Absorption and Metabolism of Resveratrol Carboxyesters and Methanesulfonate by Explanted Rat Intestinal Segments

Lucia Biasutto^{1,2}, Ester Marotta², Andrea Mattarei², Silvia Beltramello¹, Paolo Caliceti³, Stefano Salmaso³, Andreas Bernkop-Schnürch⁴, Spiridione Garbisa¹, Mario Zoratti^{1,5} and Cristina Paradisi²

¹Department of Experimental Biomedical Sciences, University of Padova, ²Dept. of Chemical Sciences, University of Padova, ³Dept. of Pharmaceutical Sciences, University of Padova, ⁴Inst. of Pharmacy - Pharmaceutical Technology, University of Innsbruck, ⁵CNR Institute of Neuroscience, Padova

Key Words

Absorption • Antioxidants • Bioavailability • Metabolism • Polyphenols • Prodrugs

Abstract

Model prodrugs of resveratrol carrying protecting substituents at the hydroxyls have been synthesised and tested. Resveratrol triacetate and resveratrol-tri-mPEG₁₉₀₀ were formed by linking methyl groups or poly(ethylene glycol) chains, respectively, via carboxyester bonds. Resveratrol trimesylate, a molecule less susceptible to hydrolytic attack, was synthesised as well. This latter compound proved to be stable *in vitro*, while the carboxyester derivatives were slowly hydrolysed in solutions mimicking the gastric or intestinal environment, and rapidly converted to resveratrol in blood. In *ex vivo* permeation experiments with explanted intestinal segments, resveratrol and its triacetate derivative appeared in the basolateral compartment essentially as a mixture of Phase II metabolites. When the PEGylated derivative was provided on the apical side, unconjugated resveratrol accounted for about 50% of the compounds in the basolateral-side chamber. The same result was obtained by providing an equivalent physical mixture

of resveratrol and PEG polymer, indicating that this behaviour is likely due to an adjuvating effect of PEG rather than to the covalent polymer conjugation. These observations suggest that the ester derivatives are rapidly hydrolysed at the intestinal surface or inside enterocytes, and are then processed as resveratrol. On the other hand, the mesylate was transported from the apical to the basolateral side without modification. It may thus be possible to enhance absorption and hinder metabolism of natural polyphenols by constructing pro-drugs incorporating bonds with appropriate resistance to enzymatic hydrolysis.

Copyright © 2009 S. Karger AG, Basel

Introduction

Polyphenols are a huge family of natural compounds exhibiting a variety of biological activities. A vast literature documents effects of potential relevance for such major health-care endeavours as protection of the cardiovascular system [1], improving performance impaired by old age or neurodegeneration [2, 3], and prevention

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2009 S. Karger AG, Basel
1015-8987/09/0246-0557\$26.00/0

Accessible online at:
www.karger.com/cpb

Dr. Mario Zoratti
CNR Institute of Neuroscience
Viale G. Colombo, 3, 35131 Padova (Italy)
Tel. +39 049 8276054, Fax +39 049 8276049
E-Mail zoratti@bio.unipd.it

and therapy of cancer [4, 5]. Several biochemical pathways underlying these effects have been identified (e.g.: cardio- and neurovascular protection [5, 6]; neuro-protection and anti-aging [7, 8]). Studies dealing with the mechanisms of the bioactivity of resveratrol, the model polyphenol used in the present work, have been summarised in recent reviews [8-11]. The 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 [12] to cyclooxygenases [13]. Effects on gene expression are important [8, 14].

A few polyphenols display high affinity for proteins. For example, epigallocatechin gallate (EGCG) binds the laminin receptor with nM affinity [15]. In many other 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 μ M [16]. Resveratrol and other polyphenols inhibit I κ B kinase (IKK), thus reducing phosphorylation and degradation of I κ B and downregulating NF- κ B. IKK is half-inhibited at a concentration of resveratrol around 1 μ M [17]. Relatively poor affinities constitute an obstacle to the utilization of these compounds, since their bioavailability is notoriously low [18-20]. Polyphenols such as quercetin and resveratrol can diffuse into enterocytes as aglycones, as shown by experiments with cultured cells. *In vivo*, they do the same after being generated from naturally occurring glycosides by the activity of glycosidases such as Lactase Phloridzin Hydrolase and Broad Specificity Glucosidase. Some evidence has been presented that they can be also taken up as glycosides, mainly via hexose carriers, to be then hydrolysed in the cytoplasm of enterocytes ([21, 22] and references therein). There, polyphenols are 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) intervene on the molecules which have entered the circulation. Only low concentrations (nM- μ M) of polyphenols, mostly in the form of conjugates, are found in plasma and lymph even after a polyphenol-rich meal. At least two dozen different quercetin derivatives have been detected in human plasma [19] and resveratrol undergoes similar processing [23-25].

“Detoxification” by conversion into sulfates and glucuronides entails faster elimination by the renal and biliary routes. The study of the properties and activities

of metabolites is still in the initial phases (e.g.: [26-34]), but it is clear that conjugation also determines at least a partial loss of bioefficacy. Since metabolites are present at much higher concentrations than the parent molecule, in some cases they may actually be more relevant than the latter in terms of biomedical activity. While these aspects remain to be investigated, a reduction or delay of phase II metabolism would allow progress in the exploitation of natural polyphenols. Furthermore, new, synthetic polyphenols are beginning to be explored as drugs (for resveratrol-inspired ones see, e.g., [35-45]). They are certain to be affected by the same (or worse) bioavailability problems as the natural compounds.

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 reactive sites with removable groups. “Capping” the hydroxyls with chemical structures less prone to hydrogen bond formation is also expected to decrease the well-known tendency of these compounds to bind aspecifically to proteins, which decreases their effective concentration and slows their distribution in the organism.

We report here the synthesis of three prodrugs of resveratrol, *in vitro* stability studies and investigations of their absorption by explanted rat intestinal segments. Resveratrol was adopted as polyphenol model because of its remarkable biomedical properties, relative structural simplicity and stability. Among the various conceivable derivatives the peracetylated molecule was chosen as a representative carboxyester. Resveratrol triacetate has recently been reported to inhibit the proliferation of human colon adenocarcinoma cells [46]. With the intent of improving passage through the intestinal barrier we also produced a PEGylated derivative. “PEGylation” often leads to the improvement of drug absorption and pharmacokinetics [47-49]. PEG is non-toxic, non-antigenic and biocompatible, is rapidly eliminated from the body, is soluble both in water and many organic solvents and has pronounced solubilizing properties. It may also increase resistance to hydrolases and stability in the gastrointestinal tract. Also in this compound the polymeric chains were linked to the polyphenol kernel via carboxy ester bonding. To test an expectedly more stable type of chemical bond we also synthesised and tested per-methylsulfonated resveratrol. Little is known about the behaviour of sulfonates in biological systems; this group may be a potentially useful prodrug building block.

The various compounds were compared to resveratrol itself in experiments with explanted intestine segments to provide information on their relative efficiency

and metabolic transformations in the specific step of permeation of the intestinal wall. To our knowledge, none of the previous studies with polyphenol prodrugs has dealt with the effects of the modifications on permeation of the intestinal epithelium (as distinct from overall bioavailability or permeation of cultured cell monolayers). Experiments involving *ex vivo* intestinal tissue have however proven useful in assessing mechanistic aspects of polyphenol absorption [19, 50-52].

Materials and Methods

Materials and instrumentation

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. Carboxyl terminating monomethoxypoly(ethylene glycol) (mPEG-COOH) was synthesised in our laboratory according to the procedure reported elsewhere [53]. Briefly, 1.9 kDa PEG-OH was activated as p-nitrophenol ester and then reacted with glycine. PEG-COOH was purified by extraction in dichloromethane. Resveratrol metabolites (R-G and R-S) were synthesised as described in the literature [54], and used as standards. ^1H NMR and ^{13}C spectra were recorded with a Bruker AC 250F spectrometer. Chemical shifts (δ) are given in ppm relative to the signal of the solvent. TLCs were run on silica gel supported on plastic (Macherey-Nagel Polygram[®]SIL G/UV₂₅₄, silica thickness 0.2 mm), or on silica gel supported on glass (Fluka) (silica thickness 0.25 mm, granulometry 60Å, medium porosity) and visualised 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. 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-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.

Synthetic procedures

3,4',5-triacetyl-resveratrol (RTA). Acetic anhydride (1.5 mL, 14.7 mmol, 16.7 eq) was added to a solution of resveratrol (200 mg, 0.88 mmol) in dry pyridine (0.5 mL), and the mixture was stirred at room temperature. After 40 min, a white solid was precipitated with ice-cold water. The precipitate was then filtered to afford 233.6 mg of the desired product (75% yield). ^1H -NMR (250 MHz, DMSO- d_6) δ (ppm): 2.28 (m, 9H, OAc), 6.90 (t, 1H, H-4), 7.12-7.38 (m, 6H, H-3', H-5', H-2, H-6, H-7, H-8); 7.62 (d, 2H, H-2', H-6', J=8.8 Hz). ^{13}C -NMR (62.9 MHz, DMSO- d_6) δ (ppm): 20.75, 20.79, 114.93, 117.09, 122.14, 126.70, 127.63, 129.44, 134.16, 139.29, 150.16, 151.12, 168.93, 169.10. ESI-MS (ion trap): m/z 355, [M+H]⁺.

mPEG₁₉₀₀-resveratrol (RmPEG). DMAP (21.4 mg, 0.17 mmol, 2 eq), DCC (180.7 mg, 0.88 mmol, 10 eq) and resveratrol (20 mg, 0.09 mmol, previously dissolved in 100 μL DMF) were added to a solution of mPEG₁₉₀₀-COOH (1 g, 0.51 mmol, 5.8 eq) in CH_2Cl_2 (1 mL). The mixture was stirred at room temperature in the dark for 2 days. The suspension was then filtered and a white solid was precipitated from the liquid phase with diethyl ether (15 mL). The solvent was decanted and the precipitation procedure repeated 3 more times. The precipitate was then dried and purified by gel filtration (Sephadex G-25 superfine, GE Healthcare) using water as eluent to afford 407 mg of product (40% yield). HPLC-UV analysis (see below) showed no free resveratrol in the final product, which consisted of mono-, di- and tri-PEGylated resveratrol in an approximately 1:8:1 ratio. The overall PEG:resveratrol molar ratio was about 2 as determined through UV-Vis spectrometric analysis of 3 different solutions of known concentration (w/v) of the product. In these assays the resveratrol content in the solutions was determined by a calibration curve obtained by plotting the absorbance at 300 nm against resveratrol concentration. The PEG concentration was assessed by the iodine assay [55] using a calibration curve obtained by plotting the absorbance at 535 nm against PEG concentration in the range 2-5 $\mu\text{g/mL}$ ($y=0.0664x+0.001$, $R^2=0.99$).

3,4',5-trimesyl-resveratrol (RTM). Methanesulfonyl chloride (2 mL, 25.8 mmol, 5.9 eq) was added to a solution of resveratrol (1 g, 4.38 mmol, 1 eq) in dry pyridine (5 mL). After stirring 3 h at 100 °C, the mixture was diluted in EtOAc (150 mL) and washed with 1 M HCl (3 $\times$ 100 mL). The organic layer was dried over MgSO_4 and filtered. The solvent was finally evaporated under reduced pressure, and the residue purified by flash chromatography using 8:1:1 CH_2Cl_2 :petroleum ether:EtOAc v/v ratio as eluent, to afford 1.05 g of the desired product (88%, white solid). ^1H -NMR (250 MHz, DMSO- d_6) δ (ppm): 3.41 (s, 3H, OCH_3), 3.49 (s, 6H, OCH_3), 7.32-7.53 (m, 5H, H-4, H-3', H-5', H-7, H-8); 7.65 (d, 2H, H-2, H-6, J=2.5 Hz), 7.75 (d, 2H, H-2', H-6', J=7.5 Hz). ^{13}C -NMR (62.9 MHz, DMSO- d_6) δ (ppm): 37.39, 37.56, 115.53, 119.05, 122.56, 126.96, 128.32, 130.27, 135.45, 140.42, 148.74, 149.68. ESI-MS (ion trap): m/z 463, [M+H]⁺.

Solubility in water

Calibration curves were built by plotting absorbance at 320 nm vs concentration for seven standard resveratrol solutions in the 10^{-6} - 10^{-4} M range, prepared from a 10^{-3} M mother solution in CH_3CN by dilution with 9:1 water: CH_3CN v/v. Aqueous saturated solutions were prepared by vigorous stirring and sonication of 4, 2, 1, 0.5 or 0.25 mg of the compound in 1 mL of water. The resulting suspensions were then centrifuged (14000g, 30 min) and the clear supernatant was transferred into a cuvette and absorbance was measured at 320 nm. The concentration of aqueous saturated solutions of the derivatives was determined by interpolation ($N = 5$). The presence of significant amounts of colloidal particles could be excluded because similar absorbance readings were obtained starting with different amounts of undissolved material. The concentration of colloids would be expected to be a function of the latter parameter. Absorbance values remained similar (after correcting for dilution) also after the addition of 10% CH_3CN to the

final solution to insure solubilisation of all organic material.

Stability in gastric and intestinal environment

The chemical stability of the compounds was tested in aqueous media mimicking gastric (0.1 N HCl) and intestinal environment (0.1 M PBS buffer, pH 6.8). A 20 μ M solution of compounds, made diluting stock solutions in DMSO, was incubated at 37°C for 6 hours. Samples were withdrawn at different reaction times and analysed by HPLC-UV (see below).

Stability in blood

Heparinised, EDTA-supplemented blood samples (1 mL) were spiked with 5 μ M compound and incubated at 37°C for 1 hour. An internal standard was then added and the samples were extracted with organic solvents. The extracts were analysed by HPLC-UV (see below).

Permeation studies

Intestine was excised from 18 h fasted rats and transferred into a saline solution (154 mM NaCl in water) at 37°C. The jejunum was cut into 1 cm long strips, 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 (248 mM NaCl, 55.3 mM Glucose, 50 mM NaHCO₃, 9.9 mM KCl, 1.9 mM MgSO₄, 40 mM Hepes, pH 6.8), and incubated in a water bath at 37°C until all chambers were assembled. The buffer was then removed and substituted with 1 mL of a 20 μ M solution of the compound to be tested in the same buffer on the apical side (dilution from a 0.2 mM stock solution in DMSO, 10% final DMSO), and with 1 mL of fresh Hepes buffer plus 10% DMSO on the basolateral one. In the controls, only Hepes buffer plus 10% DMSO was present on both sides. This relatively high content of DMSO was necessary to insure solubility of the sulfonate derivative, and was thus used in all three cases to allow a proper comparison. During the experiment, oxygen was continuously bubbled in each basolateral compartment. An aliquot of the initial apical solutions was incubated separately at 37°C for the period of the experiment, to verify the stability of each compound in the absence of jejunum. At the end of the experiment (2.5 h) 800 μ L of chamber contents on both apical and basolateral sides were collected and mixed with 8 μ L of 100 mM ascorbic acid in water and 8 μ L of 6 M acetic acid. All samples were frozen and maintained at -20°C until HPLC-UV analysis as described below.

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.

HPLC-UV analysis

Samples (20 μ L) were analyzed using a reversed-phase column (Synergi-MAX, 4 μ m, 150 x 4.6 mm i.d.; Phenomenex). Solvents A and B were 0.1% TFA supplemented H₂O and CH₃CN, 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 monitored

Scheme 1. Synthesis of the resveratrol derivatives. Reaction conditions: a) (CH₃CO)₂O (16.7 eq.), pyridine, room temperature, 40 min; b) mPEG₁₉₀₀ (5.8 eq.), CH₂Cl₂, DCC, DMAP, room temperature, 72 h; c) CH₃SO₂Cl (5.9 eq.), pyridine, 100°C, 3h.

at 320 nm. Since absorption coefficients ϵ_{320} of resveratrol and resveratrol derivatives are very similar, for quantification purposes we assumed the same absorption coefficient ϵ_{320} of resveratrol also for hydrolysis products and resveratrol metabolites. Results were expressed as percentages, where the total amounts of recovered compounds represent 100%.

LC-ESI/MS analysis

Before LC-MS analysis, samples were further concentrated under vacuum (about 10 $\times$) and analyzed using the same column, solvents and gradient profile used for HPLC-UV analyses. 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 operating in full-scan mode in both positive and negative ion mode.

Statistics

Data are presented as means $\pm$ standard deviation. Experiments were performed at least in triplicate. Statistical data analyses were performed using Student's t-test with $p < 0.1$ as a minimal level of significance, using the software Origin 7.5 (OriginLab, Northampton, MA, USA).

Results

Syntheses

The new resveratrol derivatives RTA, RmPEG and RTM were synthesised from resveratrol as shown in Scheme 1. Details about synthetic procedures, product purification and characterisation are provided in the Experimental Section.

Fig. 1. Stability of resveratrol-derivatives in media mimicking A) gastric and B) intestinal environment (at 37°C). Data are expressed as molar % of the initial amount.

Solubility in water

The solubility in water of the new derivatives differs markedly depending on the protecting group. While both RTA and RTM turned out to be poorly soluble ($\leq 2 \mu\text{M}$), the PEGylated compound is highly soluble, and saturation was not reached even dissolving 400 mg in 1 mL water ($> 70 \text{ mM}$).

Stability in gastric and intestinal-like environment

All three compounds proved to be sufficiently stable over a 6 h period, both in gastric and intestinal environment-mimicking solutions (0.1 N HCl and 0.1 M PBS at pH 6.8, respectively). RTM was stable throughout the experimental period, while RTA and RmPEG underwent slow hydrolysis of the ester bonds, with up to 20% loss of the starting compound in 6 h (Fig.1). Over this period, RmPEG hydrolysis regenerated resveratrol, while RTA hydrolysis produced intermediate products (di- and mono-acetylated derivatives).

Stability in blood

Stability studies showed that complete hydrolysis of both resveratrol carboxyesters (RTA and RmPEG) occurred over 1 hour incubation in whole blood at

Fig. 2. Composition of the basolateral compartment after 2.5 h incubation with 20 μM apical compounds (see text for details). A) Total basolateral species. Data are expressed as percentage of the total amount recovered in the apical and basolateral compartments (mean $\pm$ standard deviation, N=4 for all compounds). B) Relative amounts of basolateral resveratrol metabolites (R-3-glucuronide and R-3-sulfate), resveratrol and intact loaded compound. Data are expressed as percentages considering the total amount recovered in the basolateral compartment as 100% (mean $\pm$ standard deviation, N=4 for all compounds).

37°C, with resveratrol being the unique final released product. The sulfonyl derivative RTM, on the contrary, was stable.

Intestinal wall permeability

The susceptibility to hydrolysis of the carboxyesters might constitute an advantage, allowing regeneration of the parent compound. However, rapid *in vivo* hydrolysis may reflect in metabolism during absorption. To investigate this point, the epithelial absorption of the compounds was examined in *ex vivo* intestinal wall permeation assays using segments of excised rat jejunum mounted in Ussing chambers. This experimental system allows the study of transepithelial transport separately from processes taking place *in vivo* in the intestinal lumen, blood, or liver.

The results show that the powerful hydrolytic and conjugation enzyme activities, namely esterases and glucuronyl and sulphate transferases, associated with the intestinal membrane have strong effects on the derivative stability and metabolism. RTA and RmPEG were completely hydrolysed within 2.5 hours. The process is certainly due to enzymatic activities, since only a small amount of hydrolysis products were formed upon parallel incubation of the derivatives under the same conditions but without the intestine. Analyses of the contents of the basolateral compartments (Fig. 2) confirmed that resveratrol (i.e. the reference compound) is mainly metabolised into glucuronosyl- and sulphate- derivatives during passage across the intestinal wall. RTA produced a similar mix of basolateral resveratrol derivatives, whereas the PEGylated derivative yielded a significant amount of non-metabolised resveratrol on the basolateral side. An analogous transfer of unmetabolised resveratrol was observed when the apical-side chamber was loaded with a physical resveratrol/mPEG₁₉₀₀ mixture at the same concentration of the two components as used in the conjugate experiment (Fig. 3). In contrast RTM crossed the intestine as such (Fig. 2).

Discussion

The resveratrol conjugates investigated in this study (acetylated, PEGylated and mesylated resveratrol) constitute a new set of derivatives intended to explore the feasibility of the pro-drug approach to improve polyphenol absorption and efficacy [56].

Bond stability represents a key parameter for the preparation of effective prodrugs. A desirable property of ester-based protecting groups is that they can be removed by esterases at the action site. For example, in acetylsalicylic acid (aspirin), in which the leaving group is formally a phenoxy anion, the ester bond is stable enough for the molecule to reach cyclooxygenase and inhibit the enzyme by transferring the acetyl group to a specific serine. The resistance of the ester to hydrolysis is, in general, expected to be an inverse function of the stability of the products, i.e. mainly of the phenoxide anion leaving group. Polyphenols in which the negative charge of the deprotonated form is more widely delocalised by resonance are expected to be better leaving groups, and their ester derivatives are expected to be more fragile.

Ester precursors intended to improve bioavailability and/or stability have been obtained with several drugs bearing alcoholic or phenolic hydroxyls and with a few

Fig. 3. HPLC chromatograms (320 nm) of the basolateral compartment after 2.5 h incubation with 20 µM of: a) resveratrol; b) RTA; c) RmPEG; d) RTM; e) R and mPEG₁₉₀₀.

polyphenols [57-61]. These derivatives have been tested so far mainly on cultured cells, but peracetylated EGCG administered by intraperitoneal injection has been reported to be more effective than EGCG itself against human prostate [60] and breast [62] cancers implanted in SCID mice. In our own work, quercetin aminoacyl derivatives showed an increased protection against metabolism by epithelial cell monolayers [56].

Among the several other chemical groups that may be considered for a protective role we tested a different class of esters, namely those of sulfonic acids, using the mesylate as the representative specie. It may be worthwhile to clarify that the type of derivatives we considered are unlikely to be toxic. Alkylsulfonates ($\text{RSO}_2\text{OCH}_2\text{R}'$) are known genotoxic agents: the methylsulfonate group can be easily displaced by a nucleophile (such as a DNA base) to produce RSO_3^- and the alkylated nucleophile. However, in the case of aromatic sulphonates, such as our resveratrol derivative, an analogous reaction cannot take place. Neither displacement of the mesylate group by nucleophilic attack at an aromatic carbon nor substitution at the S-linked carbon are expected to occur under the conditions prevailing in living organisms. The only type of reaction that one might expect is the desired one: (enzyme-catalysed) hydrolysis to regenerate the phenolic component and RSO_3H .

The PEGylated derivatives were designed to improve both solubility and stability of resveratrol and to enhance its oral bioavailability. PEGylation of low molecular weight molecules has indeed been developed to tune biopharmaceutical and pharmacokinetic properties of drugs whose performance, clinical efficacy, and market potential are substantially limited by their poor oral

bioavailability, solubility, half-life, or immediate clearance by first-pass metabolism [63]. Actually, small PEGs can easily penetrate the epithelial barrier and a few studies have demonstrated that conjugation of small PEGs yields derivatives with enhanced trans-membrane penetration properties [63-65]. Accordingly, 1.9 kDa PEG was activated to produce a reversible PEG-resveratrol ester prodrug suitable for oral delivery. The product had an average substitution ratio of two PEG polymers per resveratrol molecule, which represented a compromise between the requisite for hydroxyl protection and solubilisation on one side and bulkiness on the other. The presence of a fraction of monosubstituted resveratrol explains why free resveratrol was produced by the PEGylated product, but not by RTA, in stability tests (Fig. 1).

The experiments reported here show that rat intestine possesses powerful carboxyesterase and conjugation enzyme activity involved in resveratrol and resveratrol derivative metabolism. In 2.5 hours, 64 mm² of apical intestinal surface were able to convert 100% of the carboxyesters supplied (20 nmoles) to the unsubstituted parental resveratrol. This activity readily accounts for the similarity of the transepithelial transport data obtained with RTA and RmPEG, which may well have acted only as a source of resveratrol. A first conclusion is therefore that acetylated resveratrol derivatives (and presumably other polyphenols with extensive charge delocalisation) have little chance of constituting a significant improvement over the parent polyphenol if administered orally. Due to the limited stability in blood, these derivatives seem to be not suitable for intravenous administration either.

A further observation is that PEGylation enhances resveratrol bioavailability, but this effect can be obtained by co-administration of PEG and unmodified polyphenol at the luminal side, indicating that the polymer can by itself play a role in the biotransport of this compound. The PEG effect may be interpreted in terms of the influx/efflux mechanisms involved in the transport of the drug. Resveratrol is known to cross biomembranes either passively or by exploiting active transporters [66] and to undergo efflux transport mediated exocytosis involving MRP transporters located in intestinal enterocytes [67, 68]. Although PEG-resveratrol is expected to undergo passive transmembrane diffusion, the data obtained in the present study show that PEGylated resveratrol is at least partially hydrolysed to the parent molecule by esterases. Since PEG is known to interfere with a variety of efflux mechanisms involved in drug clearance, including MRP-mediated transport, it seems reasonable to conclude that the enhanced resveratrol permeation may

be due to MRP-mediated efflux inhibition by the polymer [69, 70].

Methanesulfonates join ethers as a potentially useful class of polyphenol derivatives capable of escaping early metabolism. Permethyl ethers of polyphenols have already been considered as possible prodrugs and pharmacological agents [71, 72]. Methanesulfonates may thus be useful in pathological contexts, leading to the creation of new molecules with their own, possibly advantageous, properties.

Our results indicate however that to develop prodrugs in the traditional sense it will be necessary to identify a type of protecting group based on a bond with considerable, but not indefinite, resistance to enzymatic hydrolysis *in vivo*. They thus identify the goals and directions of future studies in this sector. The bioavailability of new compounds being developed with the intention of building on the biomedical effectiveness of natural compounds ought to be tested at an early stage, and the stability of any prodrugs should be verified in a physiologically relevant environment. PEG chains may well be one of most promising substituents to be tested once the opportune bond type is identified.

Abbreviations

DCC (N,N'-DiCyclohexylCarbodiimide); DMAP (4-DiMethylAminoPyridine); EGCG (EpiGalloCatechinGallate); EtOAc (EthylAcetate); IκB (Inhibitor of (nuclear factor) κB); IKK (Inhibitor of κB Kinase); Hepes (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid); mPEG (monomethoxypoly(ethylene glycol)); MRP (Multiple drug Resistance Protein); NF-κB (Nuclear Factor κB); PBS (Phosphate-Buffered Saline); RmPEG (mPEG₁₉₀₀-resveratrol); RTA (3,4',5-triacetyl-resveratrol); RTM (3,4',5-trimesyl-resveratrol); R (resveratrol); R-G (Resveratrol-3-Glucuronide); R-S (Resveratrol-3-Sulfate); SCID (Severe Combined ImmunoDeficiency); SULT (Sulfotransferase); TFA (TriFluoroAcetic acid); UGT (UDP GlucuronosylTransferase).

Acknowledgements

This work was supported in part by grants from the Fondazione Cassa di Risparmio di Padova and Rovigo, the Italian Association for Cancer Research (AIRC), the Italian Foundation for Basic Research (FIRB) and the University of Padova (Progetti d'Ateneo 2008).

References

- 1 Leifert WR, Abeywardena MY: Cardioprotective action of grape polyphenols. *Nutr Res* 2008;28:729-737.
- 2 Spencer JP: Food for thought: the role of dietary flavonoids in enhancing human memory, learning and neuro-cognitive performance. *Proc Nutr Soc* 2008;67:238-252.
- 3 Singh M, Arseneault M, Sanderson T, Murthy V, Ramassamy C: Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. *J Agric Food Chem* 2008;56:4855-4873.
- 4 Ramos S: Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. *Mol Nutr Food Res* 2008;52:507-526.
- 5 Yang CS, Lambert JD, Sang S: Antioxidative and anti-carcinogenic activities of tea polyphenols. *Arch Toxicol* 2009;83:11-21.
- 6 Darra E, Shoji K, Mariotto S, Suzuki H: Protective effect of epigallocatechin-3-gallate on ischemia/reperfusion-induced injuries in the heart: STAT1 silencing flavonoid. *Genes Nutr* 2007;2:307-310.
- 7 Ramassamy C: Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of their intracellular targets. *Eur J Pharmacol* 2006;545:51-64.
- 8 Harikumar KB, Aggarwal BB: Resveratrol: a multitargeted agent for age-associated chronic diseases. *Cell Cycle* 2008;7:1020-1035.
- 9 Baur JA, Sinclair DA: Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov* 2006;5:493-506.
- 10 Pervaiz S, Holme AL: Resveratrol: Its Biological Targets and Functional Activity. *Antioxid Redox Signal* DOI: 10.1089/ARS.2008.2412.
- 11 Shakibaei M, Harikumar KB, Aggarwal BB: Resveratrol addiction: to die or not to die. *Mol Nutr Food Res* 2009;53:115-128.
- 12 Illek B, Fischer H: Flavonoids stimulate Cl conductance of human airway epithelium in vitro and in vivo. *Am J Physiol* 1998;275:L902-L910.
- 13 Szewczuk LM, Forti L, Stivala LA, Penning TM: 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* 2004;279:22727-22737.
- 14 Narayanan BA: Chemopreventive agents alters global gene expression pattern: predicting their mode of action and targets. *Curr Cancer Drug Targets* 2006;6:711-727.
- 15 Umeda D, Yano S, Yamada K, Tachibana H: Green tea polyphenol epigallocatechin-3-gallate signaling pathway through 67-kDa laminin receptor. *J Biol Chem* 2008;283:3050-3058.
- 16 Al-Fayez M, Cai H, Tunstall R, Steward WP, Gescher AJ: Differential modulation of cyclooxygenase-mediated prostaglandin production by the putative cancer chemopreventive flavonoids tricrin, apigenin and quercetin. *Cancer Chemother Pharmacol* 2006;58:816-825.
- 17 Kundu JK, Shin YK, Kim SH, Surh YJ: Resveratrol inhibits phorbol ester-induced expression of COX-2 and activation of NF-kappaB in mouse skin by blocking IkappaB kinase activity. *Carcinogenesis* 2006;27:1465-1474.
- 18 Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C, Williamson G: How should we assess the effects of exposure to dietary polyphenols in vitro? *Am J Clin Nutr* 2004;80:15-21.
- 19 Manach C, Williamson G, Morand C, Scalbert A, Rémésy C: Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* 2005;81(1 Suppl):230S-242S.
- 20 Yang CS, Sang S, Lambert JD, Lee MJ: Bioavailability issues in studying the health effects of plant polyphenolic compounds. *Mol Nutr Food Res* 2008;52 Suppl 1:S139-S151.
- 21 Walle T: Absorption and metabolism of flavonoids. *Free Rad Biol Med* 2004;36:829-837.
- 22 Passamonti S, Terdoslavich M, Franca R, Vanzo A, Tramer F, Braidot E, Petrusa E, Vianello A: Bioavailability of flavonoids: a review of their membrane transport and the function of bilitranslocase in animal and plant organs. *Curr Drug Metab* 2009;10:369-394.
- 23 Wenzel E, Somoza V: Metabolism and bioavailability of trans-resveratrol. *Mol Nutr Food Res* 2005;49:472-481.
- 24 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.
- 25 Burkon A, Somoza V: Quantification of free and protein-bound trans-resveratrol metabolites and identification of trans-resveratrol-C/O-conjugated diglucuronides - two novel resveratrol metabolites in human plasma. *Mol Nutr Food Res* 2008;52:549-557.
- 26 Day AJ, Bao Y, Morgan MR, Williamson G: Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radic Biol Med* 2000;29:1234-1243.
- 27 de Pascual-Teresa S, Johnston KL, DuPont MS, O'Leary KA, Needs PW, Morgan LM, Clifford MN, Bao Y, Williamson G: Quercetin metabolites downregulate cyclooxygenase-2 transcription in human lymphocytes ex vivo but not in vivo. *J Nutr* 2004;134:552-557.
- 28 Mochizuki M, Kajiji K, Terao J, Kaji K, Kumazawa S, Nakayama T, Shimoi K: Effect of quercetin conjugates on vascular permeability and expression of adhesion molecules. *Biofactors* 2004;22:201-204.
- 29 Donnini S, Finetti F, Lusini L, Morbidelli L, Cheynier V, Barron D, Williamson G, Waltenberger J, Ziche M: Divergent effects of quercetin conjugates on angiogenesis. *Br J Nutr* 2006;95:1016-1023.
- 30 Lambert JD, Sang S, Yang CS: Biotransformation of green tea polyphenols and the biological activities of those metabolites. *Mol Pharm* 2007;4:819-825.
- 31 Lodi F, Jiménez R, Menendez C, Needs PW, Duarte J, Perez-Vizcaino F: Glucuronidated metabolites of the flavonoid quercetin do not auto-oxidise, do not generate free radicals and do not decrease nitric oxide bioavailability. *Planta Med* 2008;74:741-746.
- 32 Loke WM, Proudfoot JM, Stewart S, McKinley AJ, Needs PW, Kroon PA, Hodgson JM, Croft KD: Metabolic transformation has a profound effect on anti-inflammatory activity of flavonoids such as quercetin: lack of association between antioxidant and lipoxigenase inhibitory activity. *Biochem Pharmacol* 2008;75:1045-1053.
- 33 Murias M, Miksits M, Aust S, Spatzenegger M, Thalhammer T, Szekeres T, Jäger W: Metabolism of resveratrol in breast cancer cell lines: impact of sulfotransferase 1A1 expression on cell growth inhibition. *Cancer Lett* 2008;261:172-182.
- 34 Winterbone MS, Tribolo S, Needs PW, Kroon PA, Hughes DA: Physiologically relevant metabolites of quercetin have no effect on adhesion molecule or chemokine expression in human vascular smooth muscle cells. *Atherosclerosis* 2009;202:431-438.
- 35 Murias M, Handler N, Erker T, Pleban K, Ecker G, Saiko P, Szekeres T, Jäger W: Resveratrol analogues as selective cyclooxygenase-2 inhibitors: synthesis and structure-activity relationship. *Bioorg Med Chem* 2004;12:5571-5578.

- 36 Murias M, Jäger W, Handler N, Erker T, Horvath Z, Szekeres T, Nohl H, Gille L: Antioxidant, prooxidant and cytotoxic activity of hydroxylated resveratrol analogues: structure-activity relationship. *Biochem Pharmacol* 2005;69:903-912.
- 37 Minutolo F, Sala G, Bagnacani A, Bertini S, Carboni I, Placanica G, Protà G, Rapposelli S, Sacchi N, Macchia M, Ghidoni R: Synthesis of a resveratrol analogue with high ceramide-mediated proapoptotic activity on human breast cancer cells. *J Med Chem* 2005;48:6783-6786.
- 38 Murias M, Luczak MW, Niepsuj A, Krajka-Kuzniak V, Zielinska-Przyjemska M, Jagodzinski PP, Jäger W, Szekeres T, Jodynis-Liebert J: 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* 2008;22:1361-1370.
- 39 Lee KW, Kang NJ, Rogozin EA, Oh SM, Heo YS, Pugliese A, Bode AM, Lee HJ, Dong Z: The resveratrol analogue 3,5,3',4',5'-pentahydroxy-trans-stilbene inhibits cell transformation via MEK. *Int J Cancer* 2008;123:2487-2496.
- 40 Meng XL, Yang JY, Chen GL, Zhang LJ, Wang LH, Li J, Wang JM, Wu CF: RV09, a novel resveratrol analogue, inhibits NO and TNF-alpha production by LPS-activated microglia. *Int Immunopharmacol* 2008;8:1074-1082.
- 41 Gossiau A, Pabbaraja S, Knapp S, Chen KY: Trans- and cis-stilbene polyphenols induced rapid perinuclear mitochondrial clustering and p53-independent apoptosis in cancer cells but not normal cells. *Eur J Pharmacol* 2008;587:25-34.
- 42 Bernhaus A, Ozsvar-Kozma M, Saiko P, Jaschke M, Lackner A, Grusch M, Horvath Z, Madlener S, Krupitza G, Handler N, Erker T, Jaeger W, Fritzer-Szekeres M, Szekeres T: Antitumor effects of K1TC, a new resveratrol derivative, in AsPC-1 and BxPC-3 human pancreatic carcinoma cells. *Invest New Drugs* 2009; in press (DOI: [10.1007/s10637-008-9183-7](https://doi.org/10.1007/s10637-008-9183-7)).
- 43 Jung JC, Lim E, Lee Y, Kang JM, Kim H, Jang S, Oh S, Jung M: Synthesis of novel trans-stilbene derivatives and evaluation of their potent antioxidant and neuroprotective effects. *Eur J Med Chem* 2009;44:3166-3174.
- 44 Fan GJ, Liu XD, Qian YP, Shang YJ, Li XZ, Dai F, Fang JG, Jin XL, Zhou B: 4,4'-Dihydroxy-trans-stilbene, a resveratrol analogue, exhibited enhanced antioxidant activity and cytotoxicity. *Bioorg Med Chem* 2009;17:2360-2365.
- 45 Guha P, Dey A, Sarkar B, Dhyani MV, Chattopadhyay S, Bandyopadhyay SK: Improved antiulcer and anticancer properties of a trans-resveratrol analog in mice. *J Pharmacol Exp Ther* 2009;328:829-838.
- 46 Marel AK, Lizard G, Izard JC, Latruffe N, Delmas D: Inhibitory effects of trans-resveratrol analogs molecules on the proliferation and the cell cycle progression of human colon tumoral cells. *Mol Nutr Food Res* 2008;52:538-548.
- 47 Veronese FM, Mero A: The impact of PEGylation on biological therapies. *BioDrugs* 2008;22:315-329.
- 48 Jain A, Jain SK: PEGylation: an approach for drug delivery. *A review. Crit Rev Ther Drug Carrier Syst* 2008;25:403-447.
- 49 Bailon P, Won CY: PEG-modified biopharmaceuticals. *Expert Opin Drug Deliv* 2009;6:1-16.
- 50 Matsumoto M, Matsukawa N, Mineo H, Chiji H, Hara H: A soluble flavonoid-glycoside, alphaG-rutin, is absorbed as glycosides in the isolated gastric and intestinal mucosa. *Biosci Biotechnol Biochem* 2004;68:1929-1934.
- 51 Day AJ, Gee JM, DuPont MS, Johnson IT, Williamson G: Absorption of quercetin-3-glucoside and quercetin-4'-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. *Biochem Pharmacol* 2003;65:1199-1206.
- 52 Gee JM, DuPont MS, Day AJ, Plumb GW, Williamson G, Johnson IT: Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *J Nutr* 2000;130:2765-2771.
- 53 Sartore L, Caliceti P, Schiavon O, Veronese FM: Enzyme modification by MPEG with an amino acid or peptide as spacer arms. *Appl Biochem Biotechnol* 1991;27:45-54.
- 54 Wenzel E, Soldo T, Erbersdobler H, Somoza V: Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. *Mol Nutr Food Res* 2005;49:482-494.
- 55 Sims GE, Snape TJ: A method for estimation of poly(ethylene glycol) in plasma protein fractions. *Anal Biochem* 1980;107:60-63.
- 56 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.
- 57 Mulholland PJ, Ferry DR, Anderson D, Hussain SA, Young AM, Cook JE, Hodgkin E, Seymour LW, Kerr DJ: Pre-clinical and clinical study of QC12, a water-soluble, pro-drug of quercetin. *Ann Oncol* 2001;12:245-248.
- 58 Montenegro L, Carbone C, Maniscalco C, Lambusta D, Nicolosi G, Ventura CA, Puglisi G: In vitro evaluation of quercetin-3-O-acyl esters as topical prodrugs. *Int J Pharm* 2007;336:257-262.
- 59 Kuhn D, Lam WH, Kazi A, Daniel KG, Song S, Chow LM, Chan TH, Dou QP: Synthetic peracetate tea polyphenols as potent proteasome inhibitors and apoptosis inducers in human cancer cells. *Front Biosci* 2005;10:1010-1023.
- 60 Lee SC, Chan WK, Lee TW, Lam WH, Wang X, Chan TH, Wong YC: Effect of a prodrug of the green tea polyphenol (-)-epigallocatechin-3-gallate on the growth of androgen-independent prostate cancer in vivo. *Nutr Cancer* 2008;60:483-491.
- 61 Hirpara KV, Aggarwal P, Mukherjee AJ, Joshi N, Burman AC: Quercetin and its derivatives: synthesis, pharmacological uses with special emphasis on anti-tumor properties and prodrug with enhanced bio-availability. *Anticancer Agents Med Chem* 2009;9:138-161.
- 62 Landis-Piowar KR, Huo C, Chen D, Milacic V, Shi G, Chan TH, Dou QP: A novel prodrug of the green tea polyphenol (-)-epigallocatechin-3-gallate as a potential anticancer agent. *Cancer Res* 2007;67:4303-4310.
- 63 Riley T, Riggs-Sauthier J: The Benefits and Challenges of PEGylating Small Molecules. *Pharm Tech* 2008;32:88-94.
- 64 Gursahani H, Riggs-Sauthier J, Pfeiffer J, Lechuga-Ballesteros D, Fishburn CS: Absorption of polyethylene glycol (PEG) polymers. The effect of PEG size on permeability. *J Pharm Sci* 2009;98:2957-2856.
- 65 Ashada H, Douen T, Mizokoshi Y, Fujita T, Murakami M, Yamamoto A, Muranishi S: Absorption characteristics of chemically modified insulin derivatives with various fatty acids in the small and large intestine. *J Pharm Sci* 1995;84:682-687.
- 66 Lançon A, Delma D, Osman H, Thénot JP, Jannin B, Latruffe N: Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochem Biophys Res Comm* 2004;316:1132-1137.
- 67 Kaldas MI, Walle UK, Walle T: Resveratrol transport and metabolism by human intestinal Caco-2 cells. *J Pharm Pharmacol* 2003;55:307-312.
- 68 Henry C, Vitrac X, Decendit A, Ennamany R, Krisa S, Mérillon JM: Cellular uptake and efflux of trans-piceid and its aglycone trans-resveratrol on the apical membrane of human intestinal Caco-2 cells. *J Agric Food Chem* 2005;53:798-803.

- 69 Torres-Lugo M, Santos-Román N, Méndez J, Licha M: Poly(Ethylene Glycol) Hydrogels as Possible Multidrug Resistance Associated Protein (MRP) Inhibitors. Proceedings of the 29th Annual International Conference of the IEEE EMBS Cité Internationale, Lyon, France, August 23-26, 2007.
- 70 Werle M: Polymeric and Low Molecular Mass Efflux Pump Inhibitors for Oral Drug Delivery. *J Pharm Sci* 2008;97:60-70.
- 71 Walle T, Wen X, Walle UK: Improving metabolic stability of cancer chemoprotective polyphenols. *Expert Opin Drug Metab Toxicol* 2007;3:379-388.
- 72 Pan MH, Gao JH, Lai CS, Wang YJ, Chen WM, Lo CY, Wang M, Dushenkov S, Ho CT: Antitumor activity of 3,5,4'-trimethoxystilbene in COLO 205 cells and xenografts in SCID mice. *Mol Carcinog* 2008;47:184-196.