min at 37°C with a resting tension of 1.0 g. After this equilibration period, the rings were exposed to 60 mmol/l KCl at least three times until optimal tension was achieved. The presence of a functional endothelium in the preparations was confirmed by observing a clear relaxation response to 1 μM acetylcholine in 1 μM noradrenaline pre-contracted arterial rings. Endothelium-dependent relaxation to acetylcholine (ACh;10^{-9}–10^{-4} mol/l) was recorded. Vasodilator response to a maximal concentration of ACh was studied before and after pre-incubation with a superoxide scavenger 4,5-dihydroxy- 1,3-benzenedisulfonic acid, Tiron (10^{-2} mol/l; 30 min). Endothelium-independent relaxation were studied in response to the nitrovasodilator sodium nitroprusside (SNP; 10^{-9}–10^{-5} mol/l). The vascular relaxation curves were produced by Graph-Pad Prism, version 4.02.

ECHOCARDIOGRAPHY

Transthoracic echocardiography was performed with an Acuson Sequoia 512 Ultrasound System and an Acuson Linear 15L8 14 MHz transducer (Siemens Medical Solutions, Mountain View, CA, USA). Rats were anesthetized with xylazine 10 mg/kg i.m. (Rompun 20 mg/mL Bayer) and ketamine 40 mg/kg i.m. (Ketalar 50 mg/mL Pfizer). Normal body temperature was maintained. The measuremets were performed in a blind-fashion by the same technician during the last study week.

The M-mode echocardiographic study of the left ventricle was performed under a two dimensional (2D) control. Left ventricular end-diastolic dimension (LVEDD), end-systolic dimension (LVESD), and the thickness of intraventricular septum (IVS) and posterior wall

(PW) at end-diastole were measured. Ejection fraction was calculated by using the Teichhold method. Fractional shortening (FS) and ejection fraction (EF) were calculated from the M-mode LV dimensions using the following equations:

FS (%)={(LVEDD-LVESD)/LVEDD} x 100

EF=SV/EDV

SV=EDV–ESV

$EDV=7/(2,4+LVEDD)x(LVEDD)^3$

$ESV=7/(2,4+LVESD)x(LVESD)^3$

where:

SV=stroke volume

EDV=end diastolic volume

EDS=end systolic volume

CARDIAC SIRT1, AMPK AND P-AMPK EXPRESSION BY WESTERN BLOT

Myocardial samples were electrophoretically separated by 8 % SDS-PAGE (24 μg total protein of the whole cell lysate per lane for Sirt-1 and 45μg for AMP-K). Each lane corresponded to one rat and 4 groups were run on one gel. Proteins were transferred to a PVDF membrane (Immobilon-P®, Millipore, Bedford, MA, USA) and blocked in 5% non-fat milk-TBS-0.01% Tween-20® buffer. The membranes were probed with the primary anti-SIRT1(Abcam), anti-AMPK (Cell Signaling Technology) and anti-p-AMPK (Cell Signaling Technology) antibodies. After being probed, the membranes were stripped and re-probed for tubulin (Antialpha tubulin; Abcam) which was used as the loading control. Horseradish peroxidase-conjugated anti-rabbit secondary antibody (Chemicon, Temecula, CA, USA) was exposed to enhanced chemiluminescence solution (ECLplus, Amersham Biosciences, Buckinghamshire, UK). We quantified the relative protein expression with Fluorescent Image Analyzer (FUJIFILM Corp, Tokyo, Japan). The measurements were repeated three times, and the data are presented as means±standard error of the mean (SEM) of these experiments.

IMMUNOHISTOCHEMISTRY

The kidneys were cryosectioned at 5μm thickness and air dried. Cryosections were treated with ice-cold acetone (10 minutes), air-dried and washed with Tris-buffered saline (TBS: 0.05M Tris-buffer, 0.15 mol/l NaCl, pH 7.6). The sections were incubated for 60 min in a humid chamber at room temperature with primary monoclonal antibodies against rat monocytes/macrophages (ED1, Serotec Oxford, UK). The slides were then treated with secondary antibody (peroxidase-conjugated rabbit anti-mouse immunoglobulins; DAKO A/S, Glostrup, Denmark) for 30 minutes at room temperature. After treatment with the third antibody (HRP-F(ab')2 goat anti-rabbit) (Zymed, San Francisco, CA) for 30 minutes at room temperature, the reaction was made visible by an AEC (3-amino-9-ethyl carbazole) solution containing hydrogen peroxidase. The sections were lightly counterstained in hematoxylin, blued in tap water, and mounted with GelTol (Coulter-immunotech, Hamburg, Germany). From the processed renal samples, the relative amount of primary antibody-positive label per sample was determined by computerized densitometry (Leica IM500 and Leica QWIN software; Leica Microsystems AG, Heerbrugg, Switzerland).

RESULTS

BLOOD PRESSURE

Unexpectedly, after eight weeks of treatments, sunitinib did not produce any statistically relevant change in systolic blood pressure, in comparison to the SHR control group. Neither resveratrol nor caloric restriction regimen were able to induce a decrease in systolic blood pressure in the SHR rats.

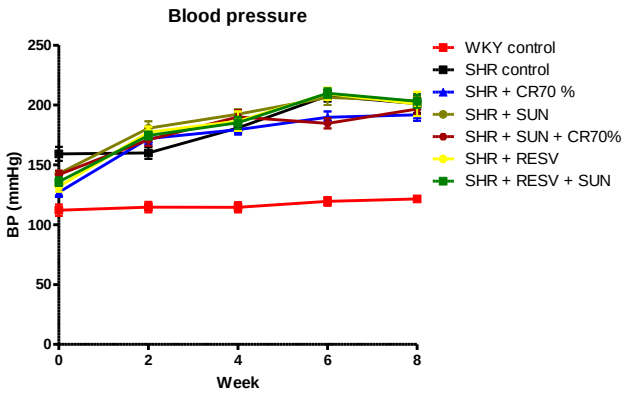


Fig. 1 SBP development in WKY and SHR controls, in SHR under caloric restriction and SHR treated with resveratrol and sunitinib

ASSESSMENT OF ENDOTHELIAL FUNCTION

The endothelium plays a crucial role in the regulation of vascular tone by releasing relaxing and contracting substances in response to different stimuli. Under several pathological conditions, including hypertension, the endothelial cells are dysfunctional. This dysfunction is characterized by impaired relaxation in response to endothelium-dependent dilators such as acetylcholine.

Endothelium-dependent relaxation in response to Ach was markedly impaired in SHR rats, in comparison with WKY rats. In the sunitinib-treated rats the Ach-induced relaxation did not significantly differ from the response in the SHR control group. Endothelium-dependent relaxation was restored by the caloric restriction regimen (Fig 2A).

The relaxations in response to NO donor SNP were comparable in all groups. Pre-treatments with Tiron improved vascular relaxation in response to ACh, but only in the caloric restriction *plus* sunitinib group we observed a statistically significant difference (Fig 2C). Resveratrol did not improve vascular relaxation (Fig 3).

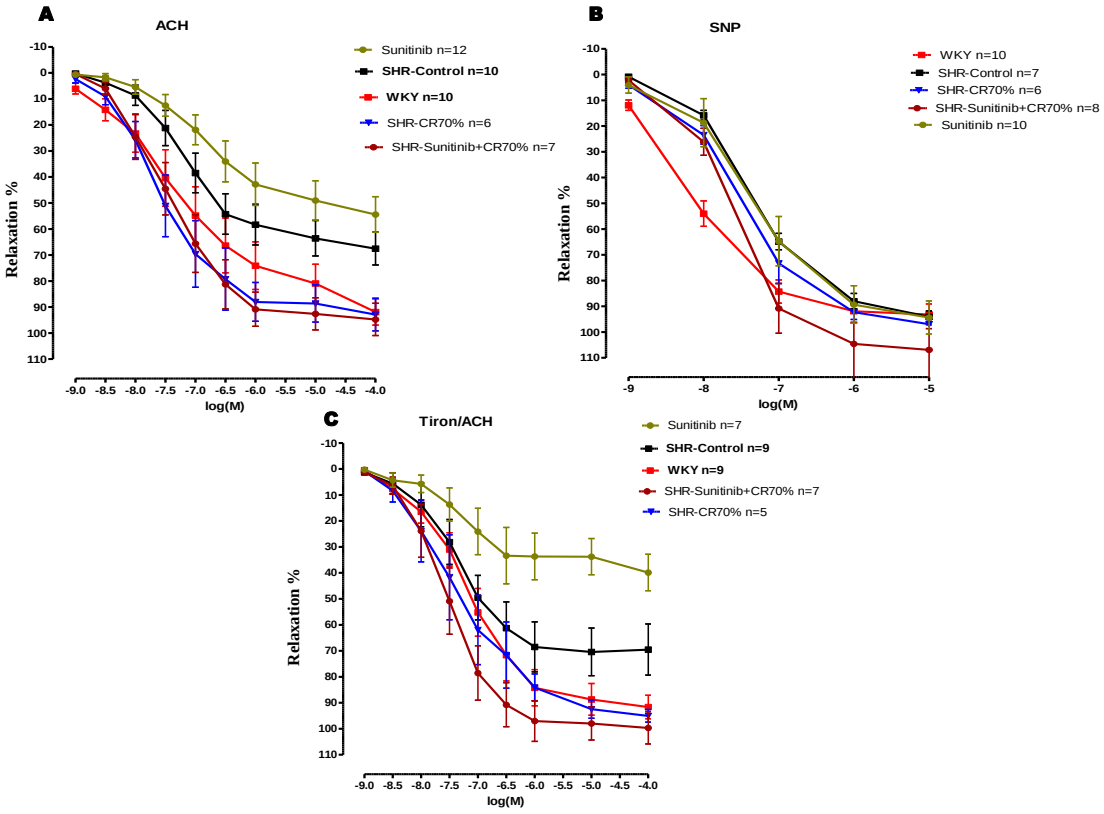


Fig. 2. **A** Effect of sunitinb and caloric restriction on endothelium-dependent relaxation stimulated by acetylcholine (Ach) in WKY and SHR rats. **B** Endothelium-independent vasodilator responses to sodium nitroprusside (SNP). On the X-axis is reported the SNP concentrations. **C** Effects of pretreatments with superoxide scavenger Tiron on endothelium-dependent dilatation stimulated by maximal dose of Ach.

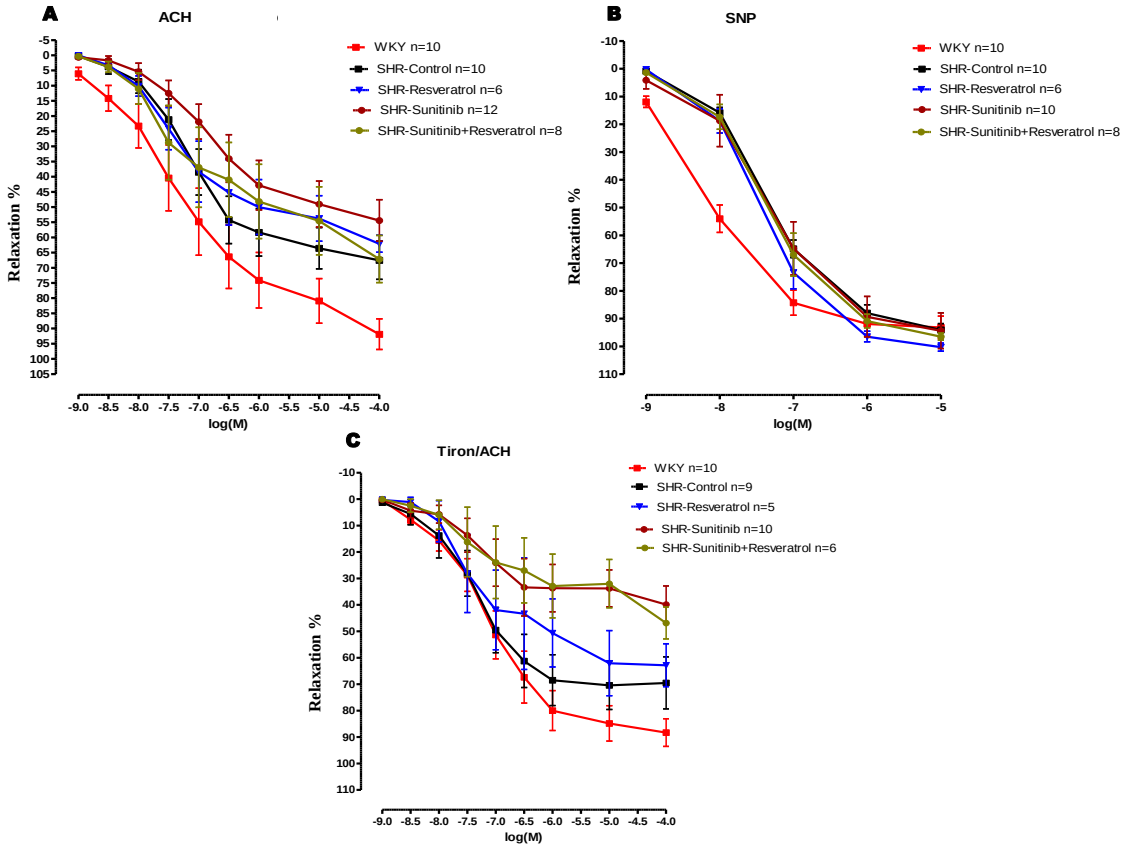


Fig. 3. **A** Effect of sunitinb and resveratrol on endothelium-dependent relaxation stimulated by acetylcholine (Ach) in WKY and SHR rats. **B** Endothelium-independent vasodilator responses to sodium nitroprusside (SNP). On the X-axis is reported the SNP concentrations **C** Effects of pretreatments with superoxide scavenger Tiron on endothelium-dependent dilatation stimulated by maximal dose of acetylcholine.

CARDIAC SIRT1 AND AMPK EXPRESSION/ACTIVATION BY WESTERN BLOT

Sunitinb treatment did not influence Sirt-1 expression in SHR rats in comparison to WKYs. It was not possible to observe any statistically significant difference between the groups treated with resveratrol and sunitinib, in comparison to the SHR control group, and between them. The caloric restriction regimen, in the sunitinib-treated group, increased the expression of Sirt-1 in comparision to the SHR *plus* sunitinib group (# p <0,05).

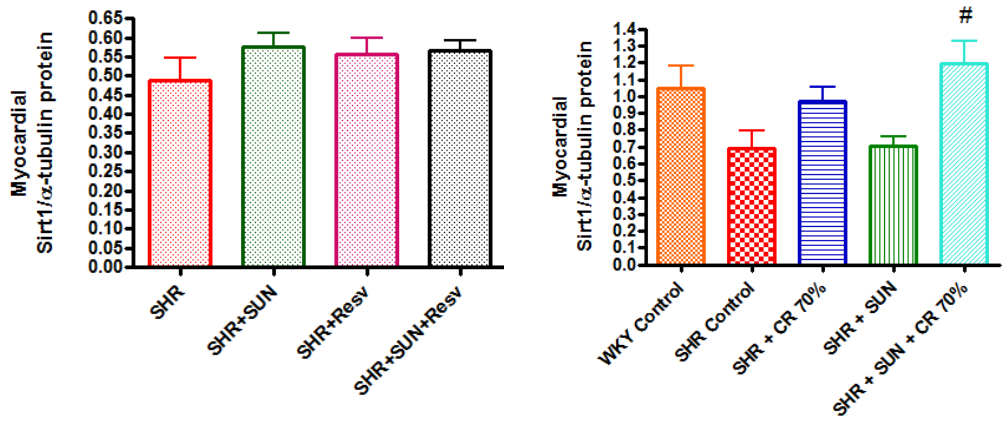


Fig. 4 SIRT-1 protein expression in WKY and SHR control, in SHR under caloric restriction and SHR treated with resveratrol and sunitinib. # $p<0.05$ in comparison to sunitinb treated SHR.

We also tested the effects of sunitinib and resveratrol on the activation of AMPK. The activation of AMPK is consistently decreased in SHR rats in comparison to WKY rats. Sunitinib did not affect the cardiac levels of phosphorylated AMPK. The resveratrol-induced increase was not statistically significant. The caloric restriction regimen did not influence AMPK activation, either in the SHR or in the sunitinib-treated rats.

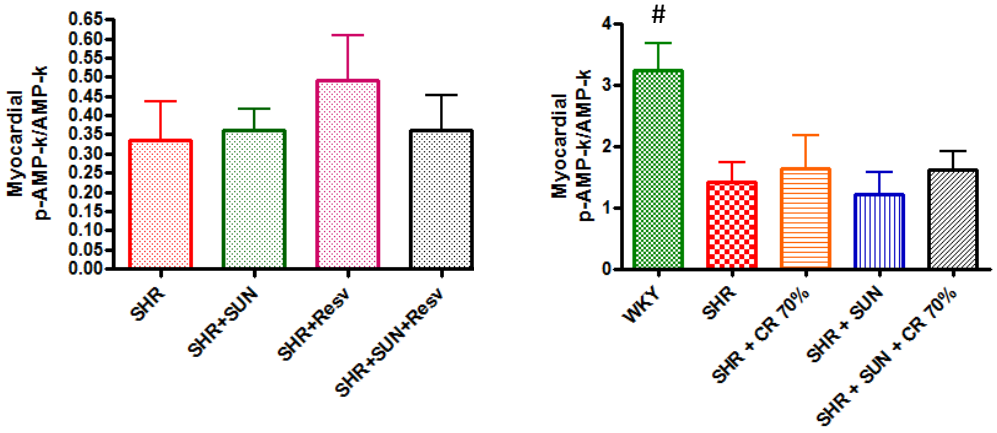


Fig. 5 AMPK activation in WKY and SHR control, in SHR under caloric restriction and in SHR treated with resveratrol and sunitinib. # $p<0.05$ in comparison to SHR.

IMMUNOHISTOCHEMISTRY

SHR rats presented a markedly inflammatory response in comparison to WKY rats. Sunitinib, at the administered dose, did not modify in a significant manner the inflammatory response - measured as monocyte/macrophage infiltration in the kidney - in comparison to the SHR control group. Neither resveratrol nor the caloric restriction

regimen were able to reduce the infiltration. The caloric restriction regimen, in sunitinib-treated rats, was able to significantly decrease the inflammatory response (# p<0,05 in comparison to sunitinib treated rats). The resveratrol *plus* sunitinib treatment produced a decrease in the monocyte/macrophage infiltration, but it was not statistically significant.

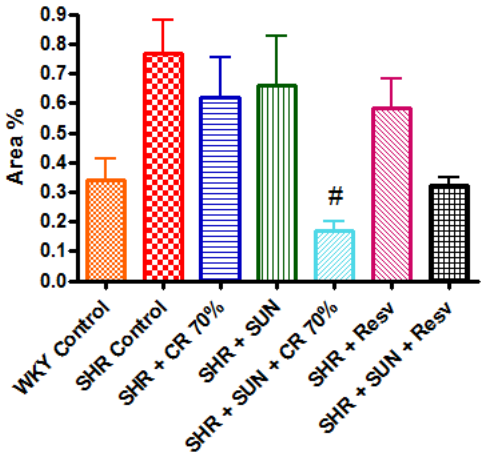


Fig. 6 Quantification of ED-1 positive labels in WKY an SHR control, in SHR under caloric restriction and in SHR treated with resveratrol and sunitinib. Values are expressed as the percentage of the immunopositive label per total surface area. # p<0.01in comparison to sunitinib-treated SHR.

EFFECTS OF RESVERATROL ON CARDIOVASCULAR PARAMETERS

In the SHR control group the heart-to-tibia-length ratio was significantly higher than in WKY controls; this difference was significantly reduced by the caloric restriction regimen. Treatment with resveratrol or sunitinib did not produce any statically significant change. In sunitinib-treated rats, the caloric restriction regimen was able to reduce the heart-to-tibia-length ratio in comparison with the group fed ad libitum.

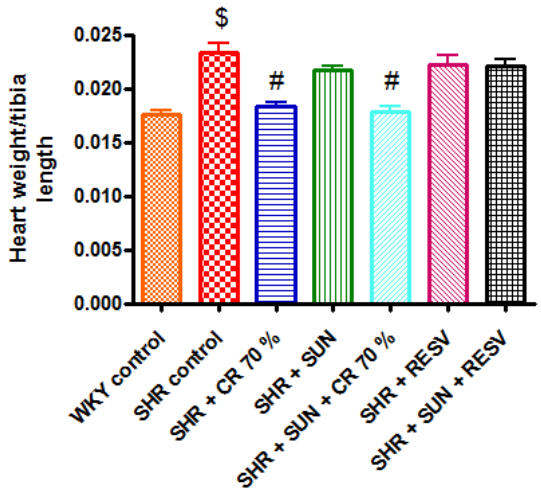
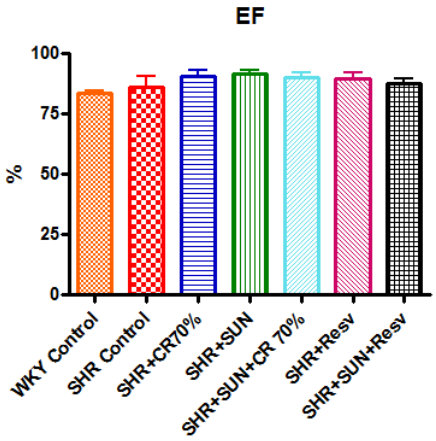


Fig. 7 Quantification of heart weight/tibia length ratio in WKY an SHR control, in SHR under caloric restriction and SHR treated with resveratrol and sunitinib. $ p<0,001 in comparison to WKY, # p<0,001 in comparison to SHR.

SHR rats did not develop systolic or diastolic dysfunction, as measured as ejection fraction (EF) and left ventricular fractional shortening (FS), and isovolumic relaxation time (IVRt), respectively. Neither systolic function nor diastolic function were statistically influenced by the treatments.



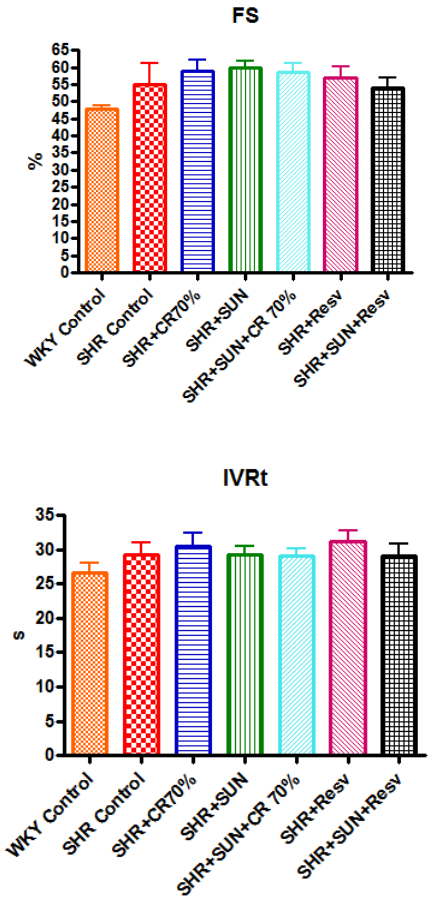


Fig. 8. Assessment of systolic and diastolic dysfunction in WKY and SHR control, in SHR under caloric restriction and in SHR treated with resveratrol and sunitinib. Ejection fraction (EF), left ventricular fractional shortening (FS), isovolumic relaxation time (IVRt)

Development of hypertrophy was present in SHRs, as characterized by increased thickness of the ventricular walls (interventricular septum and LV posterior wall) without change in LV internal dimension. The caloric restriction regimen was able to slightly reduce the interventricular septal wall thickness in sunitinib-treated rats. The other treatments did not affect these parameters of the cardiac structure.

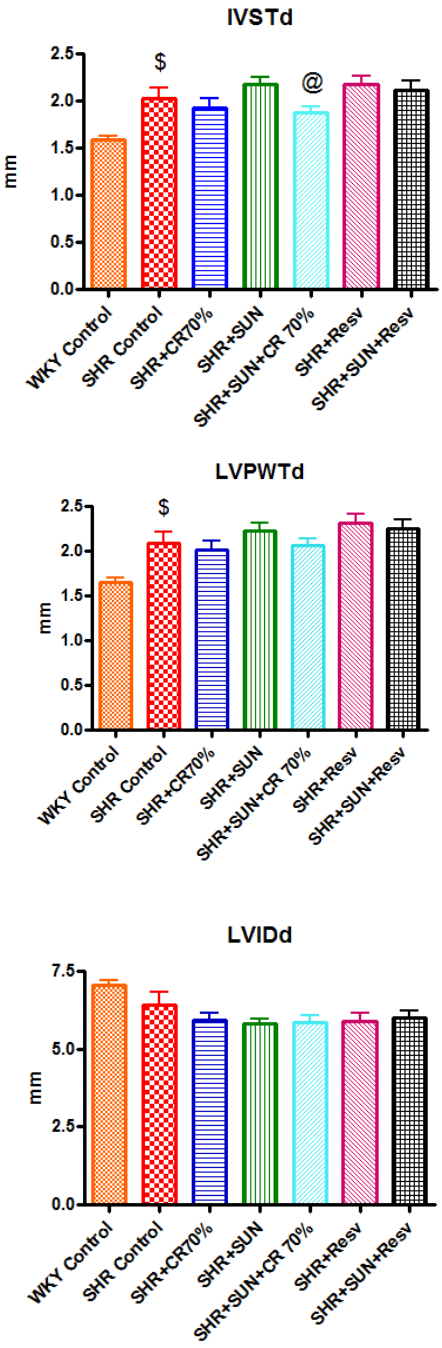


Fig. 9. Assessment of cardiac function in WKY and SHR control, in SHR under caloric restriction and in SHR treated with resveratrol and sunitinib. Interventricular septum wall thickness (IVSTd), LV posterior walls thickness (LVPWTd), LV internal dimension (LVIDd).

CONCLUSIONS

This was a preliminary study about combined effects of sunitinib and resveratrol in spontaneously hypertensive rats. To draw more significant conclusions, it will be necessary

to plan further experiments and some variables should be taken into account. In the literature contrasting data are reported about the age of onset of structural and functional changes in SHR rats: for Bell *et al*. (31), cardiac hypertrophy was increased in SHR at 16–20 weeks while Kokubo *et al*. (32) reported that "in SHR, cardiac hypertrophy develops by 4 weeks of age, and systolic and diastolic dysfunction is evident at 2 to 3 months" in comparison with the age-matched WKY. This discrepancy may be due to the biological variability that appears to exist between colonies, and the length of the treatments became an important factor to better appreciate the differences between WKY and SHR rats and between SHR and the rats under treatments. Other important variables are the dose of sunitinb, which may not have been sufficient for the full development of sunitinib-induced side effects, and the low bioavailability of resveratrol.

REFERENCES

1. Sinclair DA, Guarente L. Unlocking the secrets of longevity genes. Sci Am. 2006 Mar;294(3):48-51, 54-7.

2. Vaziri H, *et al*. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell 2001; 107: 149-59

3. Alcendor RR, *et al*. Sirt1 regulates aging and resistance to oxidative stress in the heart. Circ Res. 2007 May 25;100(10):1512-21

4. Michan S, Sinclair D. Sirtuins in mammals: insights into their biological function Biochem J. 2007 May 15;404(1):1-13

5. Lavu S, *et al*. Sirtuins-novel therapeutic targets to treat age-associated diseases. Nat Rev Drug Discov. 2008 Oct;7(10):841-53.

6. Finkel T, *et al*. Recent progress in the biology and physiology of sirtuins. Nature. 2009 Jul 30;460(7255):587-91.

7. Cardellini M. *et al*. TIMP3 is reduced in atherosclerotic plaques from subjects with type 2 diabetes and increased by SirT1. Diabetes 2009;58: 2396-2401

8. Mattagajasingh I. *et al*. SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. Proc Natl Acad Sci USA 2007; 104:

14855-60

9. Miyazaki R. *et al.* SIRT1, a longevity gene, downregulates angiotensin II type 1 receptor expression in vascular smooth muscle cells.Arterioscler Thromb Vasc Biol 2008; 28: 1263-9.].

10. Howitz K., *et al.* Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan Nature 2003 Sep 11;425(6954):191-6

11. Oliva J. *et al.* Sirt1 is involved in energy metabolism: the role of chronic ethanol feeding and resveratrol Exp Mol Pathol. 2008;85:155 – 159

12. Cohen HY, *et al.,* Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science. 2004 Jul 16;305(5682):390-2.

13. Dolinsky VW, Dyck JR. Calorie restriction and resveratrol in cardiovascular health and disease. Biochim Biophys Acta. 2011 Nov;1812(11):1477-89

14. Schmitt *et al.,* Effect of resveratrol on endothelial cell function: Molecular mechanisms Biofactors. 2010 Sep;36(5):342-9.

15. Camins A *et al.,* Sirtuin activators: designing molecules to extend life span Biochim Biophys Acta. 2010 Oct-Dec;1799(10-12):740-9.

16. Maengjo Kim, Tian R. Targeting AMPK for cardiac protection: opportunities and challenges. Journal of Molecular and Cellular Cardiology 51 (2011) 548–553

17. Ewart MA, Kennedy S. AMPK and vasculoprotection. Pharmacol Ther. 2011 Aug;131(2):242-53. Epub 2010 Nov 25.

18. Xu Q, Si LY. Protective effects of AMP-activated protein kinase in the cardiovascular system. J Cell Mol Med. 2010 Nov;14(11):2604-13

19. Baur JA, *et al.* Resveratrol improves health and survival of mice on a high-calorie diet. Nature. 2006 Nov 16;444(7117):337-42

20. Zang M, *et al.* Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice. Diabetes. 2006 Aug;55(8):2180-91

21. Dasgupta B, Milbrandt J. Resveratrol stimulates AMP kinase activity in neurons.

Proc Natl Acad Sci U S A. 2007 Apr 24;104(17):7217-22.

22. Park CE, *et al* Resveratrol stimulates glucose transport in C2C12 myotubes by activating AMP-activated protein kinase. Exp Mol Med. 2007 Apr 30;39(2):222-9

23. Hwang JT, *et al* Resveratrol protects ROS-induced cell death by activating AMPK in H9c2 cardiac muscle cells. Genes Nutr. 2008 Feb;2(4):323-6.

24. Mendel DB, *et al*. In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamics relationship. Clin Cancer Res 2003; 9: 327–37.

25. Orphanos GS, *et al*. Cardiotoxicity induced by tyrosine kinase inhibitors. Acta Oncol. 2009;48(7):964-70.

26. Chu TF, *et al*. Cardiotoxicity associated with tyrosine kinase inhibitor sunitinib Lancet. 2007 Dec 15;370(9604):2011-9

27. Monsuez JJ, *et al*. Cardiac side-effects of cancer chemotherapy Int J Cardiol. 2010 Sep 24;144(1):3-15. Epub 2010 Apr 18.

28. Kerkela R, *et al*. Sunitinib-induced cardiotoxicity is mediated by off-target inhibition of AMP-activated protein kinase. Clin Transl Sci. 2009 Feb;2(1):15-25.

29. Greineder CF, *et al*. Heart failure associated with sunitinib: lessons learned from animal models. Curr Hypertens Rep. 2011 Dec;13(6):436-41.

30. Di Lorenzo G, *et al*. Cardiovascular toxicity following sunitinib therapy in metastatic renal cell carcinoma: a multicenter analysis. Ann Oncol. 2009 Sep;20(9):1535-42

31. David Bell, *et al*. Temporal characteristics of cardiomyocyte hypertrophy in the spontaneously hypertensive rat. Cardiovasc Pathol. 2004 Mar-Apr;13(2):71-8.

32. Kokubo M, *et al* Noninvasive Evaluation of the Time Course of Change in Cardiac Function in Spontaneously Hypertensive Rats by Echocardiography Hypertens Res. 2005 Jul;28(7):601-9.

33. Haigis, M. C, Sinclair DA. Mammalian sirtuins: biological insights and disease

relevance Annu. Rev. Pathol. 2010, *5*, 253–295

34. Sangwoon Chung, *et al*. Regulation of SIRT1 in cellular functions: Role of polyphenols. Archives of Biochemistry and Biophysics 501 (2010) 79–90

Abbreviations

AMPK: AMP-activated protein kinase

AUC: Area Under Curve (in pharmacokinetics)

COX: Cyclooxygenase

DMSO: Dimethylsulfoxide

ESI: ElectroSpray Ionization

GLUT: Glucose transporter

HBSS: Hank's balanced saline solution

HEPES: N-(2-idroxyethyl)-piperazin-N'-2-ethansolfonic acid

HPLC: High Performance Liquid Chromatography

I/R: Ischemia/Riperfusion

MS: Mass Spectrometry

PEG: Polyethyleneglycol

Q: Quercetin

R: Resveratrol

SGLT: Sodium-dependent glucose cotransporters

SHR: Spontaneously Hypertensive Rats

SIRT-1: NAD-dependent deacetylase sirtuin-1

ROS: Reactive Oxygen Species

UGT: UDP-Glucuronosyltransferase

WKY: Wistar Kyoto Rats

Partecipation to congresses

- Mario Zoratti, Lucia Biasutto, Andrea Mattarei, Silvia Beltramello, Alice Bradaschia, Nicola Sassi, Ester Marotta, Spiridione Garbisa, Cristina Paradisi: Mitochondrially targeted polyphenols: antioxidants or chemotherapeutic drugs? *EMBO Workshop "Mitochondria, Apoptosis and Cancer: Targeting Mitochondria to defeat Cancer" Prague*, (Czech Republic), Oct. 1-3, 2009. Short Talk (MZ) ST9 (Abstract book, p.46).
- S. Garbisa, A. Cabrelle, V. Olivieri, A. Bradaschia, C. Gattazzo, S. Carraro, G. Binotto, F. Piazza, L. Biasutto, L. Trentin, *Hyperforin forces multiple myeloma cells to apoptosis and reduces their MMP-9 secretion.* 4th International Conference on Polyphenols and Health, Harrogate, UK, 7-11 dec. 2009. Abstracts, P75, p. 205
- Lucia Biasutto, Mario Zoratti, Andrea Mattarei, Alice Bradaschia, Silvia Beltramello, Nicola Sassi, Spiridione Garbisa: *"Developing prodrugs of polyphenols"*. 4th International Conference on Polyphenols and Health Harrogate, UK, 7-11 Dec. 2009 (Abstracts, P161, page 253).
- L. Biasutto, A. Mattarei, A. Bradaschia, S. Beltramello, M. Carraro, F. Bello, E. Marotta, S. Garbisa, C. Paradisi, M. Zoratti: "Prodrugs of resveratrol: carbamates. Resveratrol 2010-1st International Conference of Resveratrol and Health, Elsinore, Denmark, 13-15 Sept.2010
- L. Biasutto, A. Mattarei, A. Bradaschia, S. Beltramello, E. Marotta, S. Garbisa, C. Paradisi, M. Zoratti: "Prodrugs of polyphenols" WineHealth 2010 Rosazzo (UD), Italy, 3-6 october 2010 Abstracts, page 48 (oral communication).
- A. Mattarei, A. Bradaschia, L. Biasutto, E.G. Rodríguez Velo, E. Marotta, S. Garbisa, C. Paradisi, M. Zoratti: Acetal prodrugs of polyphenols5th International Conference on Polyphenols and Health. Sitges/Barcelona, Spain, Oct. 17-20, 2011. P85, Abstracts, p. 149