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CICLO XXI

COME SFRUTTARE IL POTENZIALE FARMACOLOGICO DEI POLIFENOLI VEGETALI: MODIFICAZIONI REVERSIBILI PER AUMENTARNE LA BIODISPONIBILITA'/BIOEFFICACIA

HOW TO EXPLOIT THE PHARMACOLOGIC POTENTIAL OF PLANT POLYPHENOLS: REVERSIBLE MODIFICATIONS TO INCREASE THEIR BIOAVAILABILITY/BIOEFFICACY

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Contents

Partecipation to congresses and graduated courses attended

Riassunto dell'attività svolta

Il progetto di ricerca a cui ho lavorato durante il corso di dottorato intende aprire la strada alla sfruttamento farmacologico dei polifenoli vegetali, una vasta famiglia di composti naturali presenti in molti cibi e bevande. Una grande quantità di dati dimostra che molti di essi hanno proprietà biologiche degne di nota, che potrebbero essere utili ad esempio per prevenire l'insorgere e inibire la crescita di molti tipi di cancro, per proteggere il sistema cardiovascolare o ancora per rallentare l'invecchiamento. Queste potenzialità trovano però un grosso ostacolo nella loro esplicazione a causa della scarsa biodisponibilità di questi composti. Come risultato del loro scarso assorbimento e della cospicua metabolizzazione/degradazione a livello intestinale ed epatico, solo piccole quantità di composto entrano nel circolo sanguigno, e per lo più sotto forma di metaboliti.

La prima parte del mio lavoro di dottorato ha avuto come scopo quello di aggirare la barriera della bassa biodisponibilità attraverso lo sviluppo di "precursori" di polifenoli, più resistenti alla metabolizzazione durante l'assorbimento e in grado di rigenerare il composto naturale grazie all'azione di enzimi ubiquitari. Sono stati scelti come polifenoli modello la quercetina (3,3',4',5,7-pentaidrossiflavone) e il resveratrolo (3,4',5-triidrossi-*t*-stilbene), entrambi ampiamente studiati e dotati di interessanti proprietà. Poichè le principali modificazioni metaboliche avvengono sugli ossidrili, e poiché enzimi con attività esterasica sono praticamente ubiquitari, è sembrato logico iniziare questo lavoro sviluppando dei derivati esterei.

Ho quindi sintetizzato una serie di precursori della quercetina, la maggior parte dei quali comprendeva una funzionalità amminoacidica. Ho studiato il trasporto/diffusione di questi derivati attraverso monostrati supportati di cellule epiteliali (MDCK-1, MDCK-2, Caco-2), un modello ampiamente utilizzato per ricerche di questo tipo. In questo sistema sperimentale i precursori esterei hanno dimostrato una maggiore resistenza al metabolismo rispetto alla quercetina tal quale (Biasutto, L. et al. (2007), J. Med. Chem., 50, 241-253 - Cap. 1).

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), Cell. Physiol. Biochem., in press - Cap. 2).

Per studiare la biodisponibilità dei precursori sintetizzati abbiamo però preferito saggiarne direttamente il comportamento *in vivo* attraverso studi di farmacocinetica nel ratto. A tale scopo, è stato necessario mettere a punto innanzitutto un protocollo efficace per l'estrazione dei polifenoli di interesse (quercetina e resveratrolo) dal sangue. Questo lavoro ha messo in evidenza l'opportunità di eseguire l'analisi con campioni di sangue intero anzichè sul plasma sanguigno come viene generalmente fatto, per evitare sottostime dovute all'associazione dei polifenoli con la frazione cellulare (Biasutto, L., et al., Anal. Chem., submitted - Cap. 3).

Forti dei primi risultati ottenuti con i precursori della quercetina, siamo passati a sintetizzare dei derivati esterei del resveratrolo, un polifenolo con interessanti proprietà, e dotato di una struttura chimica che evita molte difficoltà tecniche incontrate in precedenza con la quercetina (ad esempio, instabilità dei derivati a causa della presenza degli ossidrili catecolici, problemi nel sintetizzare derivati penta-sostituiti con gruppi stericamente ingombrati, suscettibilità all' ossidazione). Poiché i polifenoli sono tutti scarsamente 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 su questo composto sono ancora in corso, ma i dati disponibili 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 (Cap. 4).

Sono stati inoltre sintetizzati altri precursori del resveratrolo: due derivati esterei (con gruppi metile o polietilenglicole), e altri due meno suscettibili all'idrolisi (metil-etere e mesilato). I risultati ottenuti dagli studi di stabilità in sangue, di assorbimento con intestino *ex vivo* e di farmacocinetica hanno evidenziato l'eccessiva instabilità dei legami esterei in questi contesti biologici, che li rende poco adatti per una loro applicazione *in vivo* (Cap. 5).

Per meglio identificare e quantificare i metaboliti osservati durante gli studi di farmacocinetica e per indagare il possibile ruolo biologico dei metaboliti stessi, sono stati sintetizzati alcuni fra i più abbondanti metaboliti del resveratrolo (mono- e di-solfati, mono-glucuronidi) (Cap. 6).

Una seconda parte del progetto è nata dalla considerazione che l'azione dei polifenoli può essere esplicata/potenziata non solo cercando di innalzare il loro livello sistemico, ma anche accumulandoli specificamente in opportuni siti di azione.

I polifenoli sono infatti molecole redox-attive, e i radicali sono coinvolti in molte patologie. Sito principale di produzione dei radicali sono i mitocondri, che sono inoltre la sede di eventi chiave nella morte cellulare sia per apoptosi che per necrosi. Importante è stata anche l'osservazione (con esperimenti di patch clamp e di swelling con mitocondri isolati di fegato di ratto) che la quercetina è in grado di inibire o indurre l'apertura del poro di transizione di permeabilità mitocondriale, a seconda che prevalga la sua attività anti- o pro-ossidante (il che dipende da una serie di fattori tra cui l'abbondanza di ioni metallici e di enzimi redox, il pH, la concentrazione stessa di polifenolo, ecc.) (De Marchi, U., et al., Free Rad. Biol. Med., submitted - Cap. 7).

Sono stati quindi sintetizzati dei derivati di quercetina e resveratrolo in grado di accumularsi nei mitocondri grazie alla funzionalizzazione con un gruppo trifenilfosfonio (TPP^+), un catione in grado di diffondere attraverso le membrane biologiche e di accumularsi in regioni a potenziale negativo, quali la matrice mitocondriale. Questi composti si sono comportati come previsto in saggi *in vitro*. (Mattarei, A., et al, (2008), Chembiochem, 9, 2633-2642 - Cap. 8; Biasutto, L., et al. (2008), Bioorg. Med. Chem. Lett., 18, 5594-5597 – Cap. 9; Cap. 10).

L'assenza all'interno dalla matrice mitocondriale di enzimi metabolici (quali solfotrasferasi o glucuronil-transferasi), si sta rivelando inoltre una caratteristica potenzialmente molto utile, da sfruttare per proteggere i polifenoli e farmaci in genere da metabolizzazione (Cap. 11).

Le possibili applicazioni in campo biomedico dei derivati "mitocondriotropici" possono essere interessanti sia che prevalga una loro azione anti-ossidante/citoprotettiva che pro-ossidante/citotossica.

Una prima indagine su quale sia l'effetto biologico di questi nuovi composti è stata condotta con dei derivati della quercetina (3-QBTPI, 3-QTABTPI) e mitocondri isolati; come atteso in base alle osservazioni fatte in precedenza con la quercetina, questi composti si sono rivelati potenziali co-induttori della transizione di permeabilità mitocondriale, nonché inibitori della respirazione e dell'ATP sintasi mitocondriale (Cap. 12).

E' stato quindi condotto uno studio preliminare per saggiare la possibile azione citostatica/citotossica dei derivati mitocondriotropici e di alcuni altri costrutti recanti una catena alchilica, prodotti nel corso del progetto, su colture cellulari tumorali (C-26) e non

(MEF). Da questi esperimenti è emerso come i derivati mitocondriotropici e un'alchil quercetina abbiano una rilevante attività citotossica/citostatica su cellule a crescita veloce, mentre il loro effetto su cellule non tumorali è trascurabile se esse hanno crescita lenta (Cap. 13). Questo comportamento è tipico degli agenti chemioterapici, e andrà approfondito con studi *in vivo* per verificare l'effetto dei derivati su crescita e metastatizzazione di tumori C-26 inoculati in topi.

Prima di intraprendere questi studi sarà necessario indagare sull'assorbimento intestinale, il profilo farmacocinetico e la tossicità *in vivo* dei derivati da utilizzare. E' stata verificata per ora l'efficienza di estrazione dei composti mitocondriotropici da sangue con il metodo attualmente in uso (Cap. 14).

Summary

The research project I have been engaged in during my graduate studies is meant to open the way to the pharmacological exploitation of plant polyphenols, 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, potentially useful - for example - to prevent the onset and to inhibit the growth of several types of cancer, to protect the cardiovascular system or to slow down senescence. Applications such as these are however severely hindered by the low bioavailability of these compounds. As a result of a low level of absorption and of a rapid metabolism/degradation in the intestinal and hepatic compartments only small amounts of polyphenols are found in the bloodstream, and then mostly as metabolites.

The first part of my doctorate work was aimed at circumventing the obstacle posed by low bioavailability through the development of "pro-drugs" of polyphenols, resistant to metabolism during absorption and capable of regenerating the natural compound thanks to the action of ubiquitous enzymes. Quercetin (3,3',4',5,7-pentahydroxyflavone) and resveratrol (3,4',5-trihydroxystilbene), both much studied molecules with interesting properties, were chosen as model polyphenols for our proof-of-principle project. Since 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.

I have thus synthesised a series of quercetin precursors, most of which comprised an aminoacidic functionality. I have studied the transport/diffusion of these derivatives across supported monolayers of epithelial cells (MDCK-1, MDCK-2, Caco-2), a popular model for this type of research. In this experimental system the ester precursors turned out to be more resistant to metabolic conjugation than quercetin as such (Biasutto, L. et al. (2007), J. Med. Chem., 50, 241-253 - Chapt. 1).

The studies on absorption/metabolism 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 xenobiotic compound (in our case quercetin) (Biasutto, L., et al. (2009), Cell. Physiol. Biochem., in press - Chapt. 2).

To assess the bioavailability of the precursors we decided to test them *in vivo* performing pharmacokinetic determinations in the rat. To this end, it was first necessary to set up a protocol for the quantitative extraction and analysis of quercetin and resveratrol from blood. It soon became evident that it is more appropriate to analyse samples of whole blood, rather than blood plasma as generally done, to avoid underestimates due to the association of polyphenols with the cellular fraction (Biasutto, L., et al., Anal. Chem., submitted - Chapt. 3).

Following up on the first results with quercetin prodrugs, we went on to synthesise ester derivatives of resveratrol, a polyphenol with well-publicized properties, whose chemical structure allows one to avoid several technical difficulties encountered with quercetin (e.g., a low stability of intermediates due to the presence of a catechol group, problems in the synthesis of penta-substituted derivatives carrying sterically demanding groups, sensitivity to oxidation). Since polyphenols generally have a low solubility in water, and solubility is a key factor contributing to the bioavailability of a compound, we obtained a first resveratrol derivative functionalising the hydroxyls with glucose moieties, linked via a succinic acid group. Pharmacokinetic studies with this compound are still under way, but the available data show that absorption kinetics are delayed in comparison to resveratrol, a potentially useful outcome. The levels and the composition of the metabolite mix in the bloodstream are however analogous to those obtained using resveratrol itself. This suggests that the compound is hydrolysed to resveratrol in the gastro-intestinal tract, before absorption (Chapt. 4).

We have furthermore synthesised other resveratrol precursors: two carboxyester derivatives (carrying methyl or polyethyleneglycol groups), and two other less hydrolysis-prone ones (the per-methylether and the per-mesylate). The results of stability studies in blood, of absorption experiments using *ex vivo* intestine segments and of pharmacokinetic determinations have pointed out an excessive instability of the carboxyester linkage in these biological contexts. Its usefulness for *in vivo* applications turns out therefore to be limited (Chapt. 5).

To better identify and quantify the metabolites observed in pharmacokinetic studies, and to investigate their possible biological roles, we have synthesised some of the most abundant resveratrol metabolites (mono- and di-sulfates, mono-glucuronides) (Chapt. 6).

A second part of the project originated from the consideration that the action of polyphenols can be enhanced not only by increasing systemic levels, but also by causing them to accumulate specifically at desired sites of action. Polyphenols are redox-active

molecules, and reactive oxygen species are involved in several pathologies. Mitochondria are the major site of oxygen radical production and of key events for cell death in both the apoptotic and necrotic modes. In the genesis of this part of the project an important role was played by the observation (in patch-clamp and swelling experiments with isolated rat liver mitochondria) that quercetin can either inhibit or induce the mitochondrial permeability transition, depending on whether its anti- or pro-oxidant activity prevails (which depends on a series of factors including the abundance of metal ions and enzymes with redox activity, pH, the concentration of the polyphenol itself, etc.) (De Marchi, U., et al., Free Rad. Biol. Med., submitted - Chapt. 7).

We have thus synthesised derivatives of quercetin and resveratrol capable of accumulating into mitochondria thanks to functionalisation with the triphenylphosphonium (TPP$^+$) group, a cation capable of diffusing through biological membranes and to concentrate in regions at negative electrical potential, such as the mitochondrial matrix. These compounds behaved as expected in *in vitro* assays (Mattarei, A., et al, (2008), Chembiochem, 9, 2633-2642 - Chapt. 8; Biasutto, L., et al. (2008), Bioorg. Med. Chem. Lett., 18, 5594-5597 – Chapt. 9; Chapt. 10).

Furthermore, the absence in the mitochondrial matrix of conjugating enzymes (sulfotransferases and glucuronosyltransferases) is emerging as a potentially very useful characteristic, to be exploited in order to protect polyphenols and drugs in general from Phase II metabolism (Chapt. 11).

Interesting possible biomedical applications of mitochondriotropic derivatives can be envisioned in either case: prevalence of an anti-oxidant/cytoprotective action or, on the contrary, of a pro-oxidant/cytotoxic behaviour.

A first investigation into the biological effects of these new compounds has been carried out with some of the quercetin derivatives (3-QBTPI, 3-QTABTPI) and isolated mitochondria; as expected on the basis of the previous observations with quercetin, these compounds turned out to be potential co-inducers of the mitochondrial permeability transition, as well as inhibitors of the respiratory chain and of mitochondrial ATP synthase (Chapt. 12).

We have thus carried out a preliminary study to test the possible cytostatic/cytotoxic activity of the mitochondriotropic compounds and of a few constructs carrying an alkyl chain, produced during the project work, on cultured tumour (C-26) and non-tumoural (MEF) cells. These experiments have shown that mitochondria-targeted derivatives and an alkyl-quercetin have a relevant cytotoxic/cytostatic action on fast-growing cells, while

their effect on non-tumoural, slowly growing cells is negligible (Chapt. 13). This behaviour is typical of chemotherapeutic agents, and will need to be further investigated with studies *in vivo* to verify the possible effect of these derivatives on the growth and metastatic proliferation of tumours inoculated into mice.

Before initiating these studies it will be necessary to investigate the intestinal absorption, pharmacokinetic profile and possible *in vivo* toxicity of these molecules. At present we have verified the efficiency and applicability to mitochondriotropic compounds of the blood analysis methods currently in use (Chapt. 14).

Organization of the thesis

After a relatively brief general introduction, the bulk of this thesis is organized in chapters corresponding to individual topics of research. 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. Some of the chapters correspond to papers already published or to manuscripts under evaluation. In other cases the specific parcel of work was still unfinished as the thesis was due; the corresponding section reports the available data and comments, and mentions what remains to be done. In still other cases, the chapter deals with technical developments or reports syntheses of compounds in use for various purposes. Thus, the chapters are not homogenous in length and relevance. I hope the benefits of such an organization outweigh this disadvantage.

Introduction

Polyphenols are a vast and diversified class of plant-made molecules characterized by the presence of a few phenolic hydroxyls. They are present in a great variety of foods of vegetable origin; Fig.1 shows the structures of some of the most important polyphenol subfamilies.
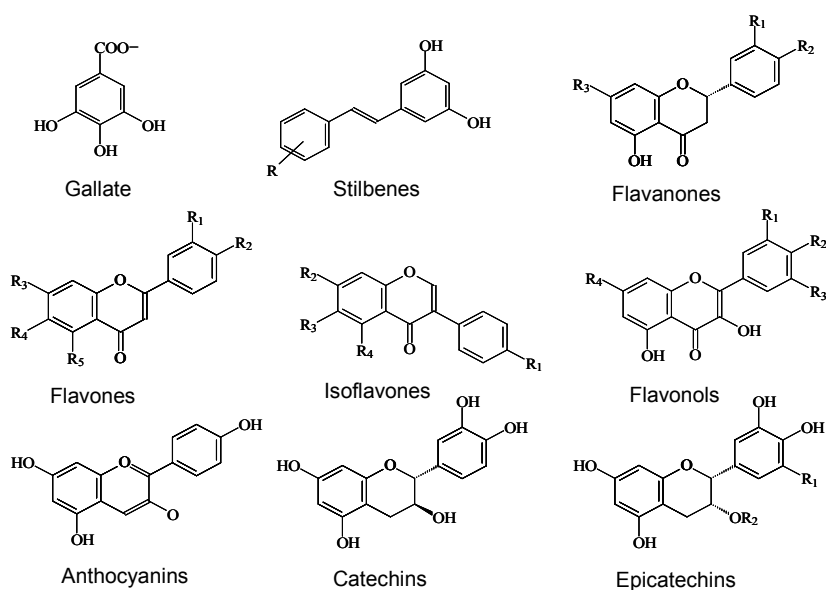
Fig. 1. Some of the most important polyphenol subfamilies.

These compounds exhibit, at least *in vitro*, a variety of activities of potential relevance for many areas of health care, such as protection of the cardiovascular system, improving performance impaired by old age or neurodegeneration, contrasting inflammation and prevention and therapy of cancer.

The biological effects of polyphenols are ascribed both to their redox properties and to interaction with proteins.

Polyphenols are redox active compounds. Their aromatic structure allows delocalization of charges or unpaired electrons, rendering them 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).

$ox = O_2^{\cdot-}, ROO^{\cdot}, Cu^{2+}, Fe^{3+}, ...$
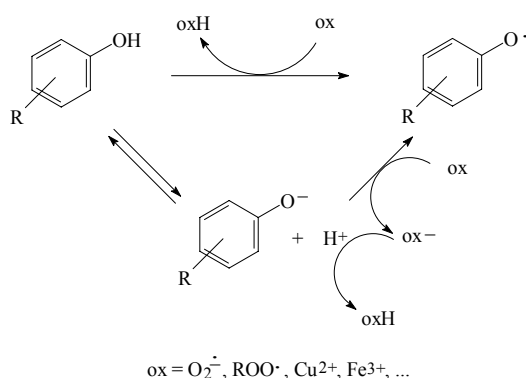
Scheme 1. Radical scavenging by a generic phenolic compound.

Anti-oxidant action has been given credit for the alleged antagonistic effects of polyphenols against processes involving radical-inflicted damage and/or radical-mediated signalling, such as aging and neurodegeneration. However, polyphenols can also act as pro-oxidants, depending on corollary factors such as their concentration, pH, and the presence of $Fe^{2+/3+}$ or $Cu^{+/2+}$. In the presence of iron or copper ions, they may act as catalysts of redox cycling leading to the oxidation of glutathione and other cellular components by atmospheric oxygen. They may thus become key participants in a "redox catastrophe" whereby an oxidative chain reaction may be amplified to the point of overwhelming the cellular redox defenses. In less extreme circumstances, the generation of H_2O_2 may have important consequences at the cellular level via redox-sensitive signalling proteins (e.g. phosphatases and kinases) and transcription factors (e.g. NFkB, AP-1). The cytotoxic pro-oxidant mode of action is as potentially useful as the anti-oxidant one, since it might be exploited to induce death of cancerous cells.

Various polyphenols are known to modulate directly a number of key proteins, including metalloproteinases, membrane receptors, channels (e.g. CFTR), cyclooxygenases, transcription factors, deacetylases (sirtuins), cytoskeleton components (tubulin) and many kinases as well as proteins of the blood (albumin, haemoglobin) and some toxins (e.g. anthrax toxin, vacA). Effects on gene expression are very important, and in fact much of the antioxidant activity of polyphenols seems to be actually mediated by redox-sensitive transcription factors and enzymes. In many cases however potentially useful interactions are relatively weak, and thus the question arises of whether the concentrations needed to exert a significant effect are reached in a physiological setting.

The notoriously low bioavailability of these compounds is a formidable obstacle for their pharmacological exploitation, and is believed to be a major reason why the epidemiological evidence for effects of polyphenol-rich diets is generally spotty and contrversial. Since they are, by definition, ready-made Phase II metabolism substrates, they are rapidly sulfated, glucuronidated by enterocyte and liver transferases, and then rapidly eliminated. Only low (nM-µM) concentrations of any given polyphenol are found in plasma and lymph even after a polyphenol-rich meal, and mostly in the form of conjugates. Low absorption and metabolism must be contrasted if the pharmacological potential of natural polyphenols is to be more fully exploited. 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".

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.

The project

My thesis developed as a proof-of-principle project intended to promote the exploitation of the biomedical potentialities of polyphenols *in vivo*, stimulating the development of a real pharmacology of these compounds.

For this work we adopted two widely used model polyphenols, quercetin and resveratrol, both credited with remarkably useful properties and widely used as representatives of the superfamily of compounds.

Quercetin   Resveratrol
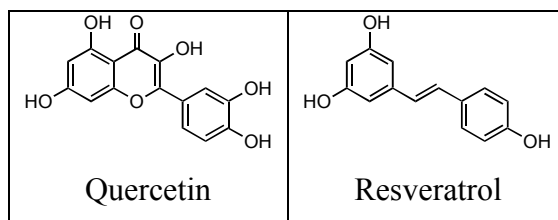
As the project developed, two main themes came into focus: one was to increase the bioavailability and efficacy of polyphenols; the other was to target these compounds to a subcellular site where they might be expected to be most effective. To increase bioavailability, we are developing derivatives protected at the sites of metabolic modification and with improved solubility characteristics, thus obtaining "pro-drugs"

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

Since Reactive Oxygen Species (ROS) in cells are mostly produced by mitochondria, the development of polyphenol-based, redox-active derivatives targeted to these organelles allows one to place these compounds in the most appropriate subcellular location for exerting their anti-oxidant (protective) or pro-oxidant (cytotoxic) effects. Importantly, mitochondrion-targeting represents also a strategy to slow metabolism, because of the lack of Phase II metabolic enzymes in the mitochondrial matrix.

1. Ester-based precursors to increase the bioavailability of quercetin

Ester-Based Precursors to Increase the Bioavailability of Quercetin

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Plant polyphenols exhibit a variety of potentially useful biochemical properties in vitro, but their evaluation and clinical exploitation in vivo is hampered by their limited bioavailability. Precursors exhibiting resistance to phase II metabolism during absorption are therefore desirable. We report here the synthesis as well as stability and solubility studies of several ester derivatives of quercetin (3,3′,4′,5,7-pentahydroxy flavone), most of which comprise an aminoacyl group. To model transepithelial absorption, we tested transport across supported tight monolayers of MDCK-1, MDCK-2, and Caco-2 cells. Quercetin itself was extensively conjugated by all three types of cells. A few of our precursors did not cross the monolayers, but others did, undergoing partial deacylation. No phase II conjugation was observed during transport of these compounds across MDCK or some Caco-2 clones. With other Caco-2 lines complete deacylation occurred, followed by metabolism of quercetin. Since elimination of residual acyl groups is expected to take place in vivo, ester derivatives of polyphenols may constitute a useful method to increase systemic aglycone concentrations.

1. Introduction

Plant polyphenols, a large and varied class of natural compounds carrying phenolic hydroxyls, exist in a sort of biomedical no man's land. A vast literature, based mostly on in vitro work, describes a variety of potentially important biochemical properties of these compounds. They have long been recognized to act as antioxidants.[1−3] Various abundant members of the family are known to interact with and modulate signal-transducing proteins ranging from channels to cyclooxygenases. Indeed, antioxidant effects are ascribed in part to modulation of transcription factors such as nuclear factor kB and activating protein-1 and to the induction of the expression of antioxidant enzymes such as glutathione-S-transferase and superoxide dismutase.[4,5]

Work with cultured cells, model organisms, and laboratory animals has given support to the notion that various dietary polyphenols—most notably flavones, isoflavones, resveratrol, and epigallocatechingallate (EGCG)[a]—can oppose cancer,[6−9] cardiovascular,[10−12] neurodegenerative,[13,14] and other diseases, and lengthen the lifespan of model organisms[15,16] and people.[17]

Statistical studies, meta-analyses, and human trials, on the other hand, have turned up limited noncontroversial evidence for significant correlations between dietary polyphenol intake and health benefits.[18−22] Even for the best-supported cases, arguably the "French paradox"[23,24] and the low incidence of breast and prostate cancers in soy-consuming populations,[25,26] doubts remain as to the significance of a diet rich in (respectively) resveratrol[27,28] and isoflavones.[29−32]

The discrepancy between in vitro results and epidemiological observations probably has multiple origins. In statistical dietary studies it is notoriously difficult to completely exclude contaminating factors. On the other hand, laboratory results present their own set of problems. Until recently, some studies may have neglected to take into account the fact that the addition of polyphenols to cell culture media may result in the production of hydrogen peroxide,[33,34] i.e., they may act as pro-oxidants (also in vivo, under overload conditions[35]). H_2O_2 is a well-known second messenger, which acts by modifying the activity of signal-transducing proteins, e.g., phosphatases, and can thereby strongly impact cell physiology, e.g., via the activation of MAP kinase cascades.[36] Dosage has been proposed to be a key factor determining the outcome of polyphenol administration,[37] and it has been repeatedly pointed out[38,39] that many in vitro studies have made use of unrealistically high concentrations of the compounds, neglecting the limited bioavailability of dietary polyphenols. This constitutes the major obstacle for a reliable assessment of their effects in vivo as well as, perhaps, for the full exploitation of their pharmacological possibilities. While there are differences depending on the class of compounds considered and on the experimental model used, polyphenols are generally treated by intestinal enterocytes as xenobiotics. According to the current model based on quercetin and resveratrol, the glycosylated derivatives present in food are hydrolyzed to aglycones and glucuronated, sulfated and methylated by detoxifying enzymes after diffusing into 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.[40,41] As a result, only very low levels (in the nanomolar to micromolar range) of polyphenolic compounds are found in blood or plasma even after a polyphenol-rich meal, mostly as metabolites.[42−46] In the case of quercetin, studies with everted intestine segments and analyses of blood and lymph samples have led to the identification of up to 23 different conjugates,[47−53] all arising via single or multiple glucuronation, sulfation, and

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 a Abbreviations: ABC/MDR, ATP-binding cassette/multiple drug resistance; Boc, *t*-butyl-oxycarbonyl; DMAP, dimethylaminopyridine; EDC, *N*-ethyl-*N*′-(3-dimethylamminopropyl)-carbodiimide; ESI, electrospray ionization; EGCG, epigallocatechingallate; HBSS, Hank's balanced saline solution; MDCK, Madin−Darby canine kidney; PBS, phosphate-buffered saline; PTFE, polytetrafluoroethylene (Teflon); Q, quercetin; TER, transepithelial resistance; TFA, trifluoroacetic acid; TOF, time of flight.

Scheme 1. Synthesis of Quercetin Derivatives
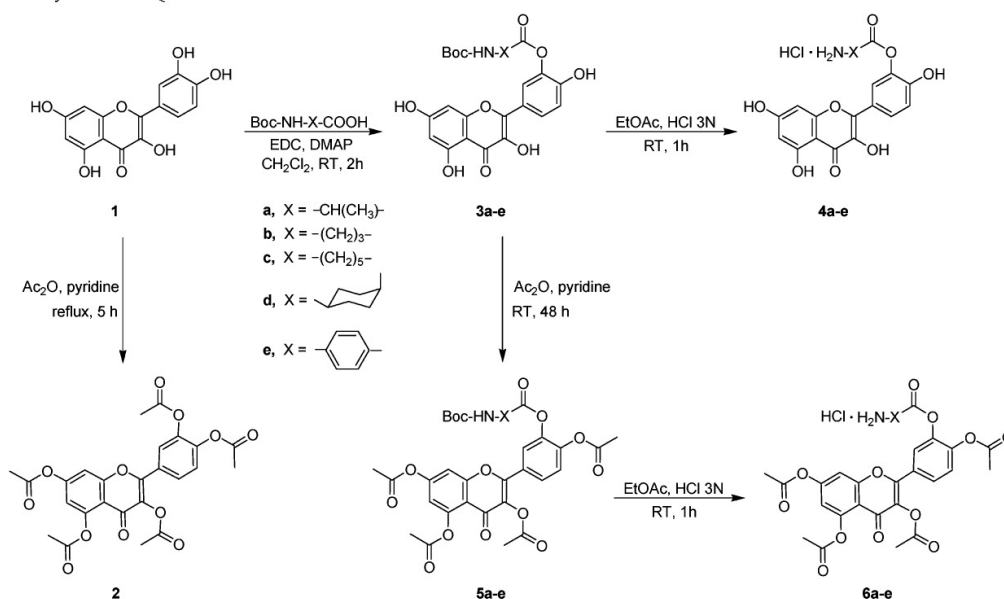
methylation of the hydroxyls. The metabolites may themselves have biological activity.[42,54,55]

In this context we have started work aiming at bypassing the modification of polyphenols by enterocytic transferases. This would improve bioavailability, increasing the levels of the aglycone in the circulatory stream and organs. In turn, this would allow more reliable assessments of the efficacy and innocuity of the compounds under various experimental conditions mimicking circumstances of (patho)physiological relevance and might eventually lead to the development of polyphenol-based molecules with preventive or curative applications. One way the bypass may be achieved is via the development of polyphenol precursors capable of withstanding transit through epithelia and of regenerating the parent compound once in circulation. This paper reports initial progress toward such goals.

Given the nature of the molecules in question, we focused on ester derivatives, also because the ubiquitous presence of enzymes with esterase activity[56–58] promises the rapid regeneration of the parent polyphenol in vivo.

Bioavailability- and/or stability-improving ester prodrugs have already been developed for many drugs with alcoholic or phenolic hydroxyls.[59,60] In the case of polyphenols, peracetylated EGCG[61,62] and quercetin,[63–66] and water-soluble 3′-(*N*-carboxymethyl)carbamoyl-3,4′,5,7-tetrahydroxyflavone (QC12)[67] have been reported. However, the effects of these modifications on transepithelial transport and metabolism have not been investigated.

Since this is intended as a proof-of-principle study, we concentrated on a model polyphenol, choosing quercetin (3,3′,4′,5,7-pentahydroxy flavone, **1**) because it is abundant in nature, readily available, present in plasma almost totally as conjugates, and because its absorption and metabolism have been studied in detail.[47–53,68–72] To evaluate the effects of derivatization on transepithelial transport, we employed supported cell monolayers of three different epithelial cell lines, namely, canine MDCK-1 and -2 and human Caco-2. The latter are often used as a stand-in for intestinal epithelium. This experimental setup has been utilized in two studies of the

transport of quercetin and its glucosides across Caco-2 monolayers. One[73] reported a rapid passage of unmodified quercetin. The other[74] found conjugation products on both sides.

We produced a series of quercetin derivatives bearing an aminoacyl substituent to promote solubility and permeation. The recent literature contains several reports of amino acid-based prodrugs. Transport of the aminoacyl esters across Caco-2 monolayers has been evaluated in a few of these investigations, and found to be improved in comparison with that of the parent drug in some cases.[75–77] Resistance to hydrolysis was reported to depend on the aminoacyl group.[78] Enhanced resistance to metabolism was also observed.[75,79]

We thus report in this paper the synthesis, characterization, and the hydrolytic and cell monolayer permeation properties of a series of quercetin esters. Most comprised an aminoacyl functionality, which we varied seeking to optimize the properties of the compounds.

2. Results

2.1. Synthesis. Scheme 1 outlines the synthesis of compounds **2**−**6**. Pentaacetylquercetin (**2**) was synthesized according to a published procedure.[63] Attempts to obtain pentaaminoacylquercetins via reaction of quercetin with excess NH_2-blocked amino acids yielded intractable mixtures of polysubstitution products. We therefore proceeded to synthesize monoaminoacyl derivatives. The reaction of Boc-protected amino acids with an excess of quercetin (**1**) resulted in esterification mainly of the B ring hydroxyls, in line with the acidity,[80] high oxidability, and reactivity of the catechol moiety. Compounds **3a**−**e** were isolated by flash chromatography and turned out to be a mixture of 3′- and 4′-substituted quercetins, in an approximately 3:1 ratio. The two isomers would be expected to have very similar properties, and in fact our strenuous attempts to separate them by chromatography were unsuccessful. Therefore, all compounds mentioned in this work were actually a mixture of two isomers. NMR characterization led to the assignment of 3′ as the major esterification site: compared to the corresponding protons in quercetin, $\delta_{H2'}$ is greater than $\delta_{H5'}$ in the major product, while the opposite is true for the minor product (not shown).

Table 1. Observed Rate Constants for the Hydrolysis of 3′-Aminoacylquercetins in 1:1 PBS/CH$_3$CN[a]

derivative	k_{obs} (s^{-1})	$t_{1/2} \times 10^{-2}$ (s)
3a	$(6.56 \pm 0.04) \times 10^{-4}$	10.7
3b	$(9.3 \pm 0.2) \times 10^{-5}$	75
3c	$(5.18 \pm 0.03) \times 10^{-5}$	132
3d	$(5.8 \pm 0.7) \times 10^{-5}$	119
4a	$(2.16 \pm 0.03) \times 10^{-2}$	0.32
4b	$(9.7 \pm 0.3) \times 10^{-2}$	0.07
4c	$(4.2 \pm 0.2) \times 10^{-4}$	16.5
4d	$(7.4 \pm 0.6) \times 10^{-4}$	9.3

[a] Error notations are average deviations ($N = 2$ or 3).

The remaining hydroxyls were acetylated with acetic anhydride in pyridine at room temperature (compounds **5a**−**e**). The Boc group was removed by treating compounds **3a**−**e** and **5a**−**e** with EtOAc/HCl to afford NH$_2$-free amino acid esters (compounds **4a**−**e** and **6a**−**e**).

2.2. Chemical Stability Studies. Assessing the stability of the compounds in aqueous solution was important both for transepithelial transport experiments and, a priori, to distinguish esterase-mediated and spontaneous hydrolysis. Hence, the chemical stability of **2**−**6** was evaluated by monitoring their UV−vis spectra in a mixture of PBS (20 mM, pH 7, $\mu = 0.1$ M)/CH$_3$CN 1:1, where CH$_3$CN was necessary to ensure the complete solubilization of all the compounds.

Compounds **2**, **3e**, **4e**, **5a**−**e**, and **6e** turned out to be stable under these conditions. All the other compounds underwent hydrolysis. Compounds **3a**−**d** and **4a**−**d** hydrolyzed to quercetin, as shown by the comparison of the final UV−vis spectrum of the reaction mixture with that of a 1:1 mix of **1** and the amino acid. The kinetic constants and estimated half-life times ($t_{1/2}$), obtained from exponential fits of plots of $A_{255.2}$ versus time, are listed in Table 1.

NH$_2$-free amino acid esters **4a**−**d** hydrolyzed significantly faster than NH$_2$-protected amino acid esters **3a**−**d**. Furthermore, the presence of the Boc-group affected stability ranking of the amino acid esters. Among Boc-protected esters the least stable was the D-alanine derivative ($t_{1/2}$: **3a** < **3b** < **3d** < **3c** < **3e**), while γ-aminobutanoic acid turned out to be the most labile substituent for esters bearing a free −NH$_2$ group ($t_{1/2}$: **4b** < **4a** < **4d** < **4c** < **4e**). 4-Aminobenzoic acid derivatives were the most stable ones in both cases.

Compounds **6a**−**d** were also unstable in the presence of PBS buffer. They underwent two consecutive hydrolytic processes, as evidenced by the appearance of two isosbestic points in the spectra recorded in the 190−500 nm range. Figure 1 illustrates a representative experiment. MS spectra of products and HPLC−UV and LC−MS analyses (not shown) led to the conclusion that the amino acid substituent was lost first, followed by an acetyl group.

A subsequent hydrolysis of the resultant triacetylquercetin was also observed at longer reaction times, but it did not influence the interpolation of the data (see the Experimental Section), as no substantial variation in absorbance was associated with this process. The kinetic constants of the first two hydrolytic processes are reported in Table 2. In all cases, hydrolysis could be blocked by acidification. Addition of 0.2% formic acid halted the progression of spectral changes (and allowed analysis of the solution). As can be appreciated from Tables 1 and 2, the stability of the compounds having free −OH and/or −NH$_2$ groups was inadequate for their utilization in transport experiments. Accordingly, they were not investigated further.

2.3. Solubility in HBSS. In Table 3 the approximate solubility in HBSS is reported for the compounds found to be stable in
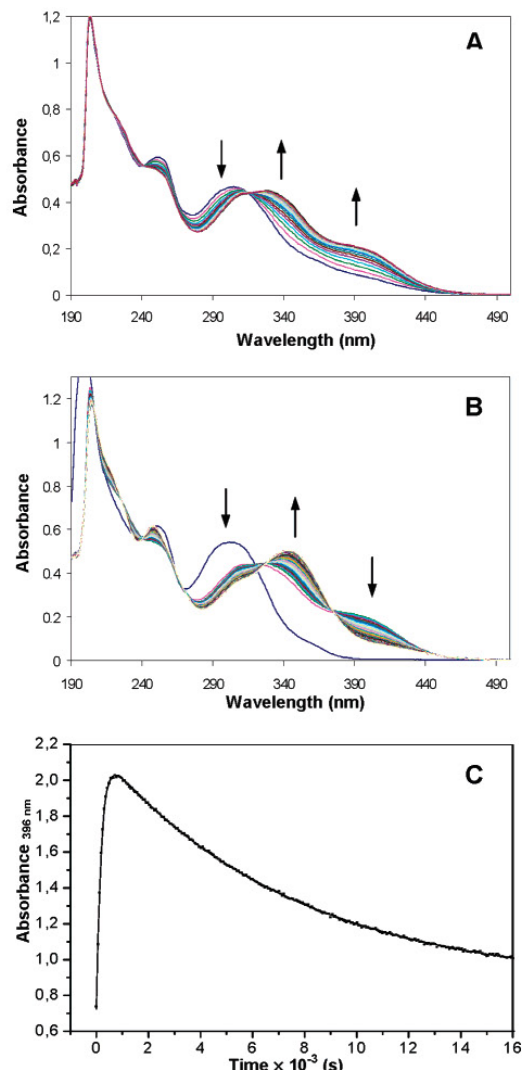
Figure 1. Hydrolysis of **6a** in PBS/CH$_3$CN 1:1. (A) UV−vis spectra recorded every minute for 30 min. (B) UV−vis spectra recorded every 10 min for 8 h. (C) Interpolation of the data with eq 2.

Table 2. Observed Rate Constants for the First and Second Hydrolysis of 3′-Amino Acid Tetraacetylquercetin Ester Hydrochlorides in 1:1 PBS/CH$_3$CN[a]

derivative	$k_{1,obs}$ (s^{-1})	$k_{2,obs}$ (s^{-1})	$t_{1/2} \times 10^{-2}$ (s)
6a	$(5.03 \pm 0.26) \times 10^{-3}$	$(1.26 \pm 0.07) \times 10^{-4}$	1.37
6b	n.d.	$(1.33 \pm 0.10) \times 10^{-4}$	n.d.
6c	$(9.6 \pm 0.9) \times 10^{-4}$	$(1.32 \pm 0.18) \times 10^{-4}$	0.72
6d	$(2.51 \pm 0.07) \times 10^{-4}$	$(1.23 \pm 0.07) \times 10^{-4}$	27.6

[a] Error notations are average deviations ($N = 2$ or 3). The tabulated $t_{1/2}$ of the compounds refers to the first process.

aqueous solution at near-neutral pH. Solubilities were in the same range as for quercetin, except in the cases of *cis*-4-aminocyclohexanecarboxylic acid and 4-aminobenzoic acid derivatives, for which they were below 1 μM. It should be pointed out that the values reported represent upper limits: part of the material in "solution" was presumably still present as colloidal particles rather than as a true solute.

Table 3. Approximate Solubilities in HBSS of Stable Compounds at Room Temperature

compound	solubility in HBSS (μM)
1	2.3
2	1.1
3e	0.4
4e	1.5
5a	12.0
5b	2.5
5c	3.9
5d	0.8
5e	0.4
6e	1.0

2.4. Transepithelial Transport. Due to their short lifetime in water solutions at pH values compatible with cell function, or to the presence of unprotected hydroxyls, compounds **3a**−**e**, **4a**−**e**, and **6a**−**d** were considered unsuitable for transepithelial transport experiments. Experiments were therefore performed only with compounds **1**, **2**, **5a**−**e**, and **6e**. Quercetin (**1**) obviously served as benchmark. To better characterize polyphenol metabolism, which depends on cell type, three different popular cellular models were employed, namely, MDCK-1, MDCK-2, and Caco-2, which form monolayers upon differentiation. A total of 223 individual transport experiments were carried out. The extent of transport depended on the size of the Transwell insert used, since the surface/volume ratio is different in the two cases (see the Experimental Section). The composition of the product mixtures in both compartments changed with time, as translocation, hydrolysis, and conjugation progressed. Analyses of the basolateral compartment from representative experiments with 24-mm supports, at 6 h, are reported in Table 4. Mass balances (i.e., the sum of the molar amounts of all compounds present in all compartments at a given time over the moles of quercetin derivative placed in the apical compartment at time zero) were in the range of 70−85% after 3 h, declining to 50−70% after 6 h. They were similar for transport experiments and for control incubations without cells. Approximately 20% of the material originally loaded in the apical compartment was found in association with the cell layer, from which it could be extracted with methanol (see the Experimental Section). The composition of this fraction was intermediate between those of the apical and basolateral compartments.

2.4.1. Quercetin (1). The "diffusion" of quercetin from the apical to the basolateral compartment was characterized to define the metabolic activities of the cells and as a reference for the performance of its derivatives. As expected, only a small percentage of **1** appeared as such in the basolateral compartment (Table 4). After 6 h, and using 24-mm inserts, this amounted to approximately 1%, 3%, and 1% of the quantity loaded in the apical compartment in the case of MDCK-1, MDCK-2, and Caco-2 cells, respectively. Quercetin sulfate, methylquercetin (probably isorhamnetin, i.e., 3′-methoxyquercetin[74]), methylquercetin sulfate, and quercetin o-quinone were the other compounds detected on the basolateral side. Figure 2A presents typical HPLC−UV$_{370}$ chromatograms from basolateral samples taken after 6 h. The o-quinone is not detected at the wavelength used in the figure (its peak absorbance is at 295 nm). Transport kinetics are illustrated by the set of chromatograms in Figure 2B. The identity of the compounds was established by comparison of retention times with standards (quercetin, 4′-methylquercetin), UV−vis spectral characteristics (quercetin sulfate,[41] quercetin o-quinone[81]), mass spectrometry, and comparison of the chromatographic profiles of untreated and sulfatase-treated samples (Figure 3). The formation of quercetin glucuronides

Table 4. Percentages of the Intact Compounds and their Metabolites in the Basolateral Compartment after 6-h Transport Experiments Across MDCK-1, MDCK-2, and Caco-2 Monolayers, using 24-mm Inserts[a]

compound loaded	basolateral species	% of apically added material at 6 h		
		MDCK-1	MDCK-2	Caco-2
1	intact compound (Q)	0.9	2.7	1.0
	Q-sulfate + methyl-sulfate	6.6	0.6	5.8
	methyl-Q	0.9	0.8	1.3
	Q-o-quinone	0.3	1.7	0.8
	total basolateral	8.7	5.8	8.9
2	intact compound		0.5	
	tetraacetyl-Q	0.4	0.9	
	diacetyl-Q	8.4	12.7	
	monoacetyl-Q	1.0	4.7	
	Q	1.6	2.5	0.4
	Q-sulfate + methyl-sulfate			5.0
	methyl-Q			0.5
	Q-o-quinone	0.6	1.2	0.5
	total basolateral	12.0	22.5	6.4
5a	intact compound			
	diacetyl-Q	2.4	8.2	
	monoacetyl-Q	2.2	2.4	
	Q	0.6	0.8	4.2
	Q-sulfate + methyl-sulfate			1.8
	methyl-Q			2.4
	Q-o-quinone	1.1	2.6	2.6
	total basolateral	6.3	14.0	11.0
5b	intact compound			
	diacetyl-Q	4.6	6.5	
	monoacetyl-Q	1.2	3.2	0.4
	Q	0.3	1.0	2.6
	Q-sulfate + methyl-sulfate			2.2
	methyl-Q			0.9
	Q-o-quinone	1.4	3.2	2.8
	total basolateral	7.5	13.9	8.9
5c	intact compound			
	diacetyl-Q	13.6	5.6	
	monoacetyl-Q	6.5	3.9	0.3
	Q	2.1	2.6	2.5
	Q-sulfate + methyl-sulfate			3.4
	methyl-Q			2.0
	Q-o-quinone	4.3	3.1	2.9
	total basolateral	22.2	12.1	8.2

[a] Representative experiments are reported. Figures given are percentages of the total amount of starting material present in the apical compartment at time zero.

by Caco-2 cells has been reported in the literature.[82] In our hands they could be detected, at low levels, in a few experiments (including the one in Figure 2), but more often they were below detection limits.

MDCK-1 and Caco-2 cells on one side and MDCK-2 cells on the other exhibited reproducible differences, in that the latter appeared to possess weaker sulfate- and methyl-transferase activities so that the major specie found on the basolateral side was quercetin itself. Caco-2 cells turned out to be complex. In the course of this work we used seven lines, which were outwardly very similar but handled quercetin and its derivatives differently. "Clone A" (actually two lines) exhibited strong phase II conjugation activity; the basolateral mixture of products obtained was similar to that of MDCK-1 cells. Five other lines, which we refer to as "clone B" for simplicity, had only weak methyl- and sulfotransferase activities, behaving much like MDCK-2 cells. These observations will be reported in detail elsewhere. Since in vivo experiments indicate that quercetin is heavily conjugated during and/or after absorption, clone A may be considered to best reflect in vivo processes. All Caco-2-related results presented in this paper were obtained with these cells.
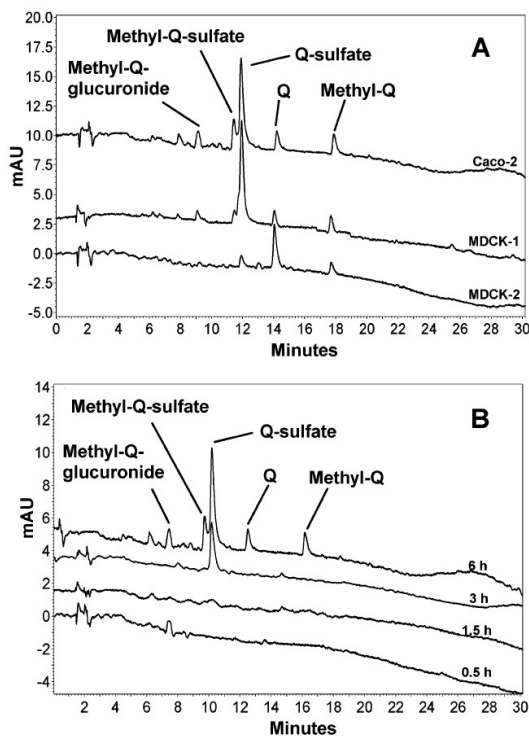
Figure 2. HPLC−UV chromatograms (370 nm) of the basolateral side in transport experiments with quercetin (**1**) (24-mm inserts): (A) after 6 h, with the three types of cell monolayers; (B) with Caco-2 cells at the indicated times.
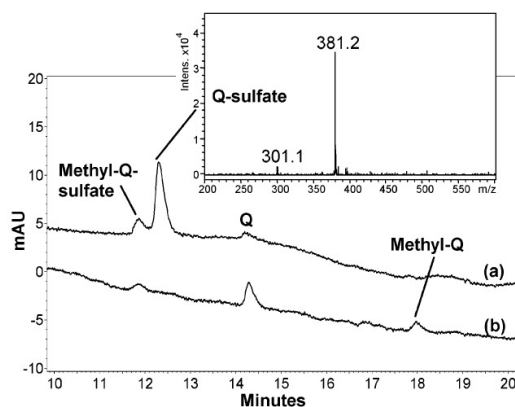

Figure 3. HPLC−UV chromatograms (370 nm) of a basolateral side sample taken after 5 h in an experiment of quercetin transport across a Caco-2 monolayer (12-mm insert), before (a) and after (b) treatment with sulfatase. Inset: negative ESI-MS spectrum of quercetin sulfate.

The composition of the apical compartment changed relatively slowly when the canine cell lines were employed. Quercetin was the major compound remaining up to at least 6 h. Minor amounts of the same metabolites found in the basolateral compartment appeared, along with some (2−3% at 6 h) quercetin *o*-quinone. With Caco-2 cells apical quercetin underwent more drastic modification, to give the usual conjugation products (Figure 4). These findings are in broad agreement with literature reports.[74,82]
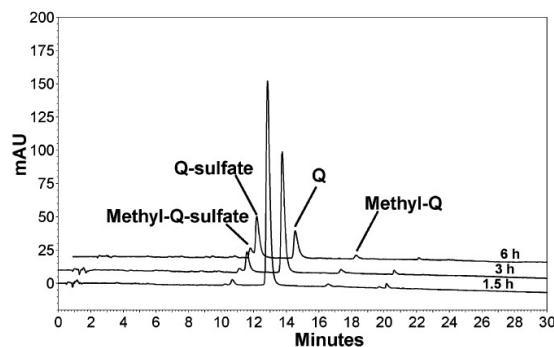

Figure 4. HPLC−UV chromatograms (370 nm) of the apical side in experiments of quercetin transport across Caco-2 monolayers using 24-mm inserts. The upper two chromatograms have been shifted to the right for clarity.

2.4.2. Pentaacetylquercetin (2). The fraction of apically loaded pentaacetylquercetin that reached the opposite compartment without undergoing alterations was also very low for all three cell lines (Table 4). With this compound, as well as the others mentioned below, a major difference between MDCK-1 and MDCK-2 cells on one side and Caco-2 cells on the other was evident. The former partially deacetylated **2** but did not generate detectable amounts of sulfated derivatives in any compartment. The latter efficiently stripped off all acyl groups, and proceeded to rapidly conjugate the aglycone, so that the major products were the same compounds observed when the transport substrate was quercetin itself (Figure 5).

The composition of the apical compartment was qualitatively similar to that of the corresponding basolateral one, except for the obvious presence of **2** itself, which declined in time becoming a relatively minor component after 6 h. With MDCK-1 and -2 cells, acetylquercetins and some quercetin were the only detectable products. Interestingly, only small quantities of tetraacetylquercetins and even lesser amounts of triacetylquercetins (at least three different isomers, when detectable) formed in either the apical or basolateral compartments, while the diacetyl specie was prominent (not shown; see Figure 7).

With Caco-2 cells, some quercetin, larger percentages of sulfated and/or methylated quercetin than in the case of MDCK monolayers, and small amounts of partially acetylated quercetins formed in the apical compartment (not shown).

2.4.3. 3′-Boc-amino Acid Tetraacetylquercetins 5a−c. The behavior of compounds **5a−c** was similar to that of **2**. Again, there was a marked difference between Caco-2 cells and the other lines. With both MDCK and "clone B" Caco-2 lines, acylated forms but no parent or sulfated compounds appeared in the basolateral compartment (Table 4 and Figure 6). The partially acetylated forms consisted mainly of diacetyl- and monoacetylquercetin, with very minor quantities of Boc-amino acid triacetylquercetin ester(s) at intermediate times and no detectable tri- and tetraacetylquercetin(s). With "clone A" Caco-2 monolayers, acylated forms were conspicuously absent, except for very small amounts of monoacetylquercetin. Sulfated and/or methylated quercetins, and quercetin itself, were the only derivatives present in quantifiable amounts (Table 4, Figure 6). With any given cell type, the chromatographic profile was qualitatively the same for the three compounds (**5a−c**).

In the apical compartment the starting compound was progressively hydrolyzed, with the loss of most of it after 6 h. Interestingly, in all cases the reaction taking place most readily was not the loss of the Boc-aminoacyl substituent, but rather
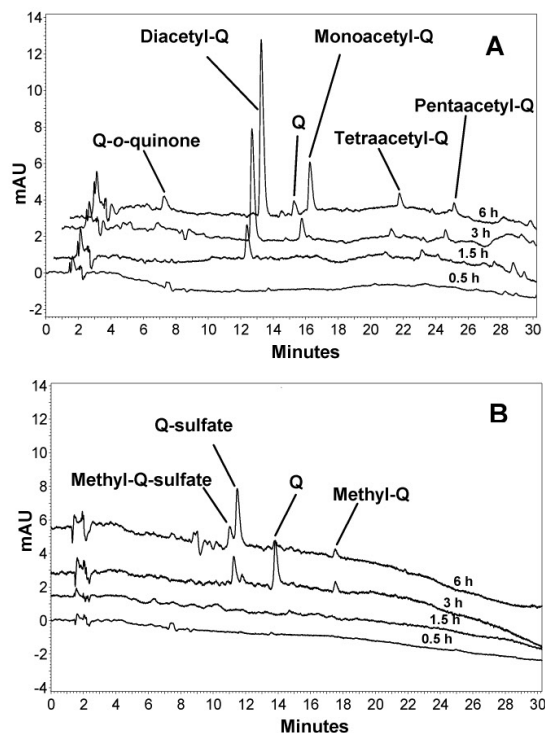
Figure 5. HPLC−UV chromatograms of the basolateral side in experiments of compound **2** transport across (A) MDCK-2 monolayers (300 nm) and (B) Caco-2 monolayers (370 nm), using 24-mm inserts. In (A) the upper three chromatograms have been shifted to the right for clarity.

that of an acetyl group, leading to the accumulation of significant amounts of Boc-amino acid triacetylquercetin ester. Again, MDCK cells did not generate detectable amounts of sulfated derivatives, while Caco-2 cells did (Figure 7).

2.4.4. 3′-Boc-amino Acid Tetraacetylquercetins 5d,e and Compound 6e. With these compounds no detectable transepi-thelial transport took place over 6 h. Little happened in the apical compartment as well. The only derivatives detected, in trace amounts, were partially deacylated compounds (not shown).

3. Discussion

The goal of the project is the development of bioavailability-enhancing precursors of polyphenols. In this phase we have taken the most straightforward approach, synthesizing first-generation ester derivatives of a model compound, quercetin, and testing them on in vitro epithelial models. While obtaining compounds simultaneously permeant, sufficiently soluble, and stable in aqueous media proved difficult, some of the molecules we tested yielded encouraging results.

The test tube chemistry of quercetin is dominated by that of its catechol group, and it is not surprising that the monoester derivatives were formed at either the 3′ or the 4′ position. The 3:1 predominance of substitution at 3′ may reflect thermodynamic control: the molecule with a free −OH in 4′ may well be slightly more stable since its conjugate base can delocalize the negative charge onto the oxygen at position 4.

The presence of a free −OH in ortho to the ester group in the catecholic moiety contributes to the instability of 3′- and 4′-acylquercetins in aqueous solution. This is an example of the "ortho effect", a case of general base catalysis: the
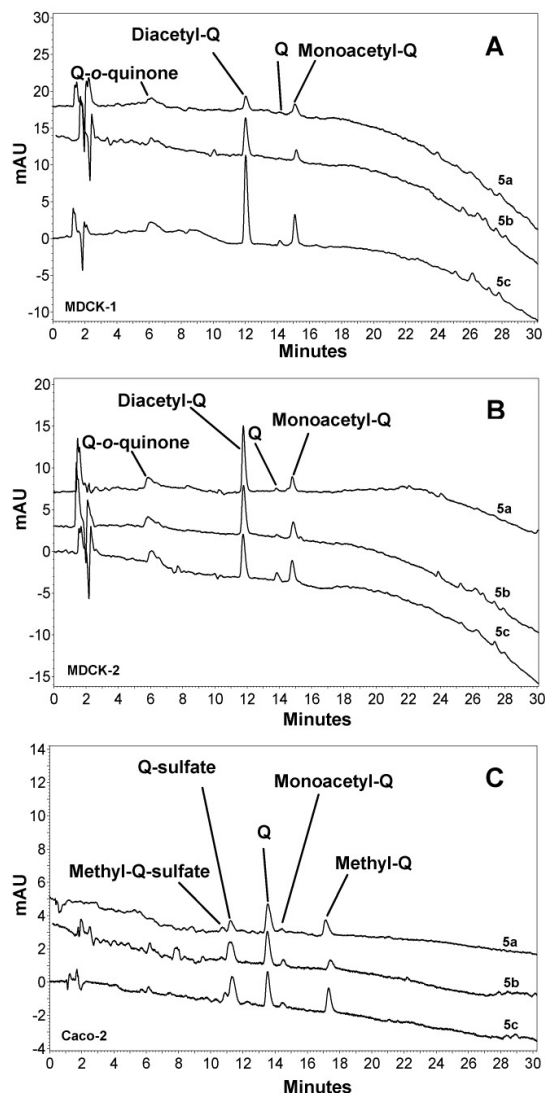

Figure 6. Comparison between HPLC−UV chromatograms of the basolateral side after 6 h in transport experiments of compounds **5a−c** across (A) MDCK-1 (300 nm), (B) MDCK-2 (300 nm), and (C) Caco-2 monolayers (370 nm).

deprotonated phenolic hydroxyl can aid the nucleophilic attack of a molecule of water to the neighboring carbonyl,[83] thus accelerating the decomposition of monosubstituted compounds (**3a−d**, **4a−d**). With persubstituted derivatives, once the first acyl (acetyl or aminoacyl) substituent is lost from a catecholic position the neighboring one is also condemned. This two-stage process could be clearly followed spectroscopically in the case of compounds **6a−d** (Figure 1). That an ionized group is involved follows from the fact that hydrolysis of monosubsti-tuted compounds, as well as the second stage of the two-phase process undergone by **6a−d**, was blocked by acid. This feature helped us to identify the products of the first step. Unfortunately, the pH values required to stop decomposition were not compat-ible with cell welfare. The "ortho effect" explains the second stage of the biphasic hydrolysis of peresterified quercetins, but a primary loss of the aminoacyl group preceded it, unless the
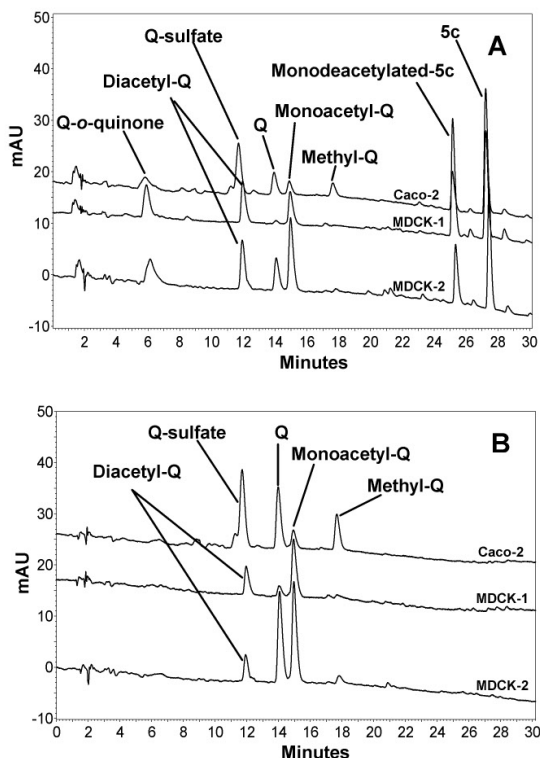
Figure 7. Comparison between HPLC–UV chromatograms of the apical side after 6 h in experiments of **5c** transport across Caco-2, MDCK-1, and MDCK-2 monolayers, recorded at (A) 300 nm and (B) 370 nm. Note that the peak of diacetylquercetin appears lower at 370 nm while those of quercetin and quercetin sulfate appear higher at this wavelength.

−NH₂ group was blocked by the *t*-butyl-*O*-carbonyl group. The destabilizing effect of the amino group probably has multiple mechanistic explanations, having different relevance for the various compounds. If close to the acyl carbonyl, as in **4a** and **6a**, the amino group, especially if protonated (−NH₃⁺), is expected to exert a "through-bond" electron-withdrawing effect, increasing the polarization of the carbonyl and therefore its susceptibility to nucleophilic attack. The Boc group, besides protecting for steric reasons, would be expected to decrease the inductive effect because its presence turns the amino group into a less basic, and less electron-withdrawing, amido group. In fact, the Boc-protected compounds (containing a free ortho −OH) hydrolyze less rapidly than the unprotected ones (compare the kinetics of **3a** and **4a**). A polarization effect can take place also "through space", thus accounting perhaps in part for the instability of compounds in which the acyl chain can fold in such a way as to bring the amino group into proximity with the acyl carbonyl, as in compounds **4c** or **6c**. Straightforward intermolecular base catalysis is however likely to be more important. In fact, in the case of **6d**, in which such a folding is unlikely, and the −NH₂ is too far from the carbonyl for a "through-bond" effect to be plausible, the rate of hydrolysis remains too high for the compound to be useful for our purposes. When also this last prohydrolyis catalytic option is removed, lowering the basicity of the −NH₂ group by turning it into an anilino group in compound **6e**, stability is finally achieved. But the solubility of **4e** and **6e**, even in the absence of the hydrophobic Boc group, is very low.

Deacylation of the water-stable compounds in the presence of cells also showed some interesting features. Triacetylquercetin, the expected product of deacylation of the two catecholic positions, was never detected in more than trace amounts, while di- and monoacetylquercetins were relatively abundant. The first process taking place was the loss of an acetyl group, yielding tetrasubstituted quercetin (Figure 7). The group lost was most likely on the A or C rings, since departure of the acetyl on the B ring would be expected to precipitate the rapid loss of the neighboring acyl group, producing trisubstituted species. Indeed, as soon as one of the B ring esters is hydrolyzed, the neighboring one follows suit, resulting in the formation of diacetylquercetin. In Caco-2 (but not MDCK) cells the two remaining ester groups of the condensed ring system are also rapidly hydrolyzed, before conjugation takes place, since we did not detect any acetylquercetin sulfate or (acetyl)-methylquercetin.

The compounds used in transport experiments did not undergo spontaneous (as distinct from cell-mediated) hydrolysis at an appreciable rate, but their stability was undermined by other processes, largely responsible for lowering the mass balance of our experiments. The amount of material used up in this manner tended to vary, suggesting that erratic processes, possibly oxidative chain reactions, occurred. No products plausibly arising from processes of this type could be detected in either transport experiment samples or in ad hoc solutions/suspensions allowed to age under air. They presumably consist of polymeric materials retained by our LC columns. Remarkably, this "loss" of material took place in water but not when the compounds were dissolved in organic solvents. A clue to what may be happening is offered by studies of the oxidation chemistry of quercetin and other polyphenols.[1,81,84−86]

The complications discussed above hindered the development of an "ideal" quercetin precursor. Nonetheless, the transport experiments were promising. They highlight the fact that different cells (even ostensibly belonging to the same line) can treat polyphenol derivatives differently. All three reference lines we used handled quercetin much like intestinal enterocytes, allowing almost only conjugated derivatives to cross the monolayer. The same products were found in cellular extracts and progressively formed in the apical compartment, confirming the existence of two-way traffic between the cellular interior and the apical extracellular space. The observation that different Caco-2 lines exhibit different levels of conjugation activities may explain discrepancies between observations by different groups.[73,74,82]

The fate of the various derivatives depended on cell type. "Clone A" Caco-2 cells proved to be the best equipped with relevant hydrolase and transferase activities. Apparently, the esters which could diffuse into the cells were rapidly stripped of protective groups and the resulting quercetin was processed. The other lines were less efficient, sufficiently so that partially deacylated molecules were the major products in the basolateral compartment. The changes in the composition of the apical compartment were consistent with this pattern. Since most of the material found in basolateral samples had been metabolized, the transport process must have involved intracellular (as opposed to paracellular) pathways. Some compounds (**5d**, **5e**, **6e**) survived for at least 6 h in the presence of the cells with little change and did not cross the monolayers in detectable amounts. This resilience may be related to their particularly low solubility in aqueous media.

The total molar fraction of permeating quercetin derivatives reaching the basolateral side was not significantly higher than
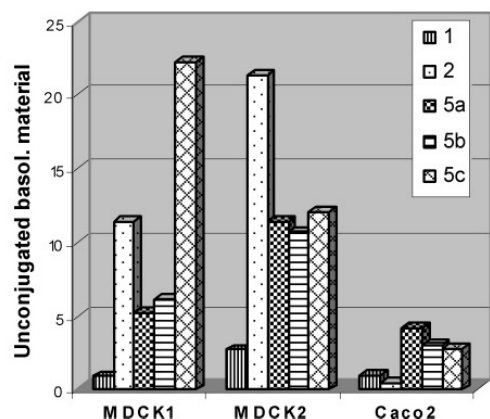
Figure 8. Use of precursors increases the portion of quercetin reaching the basolateral compartment without being conjugated. The histograms report, for the three cell lines, the molar percentage of the compounds placed in the apical compartment at time zero found to have translocated to the basolateral side after 6 h, without undergoing sulfation, methylation, or glucuronidation. Data from Table 4.

that of quercetin itself (Table 4), but in vivo the acylated species—which in favorable cases make up most of the basolateral material—would be expected to be rapidly converted to quercetin by esterases so that the net result would be an increase in the fraction of quercetin absorbed as such. This point is illustrated by Figure 8, which compares the total basolateral amounts of unconjugated compounds for the various precursors and cell lines. Esterases are found both inside and outside cells in practically all organs (especially in epithelia).[56–58] Although carboxylesterase EC 3.1.1.1 may not be present in human (as distinct from mammalian) blood,[87] the esterase activity of albumin,[87,88] which binds quercetin,[89,90] may well be sufficient for quercetin regeneration (Biasutto, L., et al., unpublished results).

A point of obvious relevance is the relationship between these observations in vitro and what may happen in vivo. Clarification of this point can only come from suitable experiments. Since Caco-2 cells are human, while MDCKs are canine (and renal), the results obtained with the former may seem more likely to predict the eventual outcome of these experiments. However, as already mentioned, several other Caco-2 clones afforded results similar to those obtained with MDCK-2 cells. Furthermore, absorption of polyphenols in the intestine, the prevalent site,[91] may or may not resemble processes handled by colonic tumoral cells. Caco-2 carboxyesterases are actually expressed in a pattern more resembling that of liver than that of the intestine.[92] For research and hypothetical treatment purposes, absorption via less aggressive epithelia may in any case be an option.

In conclusion, our results show that it may be feasible to develop ester-based precursors of naturally occurring polyphenols, capable of improving absorption through the intestinal tract or other less aggressive epithelia. In vivo tests and the development of a second generation of compounds are in the making.

4. Experimental Section

4.1. Chemistry. 4.1.1. General. Starting materials and reagents were purchased from Aldrich, Fluka, Merck-Novabiochem, J. T. Baker, Cambridge Isotope Laboratories Inc., Acros Organics, Carlo Erba, and Prolabo and were used as received. ^{1}H NMR spectra were recorded on a Bruker AC 250F spectrometer operating at 250

MHz. Chemical shifts (δ) are given in ppm relative to the signal of the solvent (δ 2.49 ppm, DMSO-d_6). Mass spectra were recorded on a Mariner ESI-TOF mass spectrometer (PerSeptive Biosystems) or an ESI MSD SL Trap mass spectrometer (Agilent Technologies). TLCs were run on silica gel supported on plastic (Macherey−Nagel PolygramSIL G/UV$_{254}$, silica thickness 0.2 mm) or on silica gel supported on glass (Fluka) (silica thickness 0.25 mm, granulometry 60 Å, 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. HPLC 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). Elemental analyses, HPLC−UV, and solubility determinations were done only for the compounds used for transport experiments (**2**, **5a−e**, **6e**). Elemental analyses were performed by the Microanalysis Laboratory of the Department of Chemical Sciences of the University of Padova. HPLC−UV was carried out as described in the transport experiments section.

4.1.2. Synthesis of Pentaacetylquercetin (2). Compound **2** was synthesized in 67% yield from **1**, according to a literature procedure.[63] Briefly, **1** (3.021 g, 8.93 mmol) in acetic anhydride (30 mL) and pyridine (2.7 mL) were heated to reflux for 5 h. Ice-water (50 g) was added to the warm mixture. The resulting precipitate was filtered and washed with water. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 2.32 (s, 3H, 3-OAc), 2.34 (s, 12H, aromatic-OAc), 7.16 (d, 1H, H-6, $J = 2.2$ Hz), 7.52 (d, 1H, H-5′, $J = 8.6$ Hz), 7.64 (d, 1H, H-8, $J = 2.2$ Hz), 7.35−7.65 (m, 2H, H-2′, H-6′). MS (ESI-TOF): m/z 513, [M + H]$^+$.

4.1.3. General Synthetic Procedure of Amino Acid Quercetin Esters. 4.1.3.1. 3′-Boc-amino Acid Quercetin Ester (3a−e). Boc-protected amino acids (Boc-D-alanine, 4-(Boc-amino)butanoic acid, 6-(Boc-amino)hexanoic acid, cis-4-(Boc-amino)cyclohexanecarboxylic acid, 4-(Boc-amino)benzoic acid) (5 mmol) were dissolved in CH$_2$Cl$_2$ (10 mL). DMAP (1 mmol) and EDC (5 mmol) were added, and the solution was stirred. Compound **1** (7.5 mmol), dissolved in DMF (5 mL), was added. The mixture was stirred for 1 h at room temperature. CH$_2$Cl$_2$ was evaporated under reduced pressure, and the mixture was dissolved in EtOAc (700 mL). The resulting solution was washed successively with 0.5 N HCl (3 × 100 mL), water (2 × 70 mL), 5% NaHCO$_3$ (3 × 100 mL), and water (2 × 70 mL), dried over Na$_2$SO$_4$, and filtered. The solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography to afford the 3′-substituted Boc-amino acid ester of quercetin containing 25% of the 4′-isomer, as determined by NMR analysis. The two isomers were not separated. Reported yields include both isomers; NMR data refer to the major one.

4.1.3.2. 3′-Amino Acid Quercetin Ester Hydrochloride (4a−e). The 3′-Boc-amino acid quercetin ester (0.1 mmol) was dissolved in 3.5 mL of a 3 N HCl solution in EtOAc. The mixture was stirred for 1 h at room temperature. The resulting precipitate was filtered and washed with diethyl ether.

4.1.3.3. 3′-Boc-amino Acid Tetraacetylquercetin (5a−e). The 3′-Boc-amino acid quercetin ester (0.6 mmol) was dissolved in CH$_2$-Cl$_2$ (12 mL). Acetic anhydride (13.70 mmol) and pyridine (1.2 mL) were added, and the mixture was stirred for 5 h at room temperature. CH$_2$Cl$_2$ was evaporated under reduced pressure, and the mixture was dissolved in EtOAc (20 mL) and washed with 0.5 N HCl (3 × 7 mL), water (2 × 5 mL), 5% NaHCO$_3$ (3 × 7 mL), and water (2 × 5 mL). The EtOAc solution was dried over Na$_2$SO$_4$ and filtered. EtOAc was evaporated under reduced pressure to afford the acetylation products.

4.1.3.4. 3′-Amino Acid Tetraacetylquercetin Hydrochloride (6a−e). 3′-Boc-amino acid tetraacetylquercetin (0.078 mmol) was dissolved in a solution of 3 N HCl in EtOAc (3 mL). The mixture was stirred at room temperature for 1 h. The resulting precipitate was filtered and washed with diethyl ether.

3′-Boc-D-alanine Quercetin Ester (3a). Eluents of flash chromatography: CH$_2$Cl$_2$/EtOAc 3:1. Yield: 28%. ^{1}H NMR (250 MHz,

DMSO-d_6) δ (ppm): 1.38−1.48 (s, 12H, CH$_3$), 4.32 (br, 1H, CH), 6.18 (d, 1H, J = 2.2 Hz), 6.41 (d, 1H, J = 2.2 Hz), 7.07 (d, 1H, H-5′, J = 8.7 Hz), 7.41 (br, 1H, NH), 7.81 (d, 1H, H-2′, J = 2.2 Hz), 7.95 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-TOF): m/z 474 [M + H]$^+$, m/z 947 [M + H]$^+$(M), m/z 418 [MH−CH$_2$= C(CH$_3$)$_2$]$^+$.

3′-D-Alanine Quercetin Ester Hydrochloride (4a). Yield: 84%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.60 (d, 3H, CH$_3$), 4.45 (q, 1H, CH), 6.21 (d, 1H, J = 2.2 Hz), 6.46 (d, 1H, J = 2.2 Hz), 7.17 (d, 1H, H-5′, J = 8.2 Hz), 8.08 (m, 2H, H-2′, H-6′), 8.64 (br, $^+$NH$_3$). MS (ESI-TOF): m/z 374 [M + H]$^+$.

3′-Boc-D-alanine Tetraacetylquercetin Ester (5a). Yield: 80%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.30−1.70 (m, 12H, CH$_3$), 2.25−2.65 (m, 12H, OAc), 4.30 (m, 1H, CH), 7.16 (d, 1H, J = 2.2 Hz), 7.54 (d, 1H, H-5′, J = 8.7 Hz), 7.59 (br, 1H, NH), 7.64 (d, 1H, J = 2.2 Hz), 7.75 (d, 1H, H-2′, J = 2.2 Hz), 7.87 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-TOF): m/z 642 [M + H]$^+$, m/z 586 [MH−CH$_2$=C(CH$_3$)$_2$]$^+$.

3′-D-Alanine Tetraacetylquercetin Ester Hydrochloride (6a). Yield: 84%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.57 (d, 3H, CH$_3$), 2.21−2.41 (m, 12H, OAc), 4.51 (br, 1H, CH), 7.18 (d, 1H, J = 2.2 Hz), 7.60 (d, 1H, H-5′, J = 8.7 Hz), 7.62 (d, 1H, J = 2.2 Hz), 7.86 (d, 1H, H-2′, J = 2.2 Hz), 7.93 (dd, 1H, H-6′, J = 8.7, 2.2 Hz), 8.59 (br, $^+$NH$_3$). MS (ESI-TOF): m/z 542 [M + H]$^+$, m/z 500 [MH− CH$_2$CO]$^+$.

3′-[4-(Boc-amino)butanoic Acid] Quercetin Ester (3b). Eluents of flash chromatography: CHCl$_3$/CH$_3$OH 9:1. Yield: 16%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.38 (s, 9H, CH$_3$), 1.74 (quintet, 2H, CH$_2$-β), 2.60 (t, 2H, CH$_2$-α), 3.03 (q, 2H, CH$_2$-γ), 6.19 (d, 1H, J = 2.2 Hz), 6.45 (d, 1H, J = 2.2 Hz), 6.91 (t, 1H, NH), 7.06 (d, 1H, H-5′, J = 8.7 Hz), 7.85 (d, 1H, H-2′, J = 2.2 Hz), 7.95 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-TOF): m/z 488 [M + H]$^+$, m/z 432 [MH−CH$_2$=C(CH$_3$)$_2$]$^+$, m/z 975 [M + H]$^+$(M).

3′-[4-Aminobutanoic Acid] Quercetin Ester Hydrochloride (4b). Yield: 80%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.89 (quintet, 2H, CH$_2$-β), 2.76 (t, 2H, CH$_2$-α), 2.88 (br, 2H, CH$_2$-γ), 6.21 (d, 1H, J = 2.2 Hz), 6.47 (d, 1H, J = 2.2 Hz), 7.14 (d, 1H, H-5′, J = 8.7 Hz), 7.87 (d, 1H, H-2′, J = 2.2 Hz), 7.94 (dd, 1H, H-6′, J = 8.7, 2.2 Hz), 7.93 (br, $^+$NH$_3$). MS (ESI-TOF): m/z 388 [M + H]$^+$.

3′-[4-(Boc-amino)butanoic Acid] Tetraacetylquercetin Ester (5b). Yield: 77%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.37 (s, 9H, CH$_3$), 1.74 (quintet, 2H, CH$_2$-β), 2.31 (s, 3H, 3-OAc), 2.33 (s, 9H, aromatic-OAc), 2.62 (t, 2H, CH$_2$-α), 3.01 (q, 2H, CH$_2$-γ), 6.92 (t, 1H, NH), 7.16 (d, 1H, J = 2.2 Hz), 7.52 (d, 1H, H-5′, J = 9.0 Hz), 7.64 (d, 1H, J = 2.2 Hz), 7.76−7.93 (m, 2H, H-2′, H-6′). MS (ESI-ion trap): m/z 678 [M + Na]$^+$, m/z 600 [MH−CH$_2$= C(CH$_3$)$_2$]$^+$.

3′-[4-Aminobutanoic Acid] Tetraacetylquercetin Ester Hydrochloride (6b). Yield: 84%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.94 (quintet, 2H, CH$_2$-β), 2.29 (s, 3H, 3-OAc), 2.33 (s, 9H, aromatic-OAc), 2.78 (t, 2H, CH$_2$-α), 2.87 (br, 2H, CH$_2$-γ), 7.17 (d, 1H, J = 2.2 Hz), 7.53 (d, 1H, H-5′, J = 8.7 Hz), 7.64 (d, 1H, J = 2.2 Hz), 7.78−8.02 (br, $^+$NH$_3$), 7.81−7.91 (m, 2H, H-2′, H-6′). MS (ESI-TOF): m/z 556 [M + H]$^+$.

3′-[6-(Boc-amino)hexanoic Acid] Quercetin Ester (3c). Eluents of flash chromatography: CHCl$_3$/CH$_3$OH 9:1. Yield: 24%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.36 (s, 9H, CH$_3$), 1.30−1.53 (m, 4H, CH$_2$), 1.63 (quintet, 2H, CH$_2$), 2.59 (t, 2H, CH$_2$), 2.85−2.98 (m, 2H, CH$_2$), 6.19 (d, 1H, J = 2.2 Hz), 6.45 (d, 1H, J = 2.2 Hz), 6.78 (t, 1H, NH), 7.06 (d, 1H, H-5′, J = 8.7 Hz), 7.85 (d, 1H, H-2′, J = 2.2 Hz), 7.96 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-TOF): m/z 516 [M + H]$^+$, m/z 460 [MH−CH$_2$= C(CH$_3$)$_2$]$^+$, m/z 1031 [M + H]$^+$(M).

3′-[6-Aminohexanoic Acid] Quercetin Ester Hydrochloride (4c). Yield: 79%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.34− 1.52 (m, 2H, CH$_2$), 1.52−1.78 (m, 4H, CH$_2$), 2.62 (t, 2H, CH$_2$), 2.78 (sextet, 2H, CH$_2$), 6.21 (d, 1H, J = 2.2 Hz), 6.47 (d, 1H, J = 2.2 Hz), 7.11 (d, 1H, H-5′, J = 8.7 Hz), 7.85 (d, 1H, H-2′, J = 2.2

Hz), 7.88 (br, $^+$NH$_3$), 7.93 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-TOF): m/z 416 [M + H]$^+$.

3′-[6-(Boc-amino)hexanoic Acid] Tetraacetylquercetin Ester (5c). Yield: 68%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.36 (s, 9H, CH$_3$), 1.27−1.53 (m, 4H, CH$_2$), 1.63 (quintet, 2H, CH$_2$), 2.31 (s, 3H, 3-OAc), 2.33 (s, 9H, aromatic-OAc), 2.62 (t, 2H, CH$_2$), 2.85−2.97 (m, 2H, CH$_2$), 6.79 (br t, 1H, NH), 7.16 (d, 1H, J = 2.2 Hz), 7.52 (d, 1H, H-5′, J = 8.2 Hz), 7.64 (d, 1H, J = 2.2 Hz), 7.77−7.91 (m, 2H, H-2′, H-6′). MS (ESI-ion trap): m/z 706 [M + Na]$^+$, m/z 722 [M + K]$^+$.

3′-[6-Aminohexanoic Acid] Tetraacetylquercetin Ester Hydrochloride (6c). Yield: 82%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.31−1.49 (m, 2H, CH$_2$), 1.49−1.74 (m, 4H, CH$_2$), 2.33 (s, 12H, OAc), 2.65 (t, 2H, CH$_2$), 2.69−2.82 (m, 2H, CH$_2$), 7.17 (br, 1H), 7.53 (br d, 1H, H-5′, J = 8.2 Hz), 7.64 (br, 1H), 7.73− 7.91 (br, 5H, H-2′, H-6′, $^+$NH$_3$). MS (ESI-TOF): m/z 584 [M + H]$^+$.

3′-[*cis*-4-(Boc-amino)cyclohexanecarboxylic Acid] Quercetin Ester (3d). Eluents of flash chromatography: CHCl$_3$/CH$_3$OH 9:1. Yield: 22%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.37 (s, 9H, CH$_3$), 1.47−1.77 (m, 6H, CH$_2$), 1.90−2.12 (m, 2H, CH$_2$), 2.80 (br, 1H, CH), 3.41 (br, 1H, CH), 6.19 (d, 1H, J = 2.2 Hz), 6.45 (d, 1H, J = 2.2 Hz), 6.83 (br d, 1H, NH), 7.07 (d, 1H, H-5′, J = 8.7 Hz), 7.81 (d, 1H, H-2′, J = 2.2 Hz), 7.94 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-ion trap): m/z 550 [M + Na]$^+$.

3′-[*cis*-4-Amino-cyclohexanecarboxylic Acid] Quercetin Ester Hydrochloride (4d). Yield: 76%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.58−1.96 (m, 6H, CH$_2$), 2.04−2.28 (m, 2H, CH$_2$), 2.96 (br, 1H, CH), 3.15 (br, 1H, CH), 7.17 (d, 1H, H-5′, J = 8.7 Hz), 7.78−7.97 (br, 5H, H-2′, H-6′, $^+$NH$_3$). MS (ESI-ion trap): m/z 428 [M + H]$^+$.

3′-[*cis*-4-(Boc-amino)cyclohexanecarboxylic Acid] Tetraacetylquercetin Ester (5d). Yield: 80%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.37 (s, 9H, CH$_3$), 1.42−1.84 (br m, 6H, CH$_2$), 1.88−2.10 (m, 2H, CH$_2$), 2.21 (s, 3H, 3-OAc), 2.32 (m, 9H, aromatic-OAc), 2.85 (br, 1H, CH), 3.41 (br, 1H, CH), 6.88 (br d, 1H, NH), 7.17 (d, 1H, J = 2.2 Hz), 7.53 (d, 1H, H-5′, J = 8.2 Hz), 7.65 (d, 1H, J = 2.2 Hz), 7.76−7.93 (m, 2H, H-2′, H-6′). MS (ESI-ion trap): m/z 718 [M + Na]$^+$, m/z 734 [M + K]$^+$, m/z 640 [MH− CH$_2$=C(CH$_3$)$_2$]$^+$.

3′-[*cis*-4-Amino-cyclohexanecarboxylic Acid] Tetraacetylquercetin Ester Hydrochloride (6d). Yield: 75%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.51−1.95 (br m, 6H, CH$_2$), 1.98−2.20 (br m, 2H, CH$_2$), 2.07 (s, 3H, 3-OAc), 2.32 (m, 9H, aromatic-OAc), 3.03 (br, 1H, CH), 4.26 (br, 1H, CH), 7.17 (d, 1H, J = 2.2 Hz), 7.55 (d, 1H, H-5′, J = 8.2 Hz), 7.64 (d, 1H, J = 2.2 Hz), 7.76− 7.94 (m br, 5H, H-2′, H-6′, $^+$NH$_3$). MS (ESI-ion trap): m/z 596 [M + H]$^+$.

3′-[4-(Boc-amino)benzoic Acid] Quercetin Ester (3e). Eluents of flash chromatography: CHCl$_3$/CH$_3$OH 9:1. Yield: 12%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.49 (s, 9H, CH$_3$), 6.18 (d, 1H, J = 1.65 Hz), 6.45 (d, 1H, J = 1.6 Hz), 7.11 (d, 1H, H-5′, J = 8.2 Hz), 7.58−7.75 (m, 2H), 7.91−8.12 (m, 4H), 9.88 (s, 1H, NH). MS (ESI-ion trap): m/z 522 [M + H]$^+$.

3′-[4-Aminobenzoic Acid] Quercetin Ester Hydrochloride (4e). Yield: 70%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 5.00 (br, $^+$NH$_3$), 6.19 (d, 1H, J = 2.2 Hz), 6.47 (d, 1H, J = 2.2 Hz), 6.63−6.81 (m, 2H), 7.11 (d, 1H, H-5′, J = 8.2 Hz), 7.76−7.88 (m, 2H), 7.91 (d, 1H, H-2′, J = 2.2 Hz), 7.97 (dd, 1H, H-6′, J = 8.2, 2.2 Hz). MS (ESI-ion trap): m/z 422 [M + H]$^+$.

3′-[4-(Boc-amino)benzoic Acid] Tetraacetylquercetin Ester (5e). Yield: 75%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.49 (s, 9H, CH$_3$), 2.18 (s, 3H, 3-OAc), 2.28−2.41 (m, 9H, aromatic-OAc), 7.16 (d, 1H, J = 2.2 Hz), 7.57 (d, 1H, H-5′, J = 8.2 Hz), 7.63−7.77 (m, 3H), 7.85−7.95 (m, 2H), 7.96−8.10 (m, 2H, H-2′, H-6′), 9.94 (s, 1H, NH). MS (ESI-ion trap): m/z 690 [M + H]$^+$, m/z 712 [M + Na]$^+$, m/z 728 [M + K]$^+$, m/z 634 [MH−CH$_2$= C(CH$_3$)$_2$]$^+$.

3′-[4-Aminobenzoic Acid] Tetraacetylquercetin Ester Hydrochloride (6e). Yield: 53%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 2.18 (s, 3H, 3-OAc), 2.31−2.38 (m, 9H, aromatic-OAc),

4.58 (br, $^+NH_3$), 6.60−6.74 (m, 2H), 7.16 (d, 1H, $J = 2.2$ Hz), 7.53 (d, 1H, H-5′, $J = 8.8$ Hz), 7.67 (d, 1H, $J = 2.2$ Hz), 7.72−7.82 (m, 2H), 7.85 (dd, 1H, H-6′, $J = 8.8, 2.2$ Hz), 7.93 (d, 1H, H-2′, $J = 2.2$ Hz). MS (ESI-ion trap): m/z 590 [M + H]$^+$, m/z 548 [MH−CH$_2$=O]$^+$.

4.2. Chemical Stability Studies. 4.2.1. UV−vis Spectrophotometry. The chemical stability of the synthesized compounds in water-containing solutions was tested following changes in the UV−vis spectra between 190 and 500 nm in PBS (20 mM, pH 7.0, $\mu = 0.1$ M with KCl)/CH$_3$CN 1:1 mixtures at 25 °C. The reaction was initiated by adding 100 μL of a freshly prepared solution of the selected substrate in acetonitrile to 3 mL of a PBS/CH$_3$CN mixture to give a final concentration in the $(3-8) \times 10^{-5}$ M range. Spectral changes were followed with a Perkin-Elmer Lambda 5 spectrophotometer equipped with water-thermostated cell holders. Quartz cells with an optical path of 1 cm were used for all measurements. The observed rate constants for the hydrolysis of **3a−d** and **4a−d** were obtained by following the absorption changes at 255.2 nm for at least $3t_{1/2}$ and by interpolating the absorbance readings versus time with eq 1.

$$A = A_\infty + (A_0 - A_\infty)e^{-k_{obs}t} \tag{1}$$

In the case of two sequential processes, as observed for derivatives **6a−d**, the reactions were followed at 396.0 nm. Interpolation according with eq 2, where [Int] is the concentration of the intermediate product, tetraacetylquercetin, and ϵ_{Int} is its molar absorbtivity, provided the observed rate constants for the two consecutive hydrolysis steps, $k_{1,obs}$ and $k_{2,obs}$. All determinations were performed at least twice.

$$A = A_0 e^{-k_{1,obs}t} + \frac{\epsilon_{Int}k_{1,obs}}{k_{2,obs} - k_{1,obs}}[Int](e^{-k_{1,obs}t} - e^{-k_{2,obs}t}) +$$
$$A_\infty \left(\frac{k_{2,obs}(1 - e^{-k_{1,obs}t}) - k_{1,obs}(1 - e^{-k_{2,obs}t})}{k_{2,obs} - k_{1,obs}} \right) \tag{2}$$

4.2.2. HPLC−UV and LC−MS. The hydrolysis of **6a−d** was monitored by HPLC−UV and LC−ESI-MS to identify the products. The reaction, carried out as described in the preceding paragraph, was quenched at different times by adding 3 μL of pure HCOOH. Chromatography was also used to verify the stability of compounds **3e**, **4e**, **5e**, and **6e**. In the case of the first two, the absorption spectra are undistinguishable from those of a 1:1 mixture of quercetin and 4-(Boc-amino)benzoic acid, the theoretical hydrolysis products. HPLC−UV analyses were performed with the Thermo Separation Products system mentioned above. The sample solution (20 μL) was injected into a reversed-phase column (Synergi-MAX, 4 μm, 150 mm × 4.6 mm i.d.; Phenomenex). Solvents A and B were H$_2$O/CH$_3$CN 9:1 and CH$_3$CN both containing 0.2% HCOOH: the acid was necessary to avoid hydrolytic processes inside the column. The gradient for B was as follows: from 20% to 30% in 15 min and then to 100% in 15 min; the flow rate was 1 mL/min. Chromatograms were obtained plotting data recorded at 300 and 370 nm. 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 from 100 to 1500 m/z in positive ion mode. The column, solvents, and gradient profile were the same as those used for HPLC−UV analyses.

4.3. Solubility in HBSS. About 1 mg of compounds **2**, **5a−e**, and **6e** was added to 20 mL of HBSS and sonicated for 10 min. The mixtures were then allowed to settle for 24 h, and the supernatants were analyzed by HPLC−UV.

4.4. Cell Assays. 4.4.1. 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$_2$PO$_4$ 0.44, Na$_2$HPO$_4$ 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, according to the producer; 0.4 μm pore size) were purchased from Cellbio.

MDCK-1, MDCK-2, and Caco-2 cell lines were kindly provided by the groups of Professors C. Montecucco, M. De Bernard, S. Garbisa, and E. Papini of the Department of Biomedical Sciences, University of Padova. Another Caco-2 clone, originally from ATCC, was purchased from the Istituto Zooprofilattico di Brescia (Italy). MDCK (Madin−Darby Canine Kidney) cells derive from dog kidney. MDCK-1 cells form tight, high-resistance (up to 20 kΩ·cm^2) epithelia. The closely related MDCK-2 line originated from the same tissue but has "leaky" tight junctions and produces monolayers with approximately 50-fold lower trans epithelial resistance (TER). The difference is due to the expression by MDCK-2 cells of zonula occludens protein claudin 2 in addition to claudins 1 and 4.[93] Caco-2 is a human colorectal adenocarcinoma line, known to exhibit heterogeneity. Its monolayers characteristically exhibit TER values of a few hundred Ω·cm^2.

4.4.2. Cell Culture and Monolayer Formation. Cells were seeded in culture flasks and passaged in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (Biospa), 1% penicillin/streptomycin solution (10 000 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). The cells were seeded onto tissue culture-treated Transwell Clear inserts at a density of 3 × 10^5 cells per cm^{-2} for MDCK-1 and Caco-2 and 5 × 10^5 cells per cm^{-2} for MDCK-2. Monolayers were grown in a humidified atmosphere of 5% CO$_2$ at 37 °C. TER was measured periodically using a Millipore Millicell-ERS epithelial volt−ohmmeter, and experiments were performed when values reached approximately 10−15 kΩ·cm^2 for MDCK-1, 100−300 Ω·cm^2 for MDCK-2, and 1.5−2 kΩ·cm^2 for Caco-2 (10−20 days postseeding). These values are in line with literature data,[94−96] indicating the formation of epithelia with the appropriate "tightness" of cell−cell junctions. This property was also assessed measuring the diffusion of Lucifer yellow from the apical to the basolateral compartment[97] (see the Supporting Information).

4.4.3. Transport across Cell Monolayers. The cell layers were washed twice with warm HBSS. The initial stock solution of compounds **1**, **2**, **5a−e**, and **6e** was freshly made in DMSO and was then diluted with HBSS to give formally 30−50 μM solutions (final DMSO concentration 0.1%) and sonicated to obtain a finer dispersion of undissolved suspended material. This 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). In most instances the compound was added simultaneously to the apical compartments of four inserts, and the apical and basolateral solutions were collected after 0.5, 1.5, 3, and 6 h. In other cases samples were obtained only at 3 or 6 h.

Apical and basolateral solutions were transferred into glass vials and frozen for further analysis by HPLC−UV and LC−MS. In some cases the Transwell inserts (cells and supporting septum) were extracted with 1 mL of methanol containing ascorbic acid (1 mM) by sonication for 20 min. The solution was filtered through 0.45 μm PTFE syringe filters (Chemtek analytica) and stored at −20 °C until analyzed. Controls showed that filtration did not result in any loss of solutes.

Control experiments were run by incubating the cells in the presence of only DMSO at a final concentration of 0.1% in HBSS to assess background signals in the HPLC analyses. Additional controls were run by incubating each compound in the absence of cells to verify the chemical stability of the compounds under the conditions used for transport experiments.

Basolateral solutions were analyzed without any treatment; apical solutions were diluted with an equal volume of CH$_3$CN and filtered through 0.45 μm PTFE filters to eliminate cell residues. HPLC−UV and LC−MS analyses were carried out with the same instruments and the same column described above for the chemical stability studies but with different solvents and a different gradient. Solvent A was H$_2$O/THF/TFA (98:2:0.1, v/v/v) and B was CH$_3$-

CN. 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 sulfated derivatives were best observed at 370 nm, acylquercetins and quercetin-*o*-quinone at 300 nm. All compounds, except the quinone, were however visible, with lower sensitivity, also at the other wavelength. LC−MS was performed on selected samples both in positive and negative ion mode.

Metabolites were identified by their UV−vis spectra, mass spectra, the use of a 4′-methylquercetin standard, and experiments with sulfatase (in the case of sulfates). These last experiments consisted in the treatment of portions of selected samples with sulfatase (*Aerobacter aerogenes*, Sigma-Aldrich), followed by comparison of HPLC traces with those of untreated portions. A solution (400 μL) of the sample was concentrated to half-volume and incubated with sulfatase (0.4 U, 25 μL) at 37 °C for 30 min. After addition of 200 μL of methanol containing 1 mM ascorbic acid, the sample was centrifuged (13 600*g*, 4 °C, 5 min), filtered through 0.45 μm PTFE syringe filters, and analyzed by HPLC−UV.

For quantification purposes, the concentration was calculated on the basis of standard curves. The quantification limit on the diode array detector was determined to be 0.2 μM. The calibration curve built using **1** was also used for quercetin sulfate, methylquercetin sulfate, and acetylquercetin, assuming for these compounds the same absorption coefficient ϵ_{370} of quercetin; the calibration curve of **2** was used for diacetylquercetin assuming ϵ_{300} to be similar to that of pentaacetylquercetin. 3′-Boc-amino acid triacetyl quercetin esters were quantified using the calibration curve of the corresponding 3′-Boc-amino acid tetraacetyl quercetin esters, assuming the same absorption coefficient at 300 nm.

Supporting Information Available: Elemental analyses of the compounds and tests of monolayer permeability to Lucifer yellow. This material is available free of charge via the Internet at http://pubs.acs.org.

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27

Supporting information

Ester-based precursors to increase the bioavailability of quercetin

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Table of contents

S1

28

Table 1S. Elemental analyses of target compounds.

Compd	Name	Formula	Calcd			Found		
			C	H	N	C	H	N
2	Pentaacetylquercetin	$C_{25}H_{20}O_{12}$	58.60	3.93	-	58.72	3.68	-
5a	3'-Boc-D-alanine tetraacetylquercetin ester	$C_{31}H_{31}NO_{14}$	58.03	4.87	2.18	57.51	4.83	2.11
5b	3'-[4-(Boc-amino)butanoic acid] tetraacetylquercetin ester	$C_{32}H_{33}NO_{14}$	58.62	5.07	2.14	58.04	5.12	2.08
5c	3'-[6-(Boc-amino)hexanoic acid] tetraacetylquercetin ester	$C_{34}H_{37}NO_{14}$	59.73	5.45	2.05	59.72	5.45	1.95
5d	3'-[cis-4-(Boc-amino) cyclohexanecarboxylic acid] tetraacetylquercetin ester	$C_{35}H_{37}NO_{14}$	60.43	5.36	2.01	59.20	5.44	1.85
5e	3'-[4-(Boc-amino)benzoic acid] tetraacetyl quercetin ester	$C_{35}H_{31}NO_{14}$	60.96	4.53	2.03	59.67	4.69	1.89
6e	3'-[4-aminobenzoic acid] quercetin ester hydrochloride	$C_{30}H_{23}NO_{12} \cdot HCl$	57.56	3.86	2.24	57.12	3.61	2.17

Tests of monolayer permeability to Lucifer Yellow

"Tightness" of supported monolayers was verified in control experiments in which 50 μM Lucifer Yellow was placed in the apical compartment. Transepithelial diffusion was evaluated by measuring the fluorescence (λ_{ex}: 428 nm; λ_{em}: 540 nm; fluorimeter: Perkin-Elmer 650-40) of suitably diluted apical and basolateral samples taken after 3 or 6 hours. With all three cell types, at these time points the portion of dye reaching the basolateral compartment was between 0.3 and 3% in the case of 1-cm^2 inserts and between 0.8 and 6% in the case of the larger ones (N = 13). By comparison, in control experiments the fraction of dye found to have diffused through the porous set itself (no cells) at the same time points was about 30 and 50%, respectively, for 1- cm^2 septa and about 41 and 55% for 4.7-cm^2 septa. It should be stressed that these data can only provide an assessment of cell monolayer aspecific (im)permeability, and have little to say about the mechanism of transepithelial "diffusion" of quercetin and its derivatives. Lucifer Yellow, like other possible permeability probes such as mannitol, is a soluble (and charged), hydrophilic molecule, while the tested compounds have low solubility, are uncharged, rather hydrophobic and were present mostly as suspended microaggregates.

S3

2. Heterogeneity and standardization of Phase II metabolism in cultured cells

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Running title: Caco-2 Phase II metabolism

Keywords

Caco-2, Phase II metabolism, sulfotransferase, glucuronosyltransferase, methyltransferase, SULT, UGT, COMT, detoxification, xenobiotics

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Abstract

Caco-2 cells are widely used for transepithelial transport and metabolism studies. We analysed the metabolites produced from quercetin (Q) during transport 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 basolateral 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 ready-made 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 $\times$ 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 $\Omega \times$ 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$\times$ 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$\times$ 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).
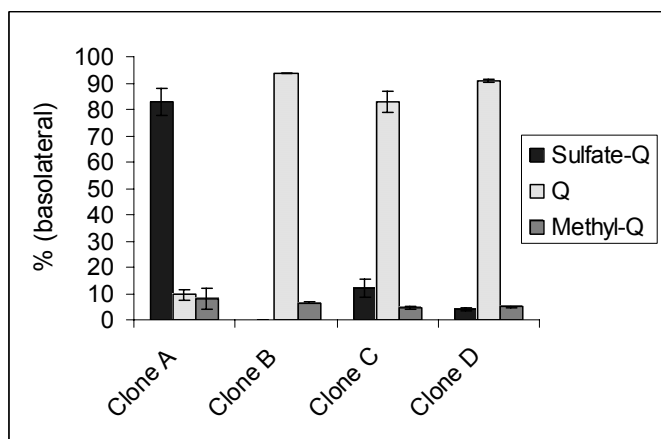

Fig. 1. A comparison of the conjugating activity of four Caco-2 clones. Relative abundance of quercetin and its major Phase II metabolites (sulfate- and methyl- quercetin) found after 6 hours in the basolateral compartment of Transwell®-supported monolayers exposed to (formally) 50 µM quercetin on the 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).
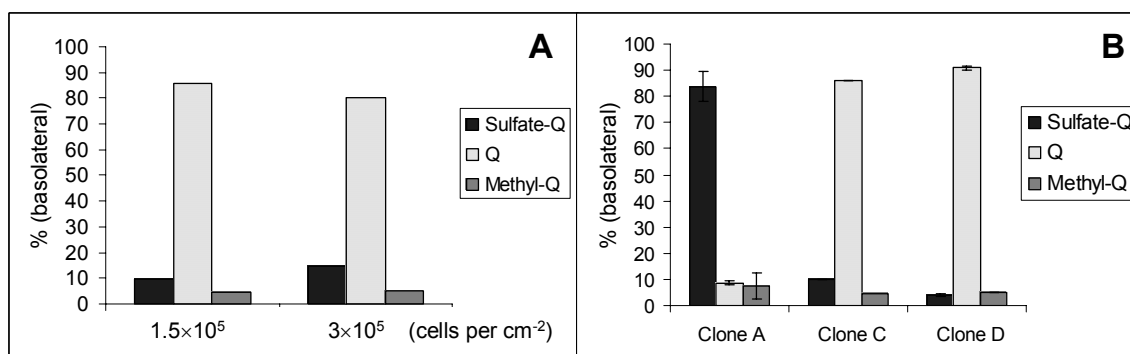
Fig. 2. Metabolic characteristics do not depend on cell density at seeding or on the value of Trans Epithelial Resistence (TER) of the differentiated monolayer. Shown is the relative abundance of quercetin and its major Phase II metabolites (sulfate- and methyl- quercetin) found after 6 hours on the 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).
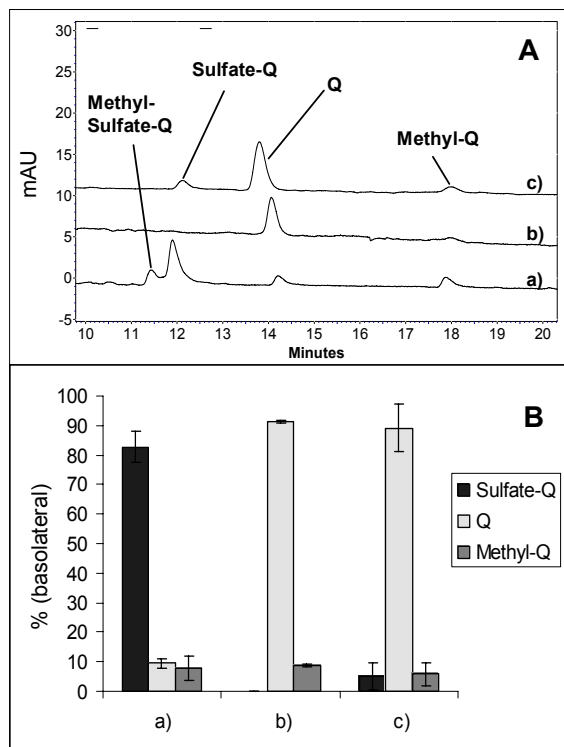

Fig. 3. Loss of metabolic activity. Relative abundance of quercetin and its major Phase II metabolites found after 6 hours on the basolateral side of Transwell®-supported monolayers exposed to (formally) 50 μM quercetin on the apical side. Experiments with clone A at different times: a) after only a 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 (see Materials and Methods). 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).
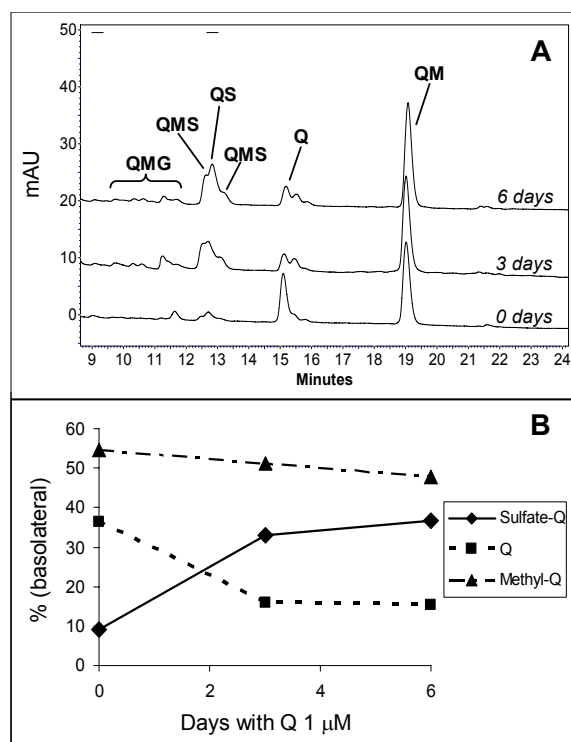

Fig. 4. Recovery of sulfotransferase activity. A) HPLC Chromatograms (370 nm) and B) relative abundance of quercetin and its major Phase II metabolites found in the medium and cells exposed to 20 μM Q for 8 hours. The data come from a representative experiment (out of 5) obtained with the same clone 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].

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3. DETERMINATION OF QUERCETIN AND RESVERATROL IN WHOLE BLOOD. IMPLICATIONS FOR PHARMACOKINETIC STUDIES

Submitted

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Abstract

Resveratrol (trans-3,4',5-trihydroxystilbene) and quercetin (3,3',4',5,7-pentahydroxy flavone) are two natural occurring polyphenols with the potential to exert beneficial health effects. Since their low bioavailability is a major obstacle to biomedical applications, an accurate evaluation of their levels in the bloodstream is important to assess delivery strategies and to verify claims of efficacy based on *in vitro* results. In the present work we optimized a simple method ensuring complete extraction and stabilization of resveratrol and quercetin from whole blood. The suitability of different protocols was evaluated measuring the recovery of polyphenol and internal standard from spiked blood samples via HPLC-UV analysis. The optimized procedure ensured a satisfactory recovery of both internal standards and compounds. We then compared analyses of plasma and of whole blood, and found that up to 70% of the analyte was unaccounted for when examining only plasma, the remainder being associated with the cellular fraction. This indicates the importance of analysing whole blood rather than plasma to avoid underestimating the polyphenol content in the bloodstream during *in vivo* bioavailability studies.

Introduction

Polyphenols are a vast class of plant-made molecules exhibiting, at least *in vitro*, a variety of properties and effects of relevance for many areas of health care.[1] These include protection for the cardiovascular and nervous systems, prevention and inhibition of cancer growth and spread, contrasting aging, reduction of chronic inflammation and lengthening of the lifespan of model organisms. These effects are ascribed in part to the redox properties of these molecules, and in part to their interaction with signalling proteins. Several such interactions have been identified, with affinities ranging from the nM (e.g. EGCG and the laminin receptor)[2,3] to the µM (e.g.: resveratrol and IKK;[4,5] quercetin and COX-1 and -2 [6]).

Quercetin and resveratrol are among the polyphenols attracting most attention, because of their relative abundance in foods and of the potential therapeutic and disease-preventing usefulness of their multiple biological effects (for recent revs. see, e.g.: refs. 7-9 for quercetin; refs. 10-12 for resveratrol). Furthermore, they have long been, quercetin in particular, favorite model compounds for studies dealing with bioavailability, absorption and metabolism.[13-16]

Several methods for the analysis of these polyphenols in biological samples (plasma, urine, tissues) have been developed.[17-23] Pharmacokinetic studies have been carried out focusing in most cases on the levels of polyphenols in plasma/serum.[24-26] Analysis of plasma, however, does not accurately report circulating levels, since quercetin[27-28] and resveratrol[29] are known to associate with cell membranes, haemoglobin and other blood proteins. This may result in an underestimate of the levels of the biologically active compound while the content in whole blood ought to more adequately reflect total uptake. Analysis of whole blood is indeed preferred for other drugs with low solubility in water and high degree of association to blood components, for example propofol[30], methotrexate[31] or cyclosporine-A.[32]

The aim of the present work was the development and optimization of a simple method for resveratrol and quercetin analysis in whole blood.

Material and methods

Instruments. HPLC-UV analyses were performed by a Thermo Separation Products Inc. system equipped with a P2000 Spectra System pump, a UV6000LP diode array detector (190-500 nm).

Chemicals. Materials were purchased from Sigma/Aldrich/Fluka/Riedel de-Haen, Merk-Novabiochem, and were used as received. Resveratrol was purchased from Waseta Int. Trading Co. (Shangai, P.R.China) and 2',5,7-trihydroxyflavone from Indofine (Hillsborough, NJ, USA).

Preparation of samples. 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 resveratrol or quercetin (dilution from 1000x stock solutions in DMSO, 0.1% final DMSO). 200 µL aliquots were taken and processed to establish the suitability of the method for the analysis of small volumes, such as blood samples collected from rats at different times during pharmacokinetic studies. Before starting the treatment, internal standard was added (4,4'-dihydroxybiphenyl and 2',5,7-trihydroxyflavone respectively for resveratrol and quercetin; dilution from 50x stock solutions in methanol, 25 µM final concentration). In the protocol eventually adopted, 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 R.T. under N_2 flow or by using a rotational vacuum concentrator (Martin Christ, RVC 2-25), and 30 µL of CH_3CN were added to precipitate residual proteins; after centrifugation, cleared samples were directly subjected to HPLC-UV analysis.

Analyte concentration in the spiked samples was 0.15, 0.2, 0.5, 2.0 and 5.0 µM. Internal standard was added at the same concentration (25 µM) in all samples.

Spiked samples were also incubated at 37°C for different time periods (5, 15, 45 and 60 minutes) before initiating treatment, to verify whether interactions with blood components varied in time, with a possible impact on recovery. Indeed we found that at the later time points of this incubation some methylation of quercetin had taken place. Recovery was thus calculated as the sum of quercetin and methylquercetin considering for the latter the same instrumental response as for quercetin.

Comparison of plasma and whole blood. Blood samples spiked with 5 µM resveratrol or quercetin (no other additions) were incubated at 37°C for 15, 30 or 45 minutes to allow time for the analytes to interact with blood components. They were then split into two equal aliquots before treatment, and plasma was obtained from one of these by centrifugation (500 g, 10 min, 4°C). Whole blood, plasma and the precipitated blood cells were treated in the same manner, as described above.

HPLC-UV analysis. Samples (20 μL) were analyzed using a reverse phase column (Gemini C18, 3 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: 30% for 5 min, from 30% to 60% in 15 min, from 60% to 100% in 5 min. The flow rate was 0.7 mL/min. The eluate was preferentially monitored at 286 nm for 4,4'-dihydroxybiphenyl, 320 nm for resveratrol, and 370 nm for quercetin and 2',5,7-trihydroxyflavone.

Calibration/standard curves. 0.15, 0.2, 0.5, 1.0, 5.0 and 25 μM solutions in $H_2O:CH_3CN$ 7:3 of resveratrol or quercetin were analyzed by HPLC-UV as described above; peak area (at 320 and 370 nm, respectively) was plotted against concentration to establish the correlation between peak area and amount analyzed.

Results and discussion

Development of the sample treatment protocol. Routine analyses in pharmacokinetic studies are best carried out using an internal standard, added in known amount and concentration to each freshly obtained blood sample. After screening a set of candidates with regard to structural similarity, stability and good resolution in the HPLC chromatogram, 4,4'-dihydroxybiphenyl and 2',5,7-trihydroxyflavone were chosen as suitable internal standards for resveratrol and quercetin respectively.

The general protocol for sample treatment included acidification with or without the use of an antioxidant (ascorbic acid) to preserve compound stability. The subsequent addition of an excess of organic solvent followed by sonication or vortexing ensured compound extraction and protein denaturation. The sample was finally cleared of cell debris and denatured proteins by centrifugation; the supernatant was collected, concentrated and analyzed by HPLC-UV. Different combinations of organic solvent (ethyl acetate, acetonitrile and acetone) and acid (perchloric acid, citric acid and acetic acid) were screened using resveratrol as model compound. The suitability of the various combinations used was evaluated measuring the recovery percentages of resveratrol and internal standard after the treatment. The acetone/perchloric acid and acetone/acetic acid/ascorbic acid combinations ensured the best recovery for resveratrol, and were therefore tested also with quercetin; the latter prevented oxidation of quercetin and was therefore adopted (see Materials and Methods for details).

Method validation. To validate the method, we considered the following parameters: recovery, precision, linearity, selectivity and sensitivity.

Recovery. Recovery was evaluated in samples spiked with an analyte concentration of 5 μM. HPLC-UV peaks of resveratrol, quercetin and their internal standards in the solutions obtained from treated blood samples were compared to those of reference aqueous solutions at the same concentration (5 μM), representing 100% recovery, taking into account the sample volume by multiplying the sample/reference peak ratio by a concentration/dilution factor, F_{cd}. The latter was calculated as follows: $F_{cd}=V_f/(V_s/5.2)$, where V_f is the final volume of the treated sample, after concentration, and V_s is the volume of the supernatant collected after sample treatment, before concentration (see Materials and Methods). Due to additions during processing the original blood volume (200 μL) increased by a factor of 5.2.

The method ensured a mean recovery of $(80\pm5)\%$ and $(87\pm9)\%$ for resveratrol and quercetin, respectively (means from 7 and 8 independent treatments, respectively). Recovery of both analytes was similar from spiked samples incubated at 37°C for different time periods (5, 30, 45 and 60 minutes) before treatment (Fig. 1). A prolonged incubation time allows analytes to interact with blood components, mimicking what happens *in vivo* in the bloodstream, and avoids a possible overestimation of the recovery from freshly spiked samples (Brockman et al., 2007). On the other hand, as reported in the Materials and Methods part, prolonged incubation times lead to some metabolization of quercetin. However, the recovery calculated as sum of quercetin and methylquercetin is completely analogous to that found at short incubation times.
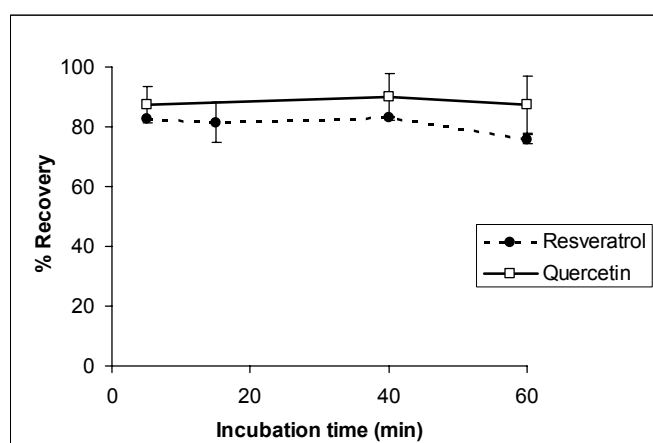

Figure 1. Percent recovery of resveratrol and quercetin from whole blood spiked with 5 μM of analyte and incubated at 37°C for the indicated time periods before treatment (mean values).

Even if structurally similar to the analyte(s), an internal standard may well associate with particulate matter to a different extent. We therefore determined the recovery of the

internal standards and analytes and the reproducibility of the ratio of recovery of analyte and standard. We found that analytes and internal standards were recovered with a reproducible ratio. This ratio can be defined as: $R_{a/is} = (A_{a,m}/A_{a,s}) \cdot (A_{is,s}/A_{is,m}) = (A_{a,m}/A_{is,m}) \cdot (A_{is,s}/A_{a,s})$, where:

$A_{a,m}$ is the measured analyte peak area in the final treated sample.

$A_{a,s}$ is the peak area of the analyte in the sample before any treatment.

$A_{is,m}$ is the measured internal standard peak area of the treated sample.

$A_{is,s}$ is the internal standard peak area in the sample before treatment, which corresponds by definition to the peak area measured for a reference aqueous solution of the internal standard at the same concentration spiked in the blood sample before treatment.

At 5 µM measured analyte $R_{a/is}$ values were 1.18 ± 0.14 (N = 7) for resveratrol / 4,4'dihydroxybiphenyl and 0.88 ± 0.06 (N = 8) for quercetin / 2',5,7-trihydroxyflavone.

The unknown quantity in pharmacokinetics samples, $A_{a,s}$, can be obtained from the formula:

$A_{a,s} = (A_{a,m}/A_{is,m}) \cdot (A_{is,s}/R_{a/is})$

$A_{a,s}$ is converted to concentration units using the calibration curves in $H_2O:CH_3CN$ for resveratrol and quercetin, which in our case were: y=182654[Res] (R^2=0.9982) and y=151698[Querc] (R^2=0.9998), respectively (concentrations expressed in µM units), giving the analyte concentration in the original sample.

Linearity. Whole blood was spiked with different concentrations of resveratrol or quercetin, and treated as described above. Plots of the ratio $A_{a,m}/A_{is,m}$ vs. analyte concentration (internal standard was always 25 µM) were linear (with a correlation coefficient of 0.9941 and 0.9983 for resveratrol and quercetin, respectively) and had y intercepts that did not differ significantly from 0, as shown in Fig. 2.
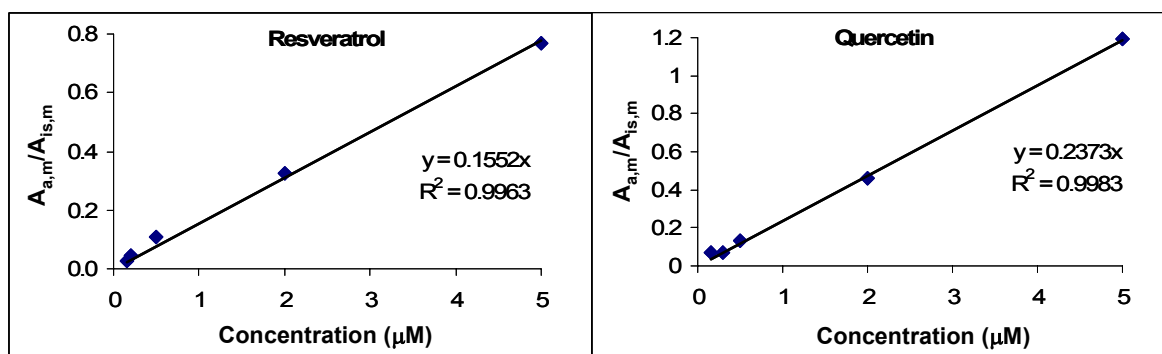

Figure 2. Linear regression of the ratio of the peak area of resveratrol and quercetin (at 320 and 370 nm, respectively) to that of their standards (at 286 and 370 nm, respectively) as a function of the concentration of the former.

These plots confirm proportionality between $A_{a,m}$ and actual analyte concentration and the constancy of $R_{a,is}$. They allow the direct measurement of the unknow concentration of analyte in pharmacokinetics samples, simply by reading the abscissa value corresponding to the ratio $A_{a,m}/A_{is,m}$.

Precision. The precision of the analytical method was determined analyzing 3 aliquots of an homogeneous sample of blood spiked with resveratrol or quercetin. The precision, expressed as coefficient of variation, was 1.6% for resveratrol, 2.7% for quercetin, 1.1% for 4,4'-dihydroxybiphenyl and 3.1% for 2',5,7-trihydroxyflavone.

Selectivity. Resveratrol, quercetin and their internal standards were quantified at 320 nm (resveratrol), 286 nm (4,4'-dihydroxybiphenyl) and 370 nm (quercetin and 2',5,7-trihydroxyflavone). The chromatograms at these wavelengths (Fig. 3) show that the analytes are both well resolved and free from interfering background peaks from the instrument and the blood matrix.

Sensitivity. Limit of detection (LD) and limit of quantification (LQ) were calculated measuring the average background response at 320 and 370 nm in 10 different HPLC runs. LD and LQ were taken to be 3 and 10 times, respectively, the standard deviation of this background response, according to the definition accepted by both IUPAC and the American Chemical Society. Interpolating the data obtained at 320 and 370 nm respectively with calibration curves in $H_2O:CH_3CN$ for resveratrol and quercetin (see above), we obtained an LD of 0.05 μM and an LQ of 0.16 μM for both compounds. LQ was confirmed analyzing a solution at the LQ concentration of resveratrol and quercetin; peak area of both the analytes at the LQ concentration fit well within the calibration curve. It should be noted that these parameters refer to the actual solution being analysed. Blood concentrations below LQ can still be quantified by the simple expedient of reducing the volume of the extract to be injected.
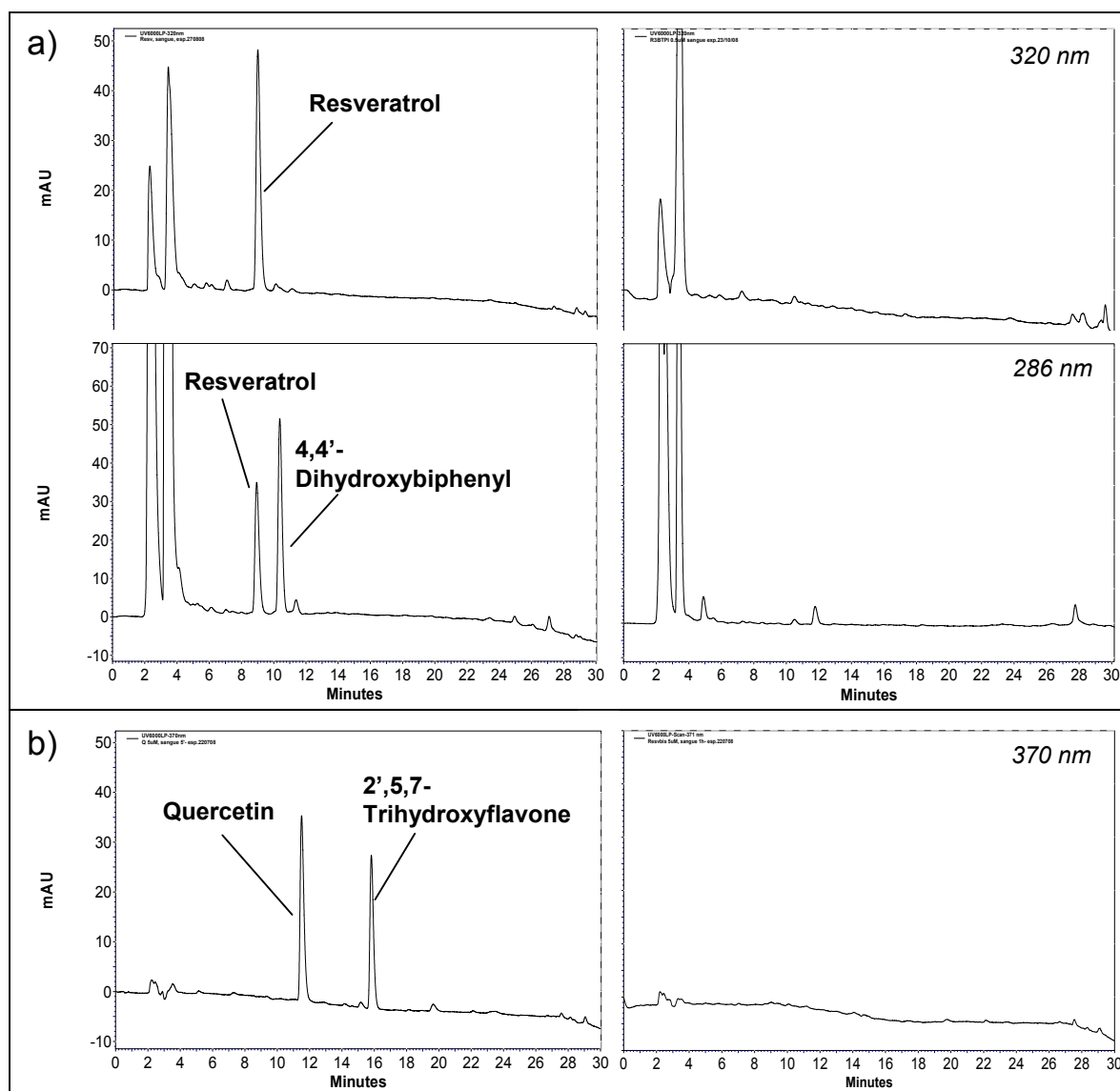
Figure 3. HPLC chromatograms of treated blood samples spiked with a) 5 μM resveratrol (recorded both at 286 and 320 nm) and b) 5 μM quercetin (recorded at 370 nm). Panels at right show the HPLC chromatogram (at the same wavelenght) of a treated blood sample without any spiking.

Analysis of whole blood vs. analysis of plasma. Finally, we analyzed samples of whole blood and plasma obtained from the same spiked blood samples. In plasma the recovery of resveratrol and quercetin was 30±11 and 50±4, respectively, while the remainder was retained in the cell pellet obtained upon plasma separation (Fig. 4). The comparison with whole blood analysis highlighted an incomplete recovery from plasma and indicated that treatment of whole blood rather than plasma is necessary to avoid underestimating the compounds in the circulatory stream. The same results were obtained incubating spiked blood samples for different times (15, 30 or 45 minutes) before separating plasma.

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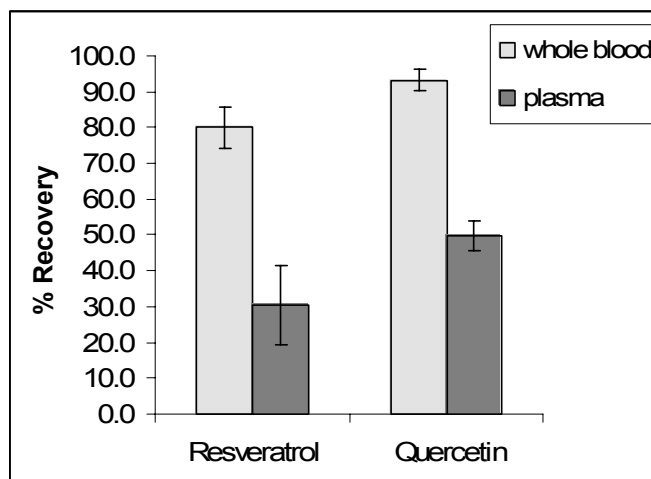
Figure 4. Mean recoveries of quercetin and resveratrol from whole blood, plasma and blood cells coming from samples spiked with 5 μM quercetin or resveratrol (N = 3).

Conclusions

The low bioavailability of polyphenols is a major obstacle to their application in therapeutic and preventive medicine. Efforts are under way to modify this situation using pro-drugs, formulations, and macromolecular vectors (liposomes, nanoparticles).[34-37] A detailed characterization of absorption and pharmacokinetics is important to evaluate the success of these efforts. Given the low solubility and the physico-chemical characteristics of most polyphenols, including resveratrol and quercetin, the vast majority of the molecules absorbed are expected to be bound to tissue (including blood) components. This applies also to plasma, since polyphenols are well known to bind avidly to albumin, and are therefore "sequestered" in plasma as well.[38-40] Our data show that treatment of whole blood instead of plasma is essential for a more complete recovery of these compounds and to avoid underestimating their levels in the bloodstream in *in vivo* bioavailability studies. The different molecular structure of the two model polyphenols used, belonging respectively to the stilbene and flavonoid sub-families, predicts the suitability of the same protocol for other classes of polyphenols.

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

Abstract

Solubility contributes importantly to overall bioavailability. We report here the proof-of-principle development of a chemical modification method of general applicability to polyphenols, which allows modulating solubility to influence absorption and overall bioavailability. Glucosyl groups were linked to a polyphenolic kernel via a succinate linker. The chemistry was optimized using 4,4'-diidroxybiphenyl and then applied to resveratrol, yielding 3,4',5-tri-(D-glucose-3-O-succinyl) resveratrol (RGS). The products were indeed water-soluble. Hydrolysis of RGS can only eventually yield, besides the polyphenol, glucose and succinate, safe molecules already abundantly present in the organism. The construct is only slowly hydrolysed in acid; in saline solution at pH 6.8 it loses a first glucosylsuccinyl group over a period of about 6 hours, and begins to regenerate resveratrol after about 28 hours. It is however completely hydrolysed by blood esterases in less than one hour. Pharmacokinetic determinations in the blood of rats showed that administration of RGS resulted in a concentration-vs-time curve shifted to longer times from administration in comparison to that obtained with an equimolar dose of resveratrol, a useful modulation of pharmacokinetics. The peak levels and metabolite mix were however analogous to those deriving from resveratrol as such, suggesting that the compound is hydrolysed in the intestinal lumen and resveratrol is then absorbed as such. This scheme is supported also by preliminary experiments with excised rat intestine mounts. The method may be advantageously employed to solubilise other polyphenols and to make food components more palatable.

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 plant polyphenols, the natural compounds of interest here. The research efforts of dozens

of groups worldwide have proven that many polyphenols possess potentially very useful biochemical properties (for resveratrol see, e.g.: 1-3). In addition to their anti- or pro-oxidant (depending on circumstances) character, many members of the family interact with an assortment of signalling proteins and transcription factors. These activities offer much promise for such major health-care endeavours as fighting aging, cancer, cardiovascular diseases and chronic inflammation. The promise however is fulfilled only to a limited extent: polyphenols are poorly absorbed, and are present in the circulatory streams at very low levels, and mostly as conjugates produced by first-pass metabolism in the intestinal enterocytes and in the liver. Poor solubility and rapid metabolism are the two major factors determining this outcome in the case of polyphenols as well as in that of many drugs and drug candidates.

Solubility is recognized as a key factor contributing to the determination of bioaccessibility and thus bioavailability (e.g. 4-7), so much so that for poorly soluble and membrane-permeant compounds a rule of thumb correlates it directly to bioavailability. Difficulties may arise both from an intrinsic (thermodynamic) low solubility of the molecule, and from unsuitable dissolution kinetics. Many of the drug candidates identified through combinatorial chemistry research or high-throughput screening methods are lipophilic – coherent with their action at or in biological membranes or membrane-associated proteins - and poorly soluble in an aqueous environment. Among the many ingenious ways, often combined into a multivalent approach, which have been adopted in an effort to bypass this obstacle are "formulation" developments (e.g. 5) such as the use of cyclodextrins (e.g. 8-11), microemulsions (e.g. 12-16), microdispersion in solids (e.g 17), polymeric micelles (e.g. 18-21), lipid-based systems (22-24), "nanosizing", i.e. approaches based on the use of extremely small drug particles (having a high surface-to-volume ratio) (e.g. 25-27) and covalent modifications of the drug such as PEGylation (e.g. 28-31), conjugation to solubilizing groups such as sugars (32,33), or the inclusion in dendrimers (e.g. 34-39). The problems and approaches to the improvement of the bioavailability of antioxidants and polyphenols via formulations and modifications have been reviewed (40,41).

The generally low solubility of polyphenol aglycones can be attributed largely to a tendency to form aggregates via hydrophobic interaction of the aromatic parts and hydrogen-bond formation between the hydroxyls. Reasoning that attachment to (a) group with high water solubility could only improve solubility, we carried out and report here the synthesis of derivatives in which a prototypical glycosyl group, glucose, is linked to a model polyphenol, resveratrol, via a dicarboxylic acid linker. In nature polyphenols occur

mainly as glycosylated derivatives (piceid is a major one of resveratrol). The current paradigm describing the absorption of these compounds in the intestine is that they are first deglycosylated at the intestinal wall, and then diffuse into the enterocytes as aglycones. Transport on the glucose transporter may take place (42-44) but it appears to be a minor mode. The presence of glycosyl groups may nonetheless improve bioavailability by influencing phenomena taking place upstream of entry into enterocytes. Several studies show this to be the case. A well-studied case is that of quercetin and its 3-O-glycosides (45-51). Kinetics and extent of absorption by rats turned out to depend strongly on the identity of the sugar group, with uptake being most efficient for the most soluble derivatives. Thus, not only the presence, but also the identity of the glycosidic residue is relevant. Consistent with the idea that what passes the surface of the intestinal epithelium is the aglycone, the metabolites found in the bloodstream were however the same regardless of which sugar was originally present. It should be stressed however that other factors, such as the food matrix (e.g. 52), also play an important and potentially confounding role. Thus, apparently contrasting data have been reported on the relative bioavailability of isoflavones and their glycosides (53-56), and it is unclear whether resveratrol or piceid, its major glycoside, has higher bioavailability (cf. 57, 58). The absorption of polyphenol from ingested glycosides may be delayed with respect to the aglycone (59, 53, 60).

Experimental

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. ^{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 (δ 2.49 ppm, DMSO-d$_6$ or δ 7.26 ppm, CDCl$_3$). 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.

Synthesis.

Diacetone-α-D-glucose-3-O-succinyl ester. Synthesis was performed modifying the procedure reported in literature (61). Briefly, DMAP (4-N,N-dimethylamino pyridine, 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 (diacetone-D-glucose, 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).
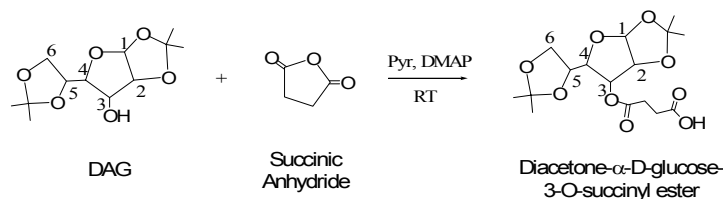
Scheme 1. Synthesis of Diacetone-α-D-glucose-3-O-succinyl ester

4, 4'-di(diacetone-α-D-glucose-3-O-succinyl)-biphenyl. DMAP (120 mg, 0.98 mmol, 1.3 eq), EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 0.58 g, 3 mmol, 4 eq) and 4,4'-dihydroxybiphenyl (140 mg, 0.75 mmol) were added to a solution of diacetone-α-D-glucose-3-O-succinyl ester (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). ESI/MS (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 TFA 12 M (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 nitrogen 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). ESI/MS (CH$_3$CN): m/z 733, [M+Na]$^+$.
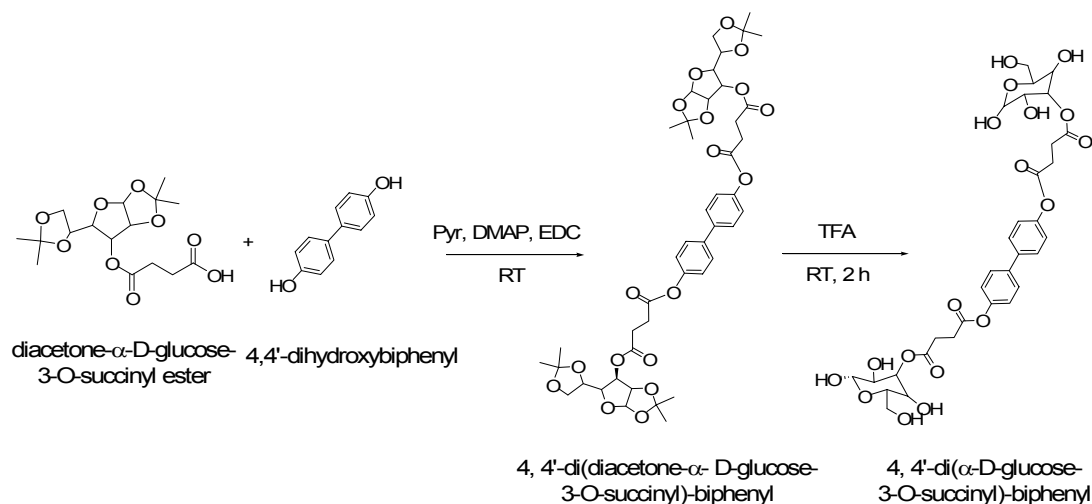
Scheme 2. Synthesis of 4, 4'-di(α-D-glucose-3-O-succinyl)-biphenyl

3, 4', 5-tri(diacetone-α-D-glucose-3-O-succinyl)-resveratrol. DMAP (120 mg, 0.98 mmol, 1.8 eq), EDC (0.58 g, 3 mmol, 5.7 eq) and resveratrol (120 mg, 0.53 mmol) were added to a solution of diacetone-α-D-glucose-3-O-succinyl ester (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 EtOAc/Exane 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). ESI/MS (CH$_3$CN): m/z 1277, [M+Na]$^+$.

3, 4', 5-tri(α-D-glucose-3-O-succinyl)-resveratrol. 3, 4', 5-tri(diacetone-α-D-glucose-3-O-succinyl)-resveratrol (150mg, 0.12 mmol) was dissolved in TFA 12 M (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). ESI/MS (CH$_3$CN): m/z 1037, [M+Na]$^+$.
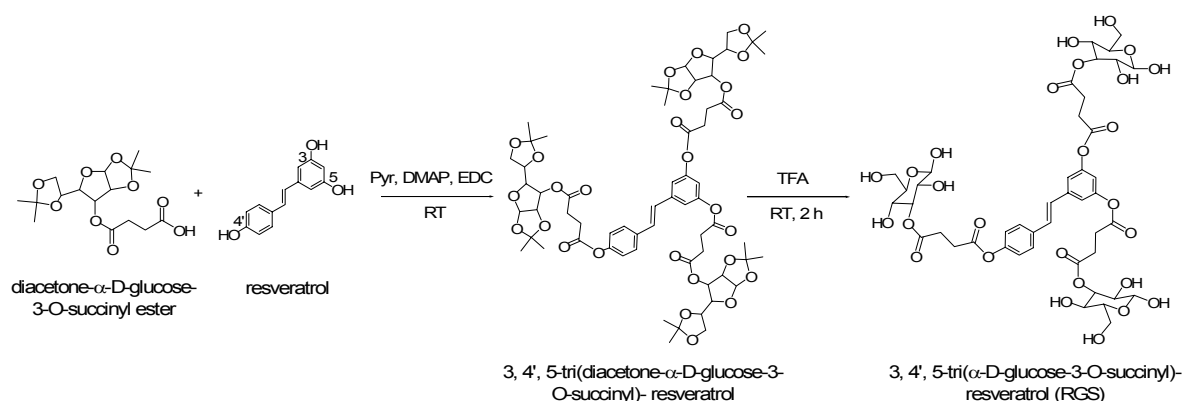
Scheme 3. Synthesis of 3, 4', 5-tri(α-D-glucose-3-O-succinyl)-resveratrol (RGS)

Stability in gastric and intestinal environment. The chemical stability of RGS 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 1:1000 a 50 mM stock solution in DMSO (i.e., 0.1% DMSO was added to ensure solubility of the hydrolisis products eventually formed), and incubated at 37°C for 48 hours; samples withdrawn at different reaction times were analyzed by HPLC-UV (see below). Hydrolysis products were identified by LC-MS analysis on selected samples.

Stability in blood. Rats were killed and blood 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 5 mM stock solutions in DMSO, 0.1% final

DMSO), incubated at 37°C for 1 hour and then treated as previously described (Chapt. 3). Cleared blood samples were finally subjected to HPLC-UV analysis (see below).

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.

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×).

Pharmacokinetic studies. Resveratrol or RGS were administered to overnight-fasted rats as a single intragastric dose. The dose was 0.22 or 0.022 mmol/kg, and compounds were dissolved in 250 μl DMSO or water (for resveratrol and RGS, respectively). 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 (150-200 μl each) were then taken from the tail tip at different time points after drug administration. A first blood sample was withdrawn before drug administration, and served as blank. Blood was collected in heparinized tubes, kept in ice and treated within 20 min. When the same rat was utilized to compare pharmacokinetics of resveratrol and RSG (see Results), a two-week recovery interval was obligatorily allowed between experiments.

Sample treatment and analysis. Blood samples were treated as described elsewhere (Chapt. 3), and then analyzed by HPLC-UV as described above. Metabolites were identified by LC-MS analysis (see above) and/or HPLC analysis couple to enzymatic treatment on selected samples (see below).

Enzymatic treatment. 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. At the moment of HPLC analysis, acetone was evaporated under a flow of nitrogen, and the concentrated samples were injected into the HPLC-UV system.

Results

Synthesis of biphenyl- and resveratrol- derivatives. Synthetic protocols were optimized using 4,4'-dihydroxybiphenyl as a model compound. This stable and simple compound provided a simplified initial system, since it has only two - equivalent –hydroxyls. The final optimized protocol was then transferred on resveratrol, and it proved to be suitable also for that compound.

The first step of the synthesis consisted on attachment of a succinyl linker to the 3-hydroxyl of a glucose molecule used in a protected form (diacetone-D-glucose, DAG) to avoid reactions of the other hydroxyls, which would have led to complex mixtures of isomers and crosslinking products. Per-esterification of 4,4'-dihydroxybiphenyl/resveratrol was then performed by activating the free carboxylic group of DAG-succinate with EDC, using DMAP as catalyst. The final step consisted in the deprotection of the glucose hydroxyl groups under acidic conditions (TFA), affording the final per-succinyl-glucose ester.

Stability. RGS underwent slow hydrolysis in 1N HCl (Fig. 1). After 6h at 37°C, 80% of the starting material was still present as such, with the main product of hydrolysis being the di-substituted derivative. At pH 6.8 in PBS product disappearance was faster (Fig. 1). Also in this case the process consisted in the loss of one entire succinyl-glucose moiety at a time. Loss of the first one was nearly complete after 6 hours, as showed by LC-MS analysis. No resveratrol was detected until 28 h. At no time were detectable amounts of products arising from the loss of glucose molecules formed.
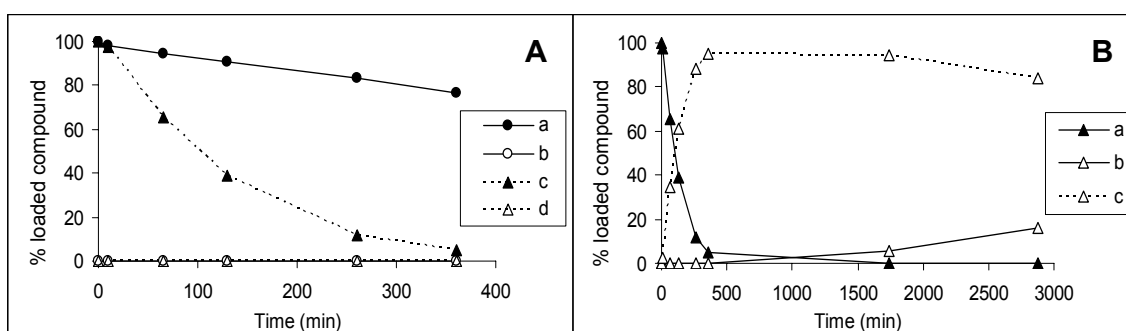

Fig.1. Stability of RGS in media mimicking gastric and intestinal environment. A) over 6 hours (37°C): a) RGS and b) resveratrol in 1N HCl; c) RGS and d) resveratrol in pH 6.8 PBS. **B)** over 48 hours (37°C): a) RGS, b) resveratrol, c) Mono- and di-glucosylsuccinylresveratrol in PBS, pH 6.8. Data are expressed as % of the initially loaded compound.

Pharmacokinetics of resveratrol and RGS. Pharmacokinetics were first carried out with resveratrol, the reference compound for further comparison and evaluation of pharmacokinetic data obtained for RGS.

On the basis of published pharmacokinetic curves (62-65), these first experiments were carried out sampling blood over a 1 hour period after intragastric administration of a 0.22 mmol/kg (50 mg/kg) or 0.022 mmol/kg (5 mg/kg) dose. Analyses revealed a relatively high peak of resveratrol sulfate- and glucuronide-conjugates. In agreement with literature reports absorption was rapid, reaching a maximum concentration of total species 10-30 minutes after administration. At all time points, intact resveratrol appeared only at very low levels, whereas metabolites prevailed. The maximum concentration of resveratrol and its metabolites depended on the administered dose, but there was also a high inter-individual variability, as evident from the size of the "error" bars in the plot of average values shown in Fig. 2A (high dose) and 2B (low dose).
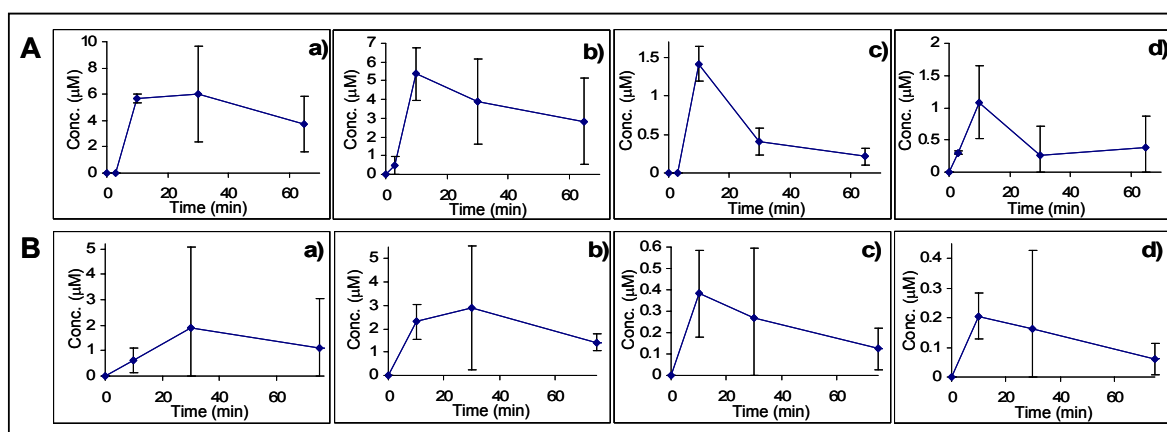

Fig.2. Pharmacokinetics of resveratrol. Administered dose was: A) 0.22 mmol/kg or B) 0.022 mmol/kg. Time profiles of the different species found in blood: a) resveratrol-3,4'-disulfate; b) resveratrol-3-glucuronide; c) resveratrol-3-sulfate; d) resveratrol. Data represent average values obtained with different rats (N=3 and 8, in A) and B) respectively).

The individual variability alluded to above means that, to be meaningful, comparisons of pharmacokinetics of different compounds must be based on a large number of experimental determinations. Alternatively, a more limited number may suffice, if data obtained using the same animal(s) are compared. We have chosen this second approach, and are in the process of collecting the data.

Initial pharmacokinetic experiments with RGS were carried out as with resveratrol (1 hour), but no detectable levels of any derivative/metabolite were found to be present in the bloodstream during the first hour, even after administration of the higher dose (0.22

mmol/kg, 223 mg/kg) For this reason, we prolonged monitoring to cover a 6 hours period, collecting also a final sample 24 h after administration. RGS, in fact, showed a delayed absorption with the maximum concentration of total species in blood reached at approximately 2 hours (lower dose, 0.022 mmol/kg) (not shown, N = 2) or at 4-5 hours (higher dose; see below) after administration. 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. The identity of the circulating species was confirmed by LC-MS analysis (not shown) and enzymatic treatment (Fig 3). Fig. 4 shows the data obtained from initial trials with the same rat, for the high dose protocol,
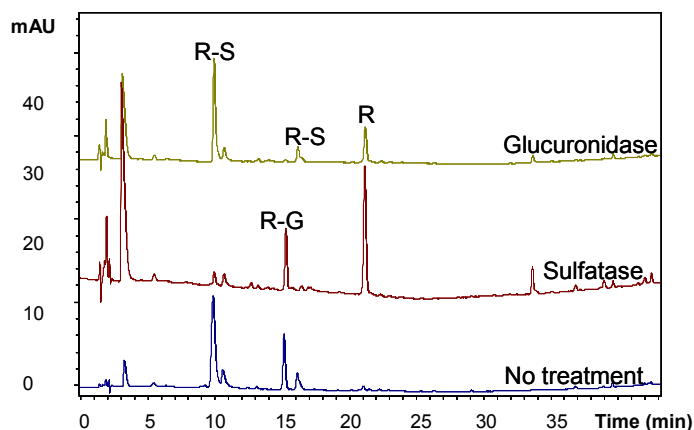
Fig.3. HPLC-UV chromatograms (320 nm) of a blood sample withdrawn 4h after intragastric administration of RGS (0.22 mmol/kg); the chromatograms represent aliquots of the same sample: without any enzymatic treatment, and after treatment with sulfatase or with glucuronidase, as indicated. R-S: resveratrol-sulfate; R-G: resveratrol-glucuronide; R: resveratrol.
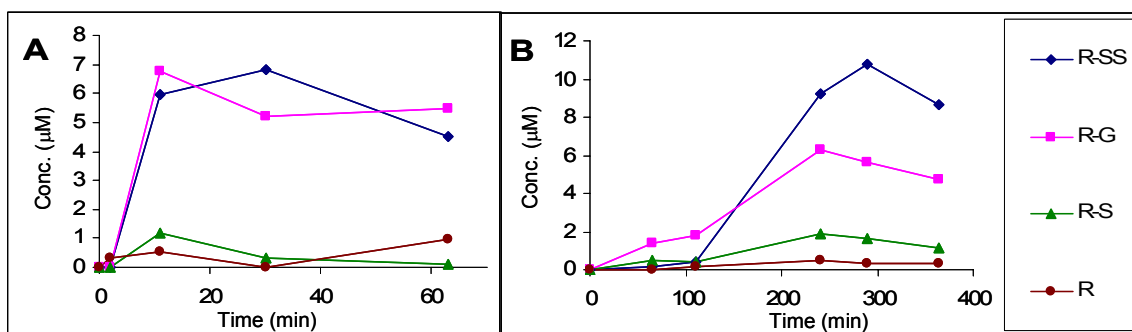

Fig. 4. Blood concentration vs. time curve for resveratrol and its major metabolites (3,4'-disulfate (R-SS); 3-glucuronide (R-G); 3-sulfate (R-S)) after intragastric administration of resveratrol (A) or RGS (B) at the 0.22 mmol/kg dose. Note the different time scales.

In upcoming determinations the duration of the experiments will be extended so as to allow a proper determination and comparison of the AUC parameter for both compounds. The delayed rise of polyphenol and derivatives is in any case confirmed.

Discussion

Most natural antioxidants, including polyphenols, are poorly soluble in water and poorly absorbed. This represents a major disadvantage for their prophylactic and therapeutic applications. RGS is soluble in water at least up to 90 mg/ml ($\approx$ 89 mM) 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 but predictably in view of the characteristics of the competing leaving groups, hydrolysis of the succinate-resveratrol bond is much faster than that of the succinate-glucose bond, so that RGS is destroyed via successive losses of whole glucosylsuccinyl groups. The results of the pharmacokinetics determinations can be summarized by stating that administration of the solubilised form of resveratrol we produced results in delayed absorption in comparison to the aglycone. Thus, administration of a proper mix of aglicone and glycosylated derivatives may produce a prompt as well as longer-lasting increase in circulating and body levels of the polyphenol and of its metabolites. The available data do allow yet a comparison of the most important parameter, the AUC, which is generally taken as a measure of bioavailability. A review of the literature has suggested that the AUC of molecules deriving from naturally occurring glycosylated isoflavones may be higher than that resulting from the intake of the aglycones themselves by a factor of 1.6-1.8 (55). If a similar enhancement were produced by our type of construct, at present it would compare favourably with a cyclodextrin-based formulation approach to resveratrol solubility increase (66). Even if it turned out that no such increase is afforded by RGS (vs. resveratrol), our observations suggest that developing a general procedure for the solubilisation of polyphenols is worthwhile.

While polyphenols generally occur in nature as glycosylated derivatives having higher solubility than the aglycone, 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 (67) , i.e. about 2×10^{-4} M (2.3 grams/liter at pH 9 and decreasing with pH: (68), http://ift.confex.com/ift/2004/ techprogram/paper_23374.htm). Quercitrin and genistin, common glucosides of quercetin and genistein

respectively, have very low solubility in cold water (67). Linkage to more than one sugar residue is expected to lead to products with higher solubility. As mentioned in the introduction, 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 (e.g. 69-82), and they are certain to be affected by the same (or worse) bioavailability problems of the natural compounds. Modulating solubility would have an impact and it provide an easier method for administration.

Modification of OHs may have a lot 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 (83, 84), 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. The parent polyphenol is regenerated rapidly. This type of molecules may therefore find relevant technological applications in the food industry, beginning in such sectors as the formulation of fortifying ingredients and supplements.

One shortcoming of RGS is the lability of the carboxyester bonds employed to attach the modifying units. Other bond types can be employed to reinforce the construct if needed. Variations on the chemical theme, leading to products with more favourable characteristics, may also include changes in the linking unit and solubilising molecule (e.g. 41). Thus, e.g., it might be advantageous to replace glucose with a non-metabolised sugar or other polyhydroxylated molecule to decrease caloric intake. The versatility of the method is another reason to believe that imitating nature's approach may be the way to go to modulate the solubility of these remarkable compounds.

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5. Resveratrol derivatives: stability and *in vivo* performance

Abstract

Model prodrugs of resveratrol carrying protecting substituents at the hydroxyls have been synthesised and tested. Two compounds, resveratrol triacetate and resveratrol-tri-PEG$_{1900}$, were formed by linking methyl groups or polymeric ethylene glycol chains, respectively, via carboxyester bonds. To compare their performance with that of molecules less susceptible to hydrolytic attack, 3,4',5-trimethoxystilbene and resveratrol trimesylate were synthesised as well. These two latter compounds proved to be stable under *in vitro* conditions, as expected. The carboxyester derivatives turned out to hydrolyse only slowly in solutions mimicking the gastric or intestinal chemical environment. In blood, however, they were rapidly hydrolysed regenerating resveratrol. Pharmacokinetics after intragastric administration and experiments with explanted intestine segments suggest that they are also hydrolysed in the intestinal lumen or at the intestinal surface, and that the specie crossing the intestinal wall is likely to be resveratrol itself. These results have prompted a move away from carboxyesters as the linkage of choice for polyphenol prodrugs.

Introduction

Polyphenols are a vast family of natural compounds exhibiting, at least *in vitro*, a variety of biomedically relevant activities. A vast literature documents effects of potential relevance for such major health-care endeavours as protection of the cardiovascular system (1-3), improving performance impaired by old age or neurodegeneration (4-12), and prevention and therapy of cancer (13-19). Several biochemical pathways underlying these effects have been identified (e.g.: cardio- and neurovascular protection: 13,14,16,20-27; neuroprotection and anti-aging: 8,28-31). Studies dealing with the bioactivity – and underlying mechanisms - of resveratrol, the model polyphenol used in the present work, are summarized in recent reviews (e.g.: 2,8,25,31-43).

The processes involved are by no means limited to the general chemical reactivity of polyphenols as anti- or pro-oxidants (for a discussion of which see, e.g., (44)). Various members of the family are known to modulate signal-transducing proteins ranging from channels (e.g. 45) to cyclooxygenases (46). Effects on gene expression are important (e.g.: 2,8,14,47-49). To mention but one example: several polyphenols, including resveratrol, inhibit STAT-3 (8,50-52), which is activated by oncogenic kinases, promotes tumorogenesis, and contributes to the febrile response in infections (e.g.: 53,54).

Some of the interactions between polyphenols and proteins display high affinity. For example, EGCG binds the laminin receptor with nM affinity (55,56). 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 μM (57). 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 (58,59).

This constitutes an obstacle to the utilization of these compounds, since their bioavailability is notoriously low (e.g.: 60-62). They are rapidly converted by conjugating enzymes in enterocytes to metabolites which are to a large extent re-exported to the intestinal lumen. Liver SULTs and UGTs then intervene on the molecules which have entered the circulation, administering other rounds of Phase II metabolism. Thus, only low (nM-μM) concentrations of any given polyphenol are found in plasma and lymph even after a polyphenol-rich meal, and mostly in the form of conjugates (e.g.: 61,63,64). At least two dozen different quercetin derivatives have been detected in human plasma (61,65-70). Resveratrol undergoes the same type of processing (71-80). A recent paper (80) reports that sulfation reduces the effectiveness of resveratrol as an inhibitor of cancerous cell growth.

"Detoxification" by conversion into sulfates and glucuronides entails faster elimination by the renal and biliary routes, and presumably at least a partial loss of bioefficacy (although the study on the properties and activities of metabolites is still in the initial phases (81-100). It must therefore be contrasted if the pharmacological potential of natural polyphenols and analogous synthetic molecules (5,101-112) is to be more fully exploited. 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".

Bioavailability- and/or stability-improving ester precursors have already been obtained for several drugs with alcoholic or phenolic hydroxyls (e.g.: 113,114). Bioavailability- and/or stability-improving ester and ether precursors of some polyphenols have already been investigated. Mulholland et al. (115) aimed at improving water solubility of quercetin with a compound (QC12) deriving from quercetin and glycine. Its oral administration to cancer patients however did not result in the appearence of quercetin or metabolites in plasma. Pentaacetylquercetin has been tested on phosphodiesterase activities (116) and on models of carcinogenesis (117), genotoxicity (118) and LPS-induced gene expression (119).

Various acylquercetins have been tested for improved skin permeability (120). Quercetin derivatives incorporating aminoacid substituents showed an increased permeability of Caco-2 cell monolayers (121). Peralkylated quercetins and several methoyflavones have been tested as inhibitors of P-glycoprotein (PgP) in cancer cells (122,123). Flavone ether derivatives have been tested against Epstain virus activation and carcinogenesis (117,124). Walle's group has reported that methylation of some mono-, di- and tri-hydroxyflavones results in higher stability towards hepatic metabolism and in enhanced permeation through Caco-2 cell monolayers (125-128). Resveratrol mimetics have been synthesized and tested in the search for improved COX-2 inhibitors (104). Permethylated resveratrol has been reported to inhibit the growth of human cancer cells and of human tumor xenografts in SCID mice (129). EGCG and some EGCG analogues have been peracetylated in order to improve EGCG stability and efficacy (130-135). Peracetylated EGCG has been recently tested in models of tumor growth and angiogenesis (132,136,137). Proteasome inhibition was tested using cultured cancer cells. Acetylation was found to favor proteasome inhibition, offering interesting perspectives for anti-cancer applications. On the contrary, proteasome inhibition was hampered by methylation of the EGCG hydroxyls (138,139).

We report here the synthesis of a few pro-drugs of resveratrol, in vitro stability studies and the initial investigations of their absorption. Resveratrol was chosen as the model polyphenol because of its remarkable biomedical properties, relative simplicity and stability. Among the various conceivable derivatives the peracetylated molecule was chosen to represent the class of carboxy ester derivatives, the most straightforward ones. Resveratrol triacetate has recently been reported to inhibit the proliferation of human colon adenocarcinoma cells (140). As mentioned, a few polyphenol derivatives of this type have already been prepared, and may represent an improvement on the parent molecule. *A priori*, one desirable property is that the protecting group be removed without difficulty by esterases. On the other hand, the hydrolysis may take place even too readily when good leaving groups are involved. Stability may vary from case to case. For example, in acetylsalycilic acid (aspirin), in which the leaving group is formally a phenate anion, the ester bond is stable enough for the molecule to reach its target (cycloxygenase) and inhibit it by transferring the acetyl group to a specific serine. Furthermore, even an esterase-sensitive bond may improve absorption by facilitating entry into intestinal epithelium cells. 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 (141-145). 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. This type of modification can 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 the performance of derivatives not subject to complications arising from hydrolysis of the protecting groups we adopted two simple ones: per-methylated and per-methylsulfonated resveratrol. The per-ether was expected to be substantially stable under our experimental conditions (e.g. 146). Little is known about the behavior of sulfonates in biological systems; this group may, *a priori*, be potentially useful in contexts such as ours.

The various compounds are to be compared to resveratrol itself in pharmacokinetics and in experiments with explanted intestine segments intended 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 studies mentioned has dealt with the effects of the modifications introduced on permeation of the intestinal epithelium *per se* (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 (61,147-152), and are expected to allow a more detailed assessment of the performance of our compounds.

Experimental

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. ^{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 (δ 2.49 ppm, DMSO-d_6 or δ 7.26 ppm, CDCl$_3$). 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. 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%). ^{1}H-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).

3, 4', 5-triPEG$_{1900}$-resveratrol (PEGResv). This compound was synthesised by Dr. S. Salmaso of the Dept. of Pharmaceutical Sciences, Padova. 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 PEG$_{1900}$-COOH (1 g, 0.51 mmol, 5.8 eq) in CH$_2$Cl$_2$ (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 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%).

HPLC-UV analysis showed no free resveratrol in the final product. The resveratrol: PEG ratio was determined through UV-Vis spectrometry. Calibration curves for resveratrol and PEG were made plotting absorbance at 300 nm and 535 nm (for resveratrol and PEG, respectively) against concentration (in the range 2-10 μg/ml). PEG determination was preceded by the iodine assay (see below). The Resveratrol:PEG ratio was determined preparing 3 different solutions of known concentration (w/v) of the product, and measuring their absorbance at 300 nm and 535 nm (after the reaction with I$_2$). Data were finally interpolated with calibration curves for resveratrol (300 nm) and PEG (535 nm).

I$_2$ assay. 10 μl of sample were diluted in 990 μl MilliQ water. 250 μl BaCl$_2$ (5g BaCl$_2$ in 100 ml HCl 1 N) and 250 μl I$_2$ (2g KI and 1.27 g I$_2$ in 100 ml water) were then added, and absorbance at 535 nm was measured after 10-15 min.

3, 4', 5- trimethoxy-resveratrol (RTMet). Methyl iodide (1.09 ml, 17.6 mmol, 4 eq) and K$_2$CO$_3$ (2.4 g, 17.6 mmol, 4 eq) were added to a solution of resveratrol (1 g, 4.4 mmol) in DMF (15 ml). The mixture was stirred 2 hours at 30°C. The mixture was 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 was purified by flash chromatography using CH$_2$Cl$_2$/Acetone 8:2 as eluent to afford 1.05 g of the desired product (88%, white solid). ^{1}H-NMR (250 MHz, DMSO-d_6) δ (ppm): 3.78 (m, 9H, OCH$_3$), 6.40 (t, 1H, H-4), 6.72-6.79 (m, 2H, H-2, H-6), 6.94 (d, 2H, , H-3', H-5', J= 7.5 Hz), 6.94-7.28 (AB system, 2H, H-7, H-8, J$_{trans}$= 15 Hz); 7.53 (d, 2H, H-2', H-6', J= 7.5 Hz).

3, 4', 5- trimesyl-resveratrol. 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 × 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 CH$_2$Cl$_2$:Petroleum ether:EtOAc 8:1:1 as eluent, to afford 1.05 g of the desired product (88%, white solid). ^{1}H-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).
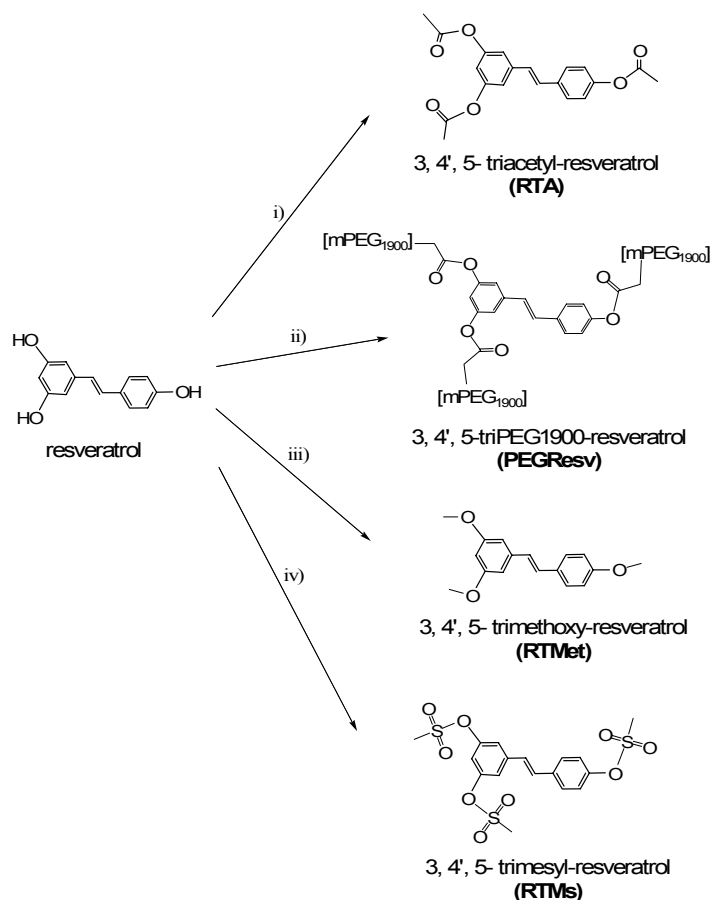
Scheme 1. Synthesis of the resveratrol derivatives mentioned in this chapter. Reaction conditions: i) Pyr, Acetic Anhydride, RT, 40 min; ii) CH$_2$Cl$_2$, DCC, DMAP, RT, 72 h; iii) CH$_3$I, K$_2$CO$_3$, DMF, 30°C, 2h; iv) Methanesulfonyl chloride, Pyr, 100°C, 3h.

Solubility in water. Calibration curves were built by plotting absorbance at 320 nm (a plateau in the UV absorption spectrum of all compounds) *vs* concentration for seven standard solutions in the 10^{-6} - 10^{-4} M range, prepared from a 10^{-3} M mother solution in CH_3CN by dilution with water:CH_3CN 9:1. The concentration of aqueous saturated solutions of the derivatives was determined by interpolation (N = 5). 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 (14000*g*, 30 min) and the clear supernatant was transferred into a cuvette; absorbance was measured at 320 nm. Since saturated solutions were prepared suspending different amounts of undissolved material, obtaining the same absorbance reading for all samples excludes the possible presence of colloidal particles (which would lead to an overestimate), since their concentration would be expected to be related to the amount of solid used in each determination.

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 (PBS buffer 0.1 M, pH 6.8). A 50 μM solution of the compound was made diluting 1:200 a 10 mM stock solution in DMSO (the presence of 0.5% DMSO ensured solubility of all compounds and hydrolysis products eventually formed), and incubated at 37°C for 6 hours. Samples withdrawn at different reaction times were analyzed by HPLC-UV(see below).

Stability in blood. Heparinized, EDTA-supplemented blood samples (1 ml) were spiked with 5 μM compound (dilution from 5 mM stock solutions in DMSO, 0.1% final DMSO), incubated at 37°C for 1 hour and then treated as previously described (Chapt. 3). Cleared blood samples were finally subjected to HPLC-UV analysis (see below).

Pharmacokinetic studies. Resveratrol or its derivatives were administered to overnight-fasted rats as a single intragastric dose (0.022 mmol/Kg), dissolved in 250 μl DMSO. Before drug administration, rats were anesthetized with isoflurane and the tip of the tail was cut off. Serial blood samples (150-200 μl each) were then taken from the tail tip at 2, 10, 30, 75, 160, 260, 370 min. and 24 hours after drug administration. A first blood sample was withdrawn before drug administration, and served as blank. Blood was collected in heparinized tubes, kept in ice and treated within 20 min.

Sample treatment and analysis. Blood samples were treated as previously described (Chapt. 3), and then analyzed by HPLC-UV (see below). Metabolites were identified by LC-MS analysis on selected samples (see below).

Permeability studies. Intestine was excised from a 24 h fasted rat, transferred into a saline solution (154 mM NaCl in water) at 37°C, and 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 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 incubated in a water bath at 37°C until all the chambers were assembled. The buffer was then removed and substituted with 1 ml of a 50 μM solution of the compound to be tested in the same buffer on the apical side (dilution from a 10 mM stock solution in DMSO, 0.5% final DMSO), and with 1 ml of fresh Hepes buffer on the basolateral one. In the controls, only Hepes buffer was present on both sides. During the experiment, air was bubbled through a syringe needle 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. 100 μl samples were collected from both compartments after 1 h of incubation. At the end of the experiment (2 h) chamber contents on both apical and basolateral sides were entirely collected. 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 analyzed by HPLC-UV as described below.

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$_2$O containing 0.1% TFA and CH$_3$CN, 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.

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 ×).

Results and discussion

Synthesis. Four resveratrol derivatives have been synthesised; in all cases all three –OH groups were covalently modified for a complete protection against metabolism during absorption. In all the synthetic protocols adopted, an excess of reagent was used to obtain the tri-functionalized derivative as the unique product. This avoided the presence of mono-

and di-substituted products in the final reaction mixture, maximizing the product yield and avoiding time-consuming separations.

Solubility in water. RTA, RTMet and RTMs turned out to have poor solubility in water, similarly to resveratrol itself (≤ 2 μM). PEGylation on the contrary greatly increased the solubility of the derivative, and saturation was not reached even dissolving 400 mg of the compound in 1 ml water (> 70 mM).

Stability in gastric and intestinal environment. All the 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 buffer, pH 6.8 respectively). RTMet and RTMs were completely stable, whereas RTA and PEG-Resv underwent slow hydrolysis of the ester bonds, with an up to 20% loss of the starting compound after 6 h (Fig.1). Formation of resveratrol, i.e. the end-product of ester bonds hydrolysis, occurred over this period only in the case of PEG-Resv. Hydrolysis of RTA was slower, generating only intermediate products (di- and mono- acetylated derivatives).
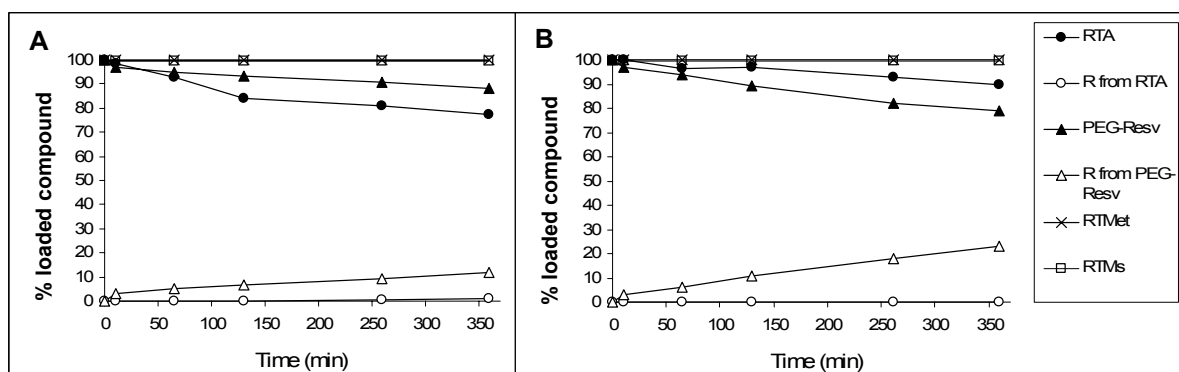
Fig.1. Stability of resveratrol-derivatives in media mimicking A) gastric and B) intestinal environment (37°C, 6 hours). Data are expressed as % of the initial loaded compound.

Stability in blood. Once absorbed, an orally administered prodrug should be able to re-generate the parent compound. Resveratrol derivatives were thus incubated in blood at 37°C for 1 hour as a way to assess their stability in the bloodstream. The blood samples were spiked with 5 μM compound in all cases. Complete hydrolysis of both resveratrol carboxyesters (RTA and PEGResv) occurred over 1 hour in whole blood, with resveratrol being the unique product. Methoxy- and sulfonyl- derivatives, on the contrary, were completely stable.

These *in vitro* stability studies did not provide a clear rationale for a selection. The two carboxyester derivatives were clearly much less stable than the methylether and the

mesylated derivative. *A priori* this might be a comparative advantage, since it allows regeneration of the parent compound which might or might not take place with the other two molecules, or a disadvantage if hydrolysis took place too rapidly *in vivo*, allowing metabolism during entero-hepatic circulation. We therefore proceeded to directly investigate the behaviour of our compounds in vivo in pharmacokinetics studies.

Pharmacokinetic studies. Because of the great inter-individual variability observed studying resveratrol absorption and metabolism (Chapt. 4), each derivative was tested twice on the same rat, which was used also to perform a control experiment with resveratrol itself (i.e. the "reference" compound). In this way sets of directly comparable data were obtained. The pharmacokinetic experiments were performed at 14 days intervals in compliance with university regulations on treatment of laboratory animals.

First experiments were performed with RTA and PEGResv. In both cases, pharmacokinetic profiles turned out to be very similar to that of resveratrol itself: the concentration of each circulating specie, as well as their combined total, reached a maximum about 10 minutes after administration. Resveratrol was present in the bloodstream only in traces, whereas metabolites prevailed (resveratrol- 3,4'-disulfate, 3-glucuronide, 3-sulfate). (Fig.1, Fig.2).
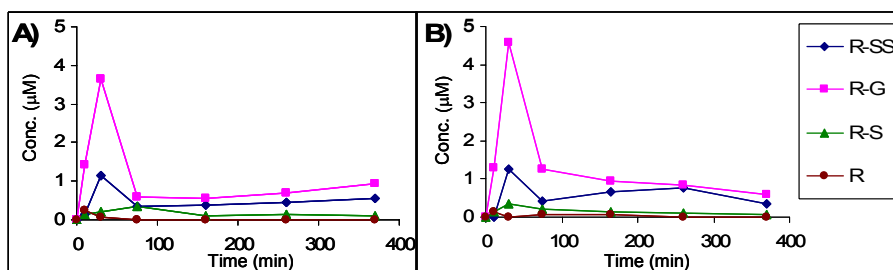
Fig. 1. Comparison of pharmacokinetic profiles obtained with the same rat, obtained after administering a single 0.022 mmol/kg intragastric dose of: A) RTA; B) Resveratrol. No resveratrol derivatives were detected in the 24 hour samples. R: resveratrol; -SS: 3,4'-disulfate; -G: 3-glucuronide; -S: 3-sulfate.
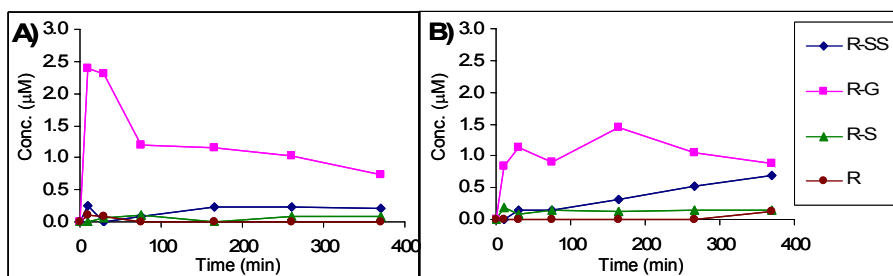

Fig. 2. Comparison of pharmacokinetic profiles obtained with the same rat, obtained after administering a single 0.022 mmol/kg intragastraic dose of: A) PEGResv; B) Resveratrol. No resveratrol derivatives were detected in the 24 hour samples. R: resveratrol; -SS: 3,4'-disulfate; -G: 3-glucuronide; -S: 3-sulfate.

The observed metabolism implies regeneration of resveratrol by hydrolysis of the ester substituents. In principle this might have taken place in the circulatory stream, after absorption of unaltered, or only partially hydrolysed, precursor. However, the similarity of the pharmacokinetic curves obtained with the pro-drugs and with resveratrol suggests instead that hydrolysis took place before adsorption, i.e. in the intestinal lumen or at the epithelial luminal surface. Given the results of stability studies, this "degradation" may well due to the activity of esterase activities associated with these compartments.

To gain a better understanding of these processes we have just begun an investigation of the absorption process using segments of excised rat jejunum mounted in Ussing chambers. This experimental system allows us to study transepithelial transport separately from other processes taking place in the intestinal lumen, in blood, or in the liver.

The first results confirmed that the intestinal wall expresses powerful esterase activities. 50 µM RTA and PEGResv (in the apical compartment) were completely hydrolysed within 1 hour. The process is certainly due to enzymatic activities, since only a small amount of hydrolysis products is formed upon incubation of the derivatives under the same conditions but without the intestine. Analysing apical and basolateral compartments after permeability experiments, we found that resveratrol (i.e. the reference compound) crossed the intestinal wall as such, i.e. without being metabolized. Since several studies report its metabolization by enterocytes (153-156), this may be a symptom of a loss of functionality caused by defective tissue oxygenation. Work is in progress to set up an apparatus insuring adequate oxygenation during all phases of the experiment.

Conclusions

While this part of the project remains to be completed, the available data clearly show that pro-drugs incorporating carboxyester bonds are too fragile *in vivo* to afford the desired level of protection to polyphenols. We have therefore decided to move on to other, more stable types of protecting / linking groups. The identification and testing of an optimal functionality for these purposes constitutes the next stage of the project.

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6. Phase II metabolites of resveratrol: syntheses, characterization and initial utilisation

Introduction

Polyphenols are a large family of natural compound with potentially useful biomedical effects. They are present in foods and are conspicuously assumed with diet, but have notoriously low bioavailability; once ingested, they are poorly absorbed through the gastrointestinal tract, and quickly converted into metabolites. Intestinal absorption begins with deglycosilation of the naturally occurring glycosylated derivatives by glycosidases at the enterocytes' surface, followed by diffusion of the generated aglycones into the cells. Aglycones reaching the cytosol are converted to methyl-, sulfonyl- or glucuronyl-conjugates and to a large extent re- exported to the intestinal lumen, where these metabolites are finally degraded by colonic microflora. A minor fraction is exported to the basolateral side, reaching the bloodstream and then the liver, where they are further metabolized. Liver enzymes operate both Phase I and II metabolism: addition of –OH groups, oxidations and dealkylations (phase I metabolism), methylation, sulfation, and glucuronidation (phase II metabolism) (rev.: 1). Gluthatione adducts are also produced. "Detoxification" by conversion into sulfates and glucuronides entails faster elimination by the renal and biliary routes (1-3). These metabolites are produced, respectively, by Sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs). SULTs (rev.: 4) include 13 different cytosolic enzymes in humans, and catalyze the transfer of a sulfonyl-group ($-SO_3^-$) from a universal donor, 3'-phosphoadenosine-5'-phosphosulfate (PAPS), to an acceptor hydroxyl-group. UGTs (rev.: 5) are integral ER membrane proteins, with the catalytic site in the ER lumen. In this case the donor molecule is uridine-5'-diphosphate-glucuronate (UDP-Glucuronate).

Methylation is another frequent Phase II conjugation, with catechol-O-methyl-transferases (COMTs) being particularly relevant in metabolizing certain flavonoids (in the case of resveratrol, however, formation of methylated derivatives have not been reported, nor have we observed it).

As the final result of low absorption and metabolism, only low (nM-μM) concentrations of any given polyphenol are found in plasma and lymph even after a polyphenol-rich meal, and mostly in the form of conjugates (e.g.: 6-8).

Resveratrol is among the polyphenols attracting most attention, because of its relative abundance and of the potential therapeutic and disease-preventing usefulness (for recent revs. see, e.g.: 9-11). Furthermore, it has long been one of the favourite model compounds for studies dealing with bioavailability, absorption and metabolism. Several different resveratrol conjugates have been detected in human plasma (12-26) These include various sulfate- and glucuronyl-derivatives, the major ones being the mono- and di-sulfates and mono-glucuronides.

The effects of polyphenols on biological systems have been explored in many *in vitro* studies, performed with cell cultures and concentrations of aglycones much higher than those reachable *in vivo*. Phase II metabolites are the most abundant species to be found in circulation after ingesting any polyphenol; thus, it would be not surprising if these conjugates turned out to play an active role in determining the beneficial health effects associated with a polyphenol-rich diet. The study of the properties and activities of metabolites is still in the initial phase, and suggests they may have their own activities (27-46).

The availability of purified, synthetic metabolites clearly would be an advantage for any project dealing with polyphenols as "nutraceuticals" or perspective drugs. For example, they could be used as standards for the identification and quantification of the species found *in vivo*, or for evaluating the suitability of various protocols; they could also be used to investigate their own biological action (e.g., assessment of their redox properties, interaction with specific proteins/transcription factors, regulation of signalling pathways).

We thus decided to chemically synthesise a series of resveratrol sulphate- and glucuronide-conjugates. Since the possible isomers formed during chemical functionalization of resveratrol are 5 at most, rather than performing regiospecific syntheses we decide to allow the reactions to proceed aspecifically, and to separate the individual products from the reaction mixture. The chemically synthesised derivatives were first used to better characterize metabolites formed *in vivo*; they were then employed to test the suitability of the protocol already adopted for blood treatment (Chapt. 3) in extracting also metabolites from blood.

Materials and methods

Materials and instrumentation. Chemicals 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. Flash chromatographic

separations were run on silica gel (Macherey-Nagel 60, 230-400 mesh) under air pressure. ^{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 solvent signal (δ 2.49 ppm, DMSO-d$_6$). HPLC-UV analyses were performed with a Thermo Separation Products Inc. system with a P2000 Spectra System pump and a UV6000LP diode array detector (190-500 nm).

HPLC-UV analysis. Samples (20 μl) were analyzed using a reversed-phase column (Gemini C18, 3 μm, 150 x 4.6 mm i.d.; Phenomenex). Solvents A and B were 50 mM Ammonium Acetate (pH 6.5) and CH$_3$CN, respectively. The gradient was optimized for each of the products to be separated, as reported in each synthetic protocol; the flow rate was 0.7 mL/min. The eluate was preferentially monitored at 286, 300 and 320 nm.

Semi-preparative HPLC. Synthesis mixtures (about 20 mg/ml, dissolved in the starting mobile phase) were purified injecting 100 μl samples in a semi-preparative HPLC column with the same stationary phase of the anaytical one (Gemini C18, 5 μm, 250 x 10 i.d. mm). Eluents and gradients were the same of the analytical HPLC; the flow rate was 3 ml/min.

Synthesis of resveratrol metabolites

**Resveratrol Sulfates.** Synthesis was performed optimizing the procedure reported in literature (22,47). Briefly, resveratrol (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, and then CHCl$_3$:MeOH 6:4 to separate monosulfates (45%) from disulfates (33%). The trisulfate was not formed in detectable amounts; reagents were used in an appropriate ratio to obtain preferentially mono- and di-substituted derivatives, which have been reported to represent the major sulfated metabolites.

Mono- and di- sulfate isomers were separated by semi-preparative HPLC (see above); 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.

Resveratrol-3-sulfate. ^{1}H-NMR (250 MHz, DMSO-d$_6$) δ (ppm): 6.53-6.66 (m, 1H, H-2, H-6), 6.70-6.80 (m, 3H, H-4, H-3', H-5'), 6.80-7.30 (AB system, 2H, H-7, H-8, J$_{trans}$=15 Hz), 7.19 (sb, OH), 7.41 (d, H-2', H-6', J$_{orto}$=10 Hz), 9.36 (sb, OH). ESI/MS (CH$_3$CN:H$_2$O 1:1, 0.1% CF$_3$COOH): m/z 309, [M+H]$^+$.

HPLC: t$_R$=20.55 min.

Resveratrol-4'-sulfate. ^{1}H-NMR (250 MHz, DMSO-d$_6$) δ (ppm): 6.13 (s, 1H, H-4), 6.41 (s, 2H, H-2, H-6), 6.82-7.05 (AB system, 2H, H-7, H-8, J$_{trans}$=15 Hz), 7.06-7.45 (m, H-3', H-5', OH), 7.19 (sb, OH), 7.47 (d, 2H, H-2', H-6', J$_{orto}$=7.5 Hz), 9.24 (sb, OH). ESI/MS (CH$_3$CN:H$_2$O 1:1, 0.1% CF$_3$COOH): m/z 309, [M+H]$^+$. HPLC: t$_R$=15.35 min.

Resveratrol-3,4'-disulfate. ^{1}H-NMR (250 MHz, DMSO-d$_6$) δ (ppm): 6.64 (m, 2H, H-2, H-6), 6.77 (s, 1H, H-4), 6.92-7.12 (AB system, 2H, H-7, H-8, J$_{trans}$=17,5 Hz), 7.06-7.38 (m, H-3', H-5', OH), 7.50 (d, 2H, H-2', H-6', J$_{orto}$=10 Hz), 9.41(s, OH). ESI/MS (LC/MS): m/z 387, [M–H]$^-$. HPLC: t$_R$=9.6 min.

Resveratrol-3,5-disulfate. ^{1}H-NMR (250 MHz, DMSO-d$_6$) δ (ppm: 6.75 (d, 2H, H-3', H-5', J$_{orto}$), 6.91-7.00 (m, 2H, H-7, H-8), 7.06 (d, 2H, H-2, H-6, J$_{meta}$= 2,5 Hz), 7.00-7.20 (sb, H-4, OH), 7.44 (d, 2H, H-2', H-6', J$_{orto}$). ESI/MS (LC/MS): m/z 387, [M–H]$^-$. HPLC: t$_R$=11.6 min.
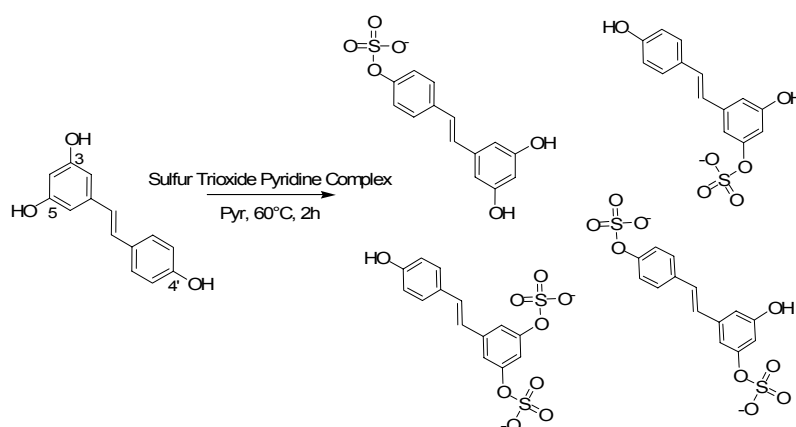
Scheme 1. Synthesis of resveratrol sulfates

Resveratrol Glucuronides. Synthesis was performed optimizing the procedure reported in literature (22,48). Briefly, resveratrol (2.28 g, 10 mmol), acetobromo-alpha-D-glucuronic acid methyl ester (2 g, 6.3 mmol) and metallic Na (0.23 g, 1 mmol) were dissolved in methanol (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 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, as reagents were used in an appropriate ratio

to obtain preferentially mono- substituted derivatives, which have been reported to represent the major glucuronyl-derivatives. 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 (see below); the gradient for B was as follows: 18% for 10 min, from 18% to 90% in 5 min, then 90% for 2 min.

Resveratrol-3-glucuronide. ^{1}H-NMR (250 MHz, MeOD) δ (ppm): 3.51 (m, 3H, CH), 3.73-3.82 (m, 1H, CH), 6.50 (t, 1H, H-4, J$_{meta}$=2.5 Hz), 6.60-6.65 (m, 1H, H-2), 6.73-6.80 (m, 3H, H-6, H-3', H-5'), 6.80-7.08 (AB system, 2H, H-7, H-8, J$_{trans}$=15 Hz), 7.37 (d, 2H, H-2', H-6'), J$_{orto}$=10 Hz). ESI/MS (LC/MS): m/z 405, [M+H]$^+$. HPLC: t$_R$=8.55 min.

Resveratrol-4'-glucuronide. ^{1}H-NMR (250 MHz, MeOD) δ (ppm): 3.51 (m, 3H, CH), 3.70-3.80 (m, 1H, CH), 6.17 (t, 1H, H-4, J$_{meta}$=2,5 Hz), 6,47 (d, 2H, H-2, H-6, J$_{meta}$=2,5 Hz), 6.80-7.04 (AB system, 2H, H-7, H-8, J$_{trans}$=15 Hz), 7.09 (d, 2H, H-3', H-5', J$_{orto}$), 7.44 (d, 2H, H-2', H-6', J$_{orto}$=10 Hz). ESI/MS (LC/MS): m/z 405, [M+H]$^+$. HPLC: t$_R$=5.05 min.
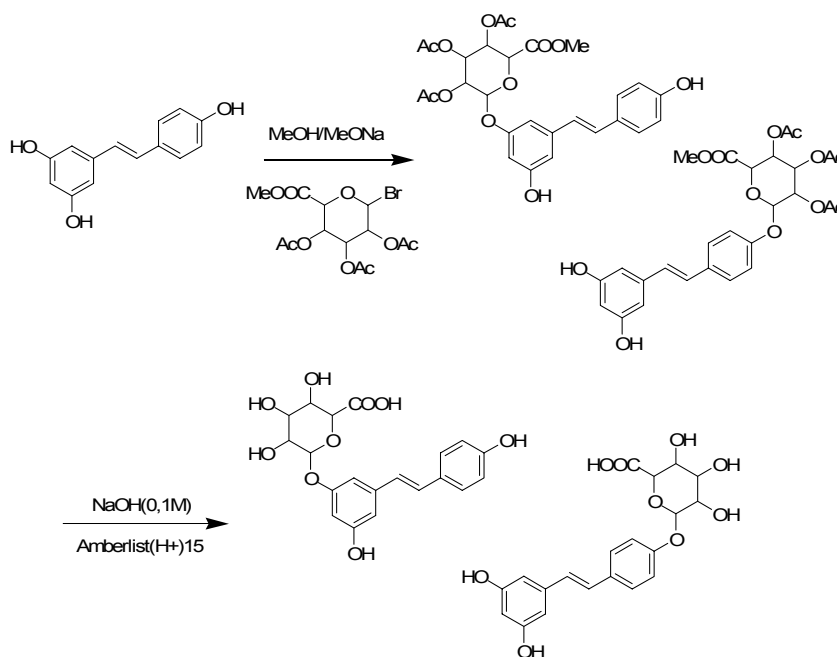
Scheme 2. Synthesis of resveratrol glucuronides

Chromatographic comparison of synthetic conjugates with metabolites observed *in vivo*. Synthesized resveratrol conjugates were dissolved in a water:CH_3CN 7:3 (5 µM final concentration) and then analysed by HPLC-UV (see above). 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 30% in 15 min, from 30% to 60% in 15 min, from 60% to 100% in 5 min (2 min). The same HPLC protocol was used to analyze a representative blood sample coming from a pharmacokinetic study and containing all the species observed until now during this type of studies. Chromatograms at 320 nm were then compared.

Extraction from blood samples. Freshly withdrawn blood samples (1 ml) were spiked with 3,4'-resveratrol-disulfate, 3-resveratrol-glucuronide or 3-resveratrol-sulfate (dilution from 1000× stock solutions in DMSO, 0.1% final DMSO). 200 µl aliquots were then treated as described in Chapter 3 using 4,4' dihydroxybiphenyl as internal standard, and then analyzed by HPLC-UV. Analyte concentration in the spiked samples was 0.15, 0.2, 0.5, 1 or 5 µM; internal standard was added at the same concentration (25µM) in all samples.

Results

Synthesis of resveratrol metabolites. Resveratrol has a relatively simple molecular structure with three hydroxyl-groups, two of them being equivalent. Its functionalization may lead to a few derivatives (5 in total), and the relative abundance of mono-, di- and tri-substituted species can be adjusted varying the number of equivalents of the reagents being used. Pure products can thus be obtained through separation by preparative HPLC without the use of any protecting groups for resveratrol. Derivatization with glucuronic acid required the use of methyl acetobromo-α-D-glucuronate, i.e. the corresponding molecule with a bromo- substituted reacting position and all the other hydroxyls protected; this avoids any interference of glucuronic acid hydroxyls during the reaction. Protective groups were finally removed after the alkylation step.

^{1}H-NMR allowed the individuation of the conjugation position(s) of the chemically synthesised derivatives; 4'-conjugation produces an highly symmetrical molecule, in which H-2 and H-6 are equivalent (A_2B spin system). In contrast, the 3- isomer (as well as 3,4'-disubstituted derivatives) constitutes an ABC spin system, and H-2 and H-6 give separate signals. (Fig.1)
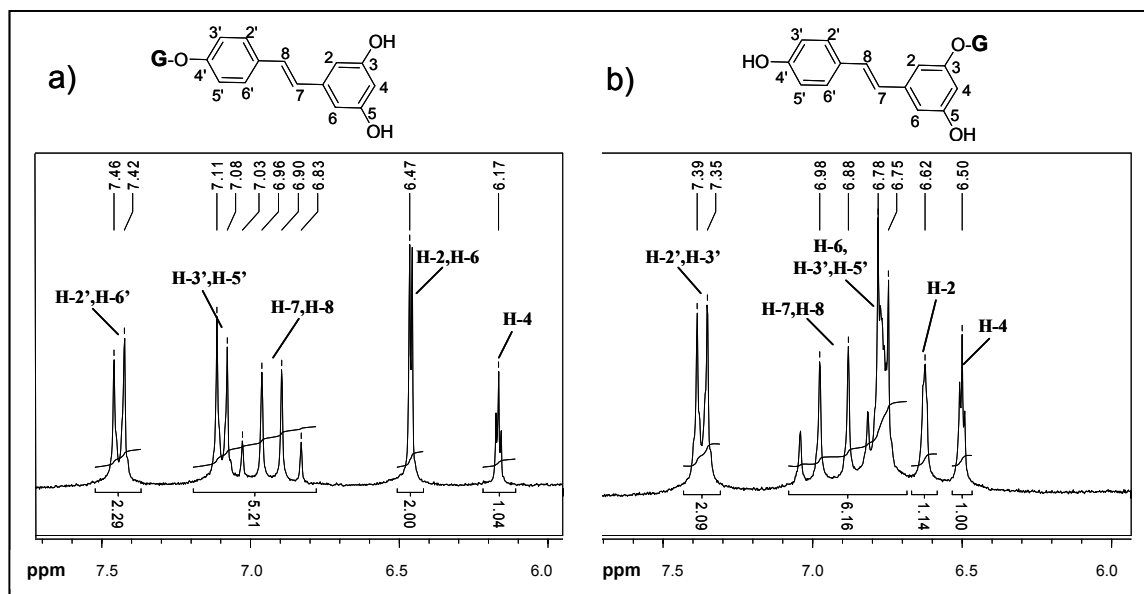
Fig. 1. Identification of conjugation position by ^{1}H-NMR (MeOD, 250 MHz). Comparison of resveratrol proton signals in: a) resveratrol-4'-glucuronide; b) resveratrol-3-glucuronide. **G**: glucuronyl-. Similar results were obtained also for the sulfates.

Comparison of synthetic conjugates with metabolites observed *in vivo*. HPLC-UV chromatograms of the synthetic derivatives and of a blood sample withdrawn from a rat 30 min. after a single intragastric dose of resveratrol (0.22 mmol/kg) were compared. Previous LC-MS analysis and enzymatic treatment (with sulfatase and/or glucuronidase) of the same blood sample allowed the identification of the HPLC-UV peaks as "sulfates" or "glucuronides", but nothing was known about their conjugation position. Co-elution with the synthetic derivatives allowed the complete characterization of the peaks, identified as resveratrol 3,4'-disulfate, 3-glucuronide and 3-sulfate.

Synthetic conjugates identified as *in vivo* metabolites were thus selected to be employed in future studies on their properties. We started using them as "standards" to evaluate the suitability/efficiency of the blood analysis protocols optimized for resveratrol.
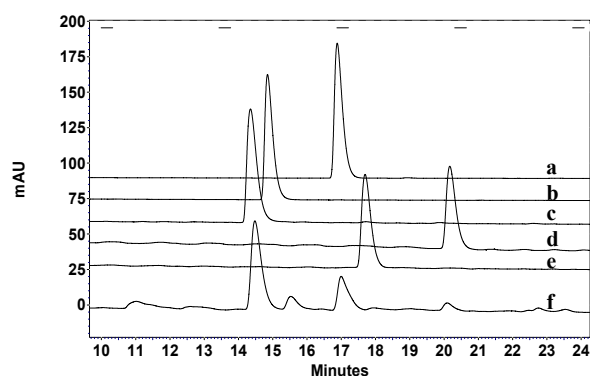
Fig. 2. Comparison between synthetic resveratrol conjugates and *in vivo* metabolites. HPLC chromatograms recorded at 320 nm of: a) R-3-glucuronide; b) R-4'-glucuronide; c) R-3,4'-disulfate; d) R-3-sulfate; e) R-4'-sulfate; f) rat blood sample collected 30 min after administration of 0.22 mmol/kg resveratrol.

Extraction from blood samples. We verified the suitability of the protocol validated for resveratrol (Chapt. 3) in extracting also its metabolites from whole blood; for this purpose blood samples were spiked with a known amount of resveratrol-3,4'-sulfate, 3-glucuronide or 3-sulfate, and then treated as previously described. The method was found to ensure also for the metabolites a constant recovery ratio of the analyte to the internal standard: 0.90 ± 0.01 (N = 3), 0.64 ± 0.04 (N = 6) and 0.90 ± 0.02 (N = 3), for 3,4'-disulfate, 3-glucuronide and 3-sulfate, respectively. This ratio allows us to determine the unknown amount of analyte in a blood sample by measuring the recovery of the internal standard, which was added in a known amount. It must be stressed that recovery ratios obtained with the metabolites and with resveratrol itself (1.18 ± 0.14) are constant, but different from one analyte to the other. The differential recoveries must be taken into account when quantifying the circulating species to obtain a correct evaluation of their relative abundance during pharmacokinetic studies.

Conclusions and perspectives

We have synthesised a series of resveratrol sulfate- and glucuronyl- derivatives. ^{1}H-NMR analysis allowed us to identify the conjugation position(s), and chromatographic comparison with the species found in the bloodstream during pharmacokinetic studies in rat allowed the complete identification of the *in vivo* species (i.e. the type and position of conjugation). Besides helping qualitative analysis in this way, availability of synthetic metabolites has allowed a more precise quantification of their concentration in blood samples.

Polyphenols are redox- active compounds, and there is a tendency to ascribe their biological effects to this characteristic. As mentioned in the introduction, the concentrations of parent polyphenol are however generally much lower than those of the metabolites. To progress in our understanding of the role of the latter it will be useful to investigate their redox properties as well. Can they act as reducing agents and/or radical scavengers (or as pro-oxidant agents) in the same way as the parent aglycone? To our knowledge, no data are available on this aspect. Future experiments will therefore determine some redox parameters of the metabolites and compare them to those of resveratrol. This will be done using cyclic voltammetry and spectrophotometric radical quenching assays. The investigation will be extended to our other model polyphenol, quercetin, the synthesis of whose metabolites is planned. Another interesting perspective would be testing the ability of Phase II conjugates to interact with transcription factors and

other proteins known to respond to administration of the parent compound. The question to be addressed might be phrased as: what part of the biological effects ascribed to a given polyphenol is actually due to interactions of the polyphenol as such, and what part instead to interactions of the more abundant (if presumably less active in most cases) metabolites? This type of investigation is susceptible of almost unlimited expansion, given the number of polyphenols known to be biologically active (consider for example the so-called "phytohormones"). Contacts have been made with the group of Prof. R. Zenobi (Zürich) for a possible cooperative venture on these themes.

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7. Quercetin can act either as an inhibitor or an inducer of the Mitochondrial Permeability Transition Pore: a demonstration of the ambivalent redox character of polyphenols

Submitted

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Abstract

The Ca^{2+}- and oxidative stress-induced Mitochondrial Permeability Transition (MPT) plays an important role in phenomena ranging from tissue damage upon infarction to muscle wasting in some forms of dystrophy. The process is due to the activation of a large pore in the inner mitochondrial membrane. Antioxidants are considered a preventive and remedial tool, and mitochondria-targeted redox-active compounds have been developed. Plant polyphenols are generally considered as antioxidants, and thus candidates to the role of mitochondria-protecting agents. In patch-clamp experiments, easily oxidizable polyphenols induced closure of the channels. In swelling experiments with suspensions of mitochondria, high (20-50 μM) concentrations of quercetin, the most efficient inhibitor, promoted instead the onset of the MPT. Chelators of $Fe^{2+/3+}$ and $Cu^{+/2+}$ ions counteracted this effect. Fluorescent indicators of superoxide production confirmed that quercetin potentiates O_2^- generation by isolated mitochondria and cultured cells. Since this was not affected by chelating Fe and Cu ions, the MPT-inducing effect can be ascribed to a "secondary", metal ion-catalyzed production of ROS. These results are a direct demonstration of the ambivalent redox character of polyphenols. Their mode of action *in vivo* cannot be taken for granted, but needs to be experimentally verified.

Keywords: Mitochondrial Permeability Transition Pore; Polyphenols; Quercetin; Reactive Oxygen Species; ROS; Patch-clamp

Introduction

By the laws of thermodynamics, any complex structure depends for its very existence on the continuous input of energy. If this input takes place through a specialized device, this device is both a key element and a dangerous one, since a potential for accidents exists anywhere energy is handled. An example is provided by mitochondria, where a fraction of the electrons transported by the respiratory chain is delivered to oxygen in a process generating superoxide anion, $O_2\cdot^-$, and hence H_2O_2 and reactive oxy and peroxy radicals ([1] and refs. therein) While this fraction may be as high as 2-3% under in vitro conditions, chronic production of reactive oxygen species (ROS) by mitochondria *in vivo* is probably much lower, difficult to estimate and variable [1]. Nonetheless, its relevance and potential impact is clearly demonstrated, among much other evidence, by the phenotype of transgenic mice or cells depleted of or sovraexpressing Mn-SOD [2,3].

Production of ROS by mitochondria is relevant under frankly pathological circumstances, including the two main causes of death in the western world, cancer and ischemias. Much evidence links mitochondrial ROS to cancer. Besides being a factor in carcinogenesis [4] mitochondrial ROS have been linked to metastatic aggressiveness and angiogenesis in the tumoral mass ([5]; but see [6]). These effects are mediated by the activation of enzymes such as metalloproteases [7] and transcription factors, most notably HIF [8-10]. The intermediacy of ROS in ischemia/reperfusion (I/R) damage is also well established [11,12,1]. The downstream effector in this case is, to a considerable extent, the Mitochondrial Permeability Transition (MPT) [13-16].

The latter phenomenon (revs.: [17-21]) is at the focus of this paper. Briefly stated, it consists in the opening of an unspecific permeation pathway capable of admitting solutes up to 1.5 KDa, the Mitochondrial Permeability Transition Pore (MPTP), in the inner mitochodnrial membrane (IMM). This causes depolarization, uncoupling, loss of metabolites and respiration factors such as NADH from the mitochondrial matrix, ATP depletion, and, if prolonged, leads to necrotic cell death [14,15]. The MPTP also intervenes in apoptosis by causing cristae remodelling and thus making cytochrome c available for release [22,23].

The molecular nature of the pore is uncertain. The classical view envisioning a supramolecular complex centered around interacting adenine nucleotide transporter (ANT) and porin (VDAC) is in crisis following reports that the MPT can take place also in mitochondria not expressing the ANT [24], VDAC-1 [25,26] or cyclosporin A (CSP) sensitivity-conferring Cyclophilin D (CypD) [27,28]. The basic model however may still

hold, by substituting another mitochondrial carrier (like the phosphate carrier (PiC): [21,29, 30] and the still-misterious "maxi-chloride channel" [26] for the ANT and VDAC respectively.

In studies with isolated mitochondria the MPT is most often studied by following its propagation in the population of suspended organelles by monitoring light scattering. This is possible because opening of the MPTP induces colloidosmotic swelling. This method is not well suited to provide information on pore dynamics: swelling of a single mitochondrion takes place in the time range of 1 second [31] and once the mitochondrion has swollen, it does not report on the state of the MPTP. This latter type of information can instead be obtained by patch-clamping the membrane of mitoplasts. This approach has allowed a detailed characterization of a high-conductance pore whose pharmacology matches that of the MPTP [17,28,32]. The main characteristics of the channel can be thus summarized: 1) A maximal conductance in the 0.9-1.3 nS range (in symmetrical 150 mM KCl) with substates. 2) A characteristic substate with a conductance approximately one-half of maximal whose presence and characteristics are consistent with, although they do not prove, a dimeric structure of the MPTP [26]. 3) Moderate anionic selectivity. 4) Voltage-dependence: closure induced by relatively low voltages of either sign, with prompt reopening (ms time scale) when the voltage is turned off (unless long-lasting inactivation sets in).

A number of conditions and chemicals are known to affect the MPT, either increasing or decreasing the probability of MPTP opening, or inducing its closure once open. Matrix Ca^{2+} is a key effector: in vitro the MPT is difficult to induce unless at least μM levels of this ion are present in the mitochondrial suspension. While the details of its action are unknown, it may act by binding to cardiolipin (CL), thereby altering the susceptibility of the membrane proteins to conformation or interaction changes prompted by the co-inducer, or, importantly, to oxidation [33-35]. This model can explain the apparently unsaturable competition between Ca^{2+} and MPTP inhibitors such as CSP or ADP [36]. Activation by ROS has long been known and indeed it is considered to be an essential feature of the MPT [37-39]. Enhanced ROS production by Ca^{2+}-treated mitochondria has been observed (e.g. [40]). Believed to be caused by Ca^{2+}-induced alterations of membrane lipid-protein interactions, it is a likely mechanism for the transduction of Ca^{2+} binding into MPT opening [37,38,40-43]. The role of thiols in permeability transition onset has been carefully characterized by Bernardi's group [44-47]. At least two classes of dithiol sites can be recognized, differing in terms of accessibility to reagents and to mitochondrial pools

of glutathione and pyridine nucleotides. Another mechanistic model, not necessarily alternative, envisions again an important role of cardiolipin: its conversion to CL hydroperoxide might favour the formation of the MPTP by IMM carriers [48], and the detachment of Cytochrome c, which can thus take part in apoptosis [49, 50]. Indeed, the mitochondrial carriers candidate to the role of components of the MPTP require CL for proper function (e.g.: ANT [51], PiC [52]).

The MPT is not only a target for cell-, organ- and life-saving efforts (e.g. [20,53-56]); it can also represent a tool for the elimination of unwanted, i.e., cancerous, cells. Its induction by redox stress is one of the strategies considered in the emerging field of mitochondrial medicine [57,58]. Cancer cells live under intrinsic redox stress and may thus be more vulnerable than normal ones [59].

Manipulation of the redox state of mitochondria to either inhibit or enhance MPT occurrence thus appears to be a worthwhile approach to the prevention and/or treatment of major pathologies. Since cells maintain mM levels of "redox buffers" (e.g. glutathione) this perspective implies that a way is found to obtain sufficient concentrations of redox-active compounds in mitochondria. Hence the importance of developing mitochondrion-targeted redox-active constructs, which has stimulated ground-breaking research aiming at the delivery of ROS-abating compounds (revs.: [60-67]). Conjugation to membrane-permeant cations, the most widely used technique, results in accumulation according to Nernst's law. We have recently used conjugation to triphenylphosphonium to produce mitochondriotropic derivatives of quercetin [68], a flavonol, and resveratrol [69], a stilbene, two polyphenols widely used as model compounds and endowed with remarkable biochemical activities.

From the point of view of redox action polyphenols are ambivalent compounds [70,71]. The same chemical properties that confer them anti-oxidant character may lead, under different conditions, to a pro-oxidant effect [72,73]. Some polyphenols, including flavonols, inhibit peroxidises [74,75], and may alter the redox poise of cells by acting on enzymes of this class, including the mitochondrial thioredoxin-peroxiredoxin system and mitochondrial phospholipid hydroperoxide glutathione peroxidise. They may thus hinder or enhance the permeabilization of the IMM, a potentially useful outcome either way.

To gain a clue as to how polyphenols might act on the MPTP, we have studied the effects of some representative members of the family on it, observing the activity of the pore at the single channel level in patch-clamp experiments. We have then used the induction of swelling of isolated mitochondria to verify the effects of one of the compounds most

efficacious in electrophysiological experiments, quercetin, on suspensions of isolated organelles. The two experimental approaches led to apparently contradictory results, underscoring the importance of carefully considering experimental conditions when evaluating the biomedical effects of these potentially extremely useful natural compounds.

Materials and Methods

Materials. Daidzein was purchased from LKT Laboratories (St. Paul, MN, USA), Galangin was from Indofine (Hillsborough, NJ, USA). All other reagents were from Sigma/Aldrich/Fluka/Riedel de Haen or from sources specified in the text.

Cells and mitochondria. Human Colon Tumor (HCT116) cells [76] (kindly provided by B. Vogelstein) were grown in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) plus 10 mM HEPES buffer, 10% (v/v) fetal calf serum (Invitrogen), 100 U/mL penicillin G (Sigma), 0.1 mg/mL streptomycin (Sigma), 2 mM glutamine (GIBCO) and 1% nonessential amino acids (100X solution; GIBCO), in a humidified atmosphere of 5% CO_2 at 37 °C. HCT116 mitochondria were isolated as described in [77]. Rat liver mitochondria were prepared by standard differential centrifugation procedures and obtained as a suspension in 0.25 M sucrose, 5 mM Hepes/K^+, pH 7.4.

Electrophysiology. Patch-clamp experiments on mitoplasts were carried out essentially as described by [78]. A few µl of mitochondrial suspension were added to 1 ml of standard electrophysiology medium (150 mM KCl, 0.5 mM $CaCl_2$, 1 mM Pi, 20 mM Hepes, pH 7.4) in the patch-clamp dish, and allowed to settle for approximately 10 min. The dish was then extensively perfused with the same medium to remove the objects not attached to the bottom. At variance from [78], mitoplasts were not produced by osmotic shock, but by "spontaneous" swelling in the presence of Ca^{2+} and Pi. Under these permeability transition-inducing conditions the mitochondria swell producing objects with a diameter 1-3 µm on which seals were established under symmetrical ionic conditions. Pipette resistance was 4.6±0.4 MΩ (N=49). Connection to the Ag/AgCl ground electrode compartment was via a 1 M KCl agar bridge. For inhibition, a few µL of concentrated inhibitor solution were added, and the bath contents were thoroughly mixed by withdrawing and re-adding aliquots with a Gilson pipette. Seal configuration was mitochondrion-attached, as confirmed by the polarity of the voltage dependence of the 107 pS channel [79]. Voltage was controlled by an Axopatch 200 unit, and Axon pClamp software was used for voltage control and data analysis. The voltages reported in this paper are those applied to the patch-clamp pipette interior. Current (cations) flowing from the

pipette to the ground electrode is considered as positive and plotted upwards. The voltage was either controlled manually or applied as trains of sequential square pulses to ±20 mV with alternating polarity and a 0.1-sec. "rest" period at 0 mV between pulses. All data were filtered at 10 KHz and recorded on tape using a VR-10B (Instrutech) adaptor, and recovered later for off-line analysis.

Mitochondrial swelling and respiration assays. Mitochondrial volume changes were followed as pseudo-absorbance decrease at 540 nm in a multi-sample Kontron Uvikon 922 UV/Vis spectrophotometer. The experiments were initiated by the dilution of the mitochondrial suspension into 250 mM sucrose, 10 mM Hepes/K^+, 5 mM succinate/K^+, 1.25 μM rotenone, 1 mM P_i/K^+, pH 7.4 (suspension buffer), supplemented with the desired concentration of $CaCl_2$ and/or inhibitors. T: 20°C. Swelling traces shown in the same figure were recorded simultaneously. Respiration was monitored as [O_2] decrease in a stirred, closed, thermostatted chamber containing a Clark electrode.

Fluorescence assays. The formation of fluorescent, oxidized species from dihydroethidine (HE) (Invitrogen/Molecular Probes) was used to monitor the production of O_2^- in RLM suspensions [80]. Stock solutions were prepared as detailed in [81] and used as described in [82]. RLM were incubated at 0.5 mg.prot.ml^{-1} in suspension buffer in a stirred quartz cuvette in a Shimadzu RL-5000 spectrofluorimeter for 2-4 min. to check for signal stability. Quercetin was then added, followed by HE. Excitation was at 470 nm (3 nm slits) and emission was monitored at 585 nm (10 nm slits). O_2^- generation in cells was detected using the mitochondriotropic probe MitoSOX Red® (Invitrogen/Molecular Probes) used as specified by the producer. Cells were sown on coverslips and cultured for three days. They were then placed in an holder, incubated for 10 min. with 1 μM MitoSOX Red in HBSS (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)), then washed three times, covered with 1 mL HBSS, and placed on the microscope stage. Additions were performed by withdrawing 0.5 ml of incubation medium, adding the desired solute to this aliquot or to 0.5 ml of fresh medium with identical composition, mixing, and adding back the solution into the chamber at a peripheral point. Excitation was at 500-520 nm, and fluorescence was collected at $\lambda > 570$ nm. Images were automatically acquired at 1 min intervals using an Olympus Biosystems apparatus comprising an Olympus IX71 microscope and MT20 light source, and processed with Cell$^{R©}$ software. Images (Fig. 6) are presented using the same display parameters; fluorescence intensities can thus be compared.

Results

Inhibition of the MPTP by readily oxidizable polyphenols. The most straightforward approach to an assessment of the effects of polyphenols on the MPTP is to observe the pore itself at the single-channel level in patch-clamp experiments, recording what happens when a polyphenol is added. We performed experiments of this type with a series of polyphenols (Table I), using mitochondria isolated from rat liver (RLM) or from cultured HCT116 cells. Figures 1-3 and Supplementary Fig. 1 illustrate representative experiments.
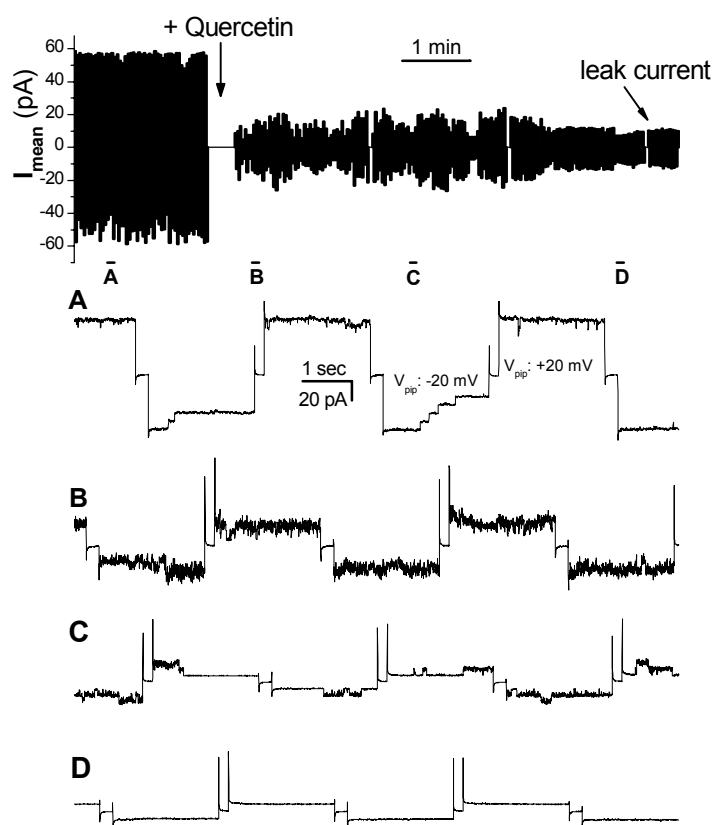
Figure 1. Inhibition of the MPT pore by quercetin in patch-clamp experiments. In this representative experiment on an HCT116 mitoplast, 20-mV, 1-second voltage pulses of alternating polarity were applied sequentially, with a 0.1 sec. interval at zero voltage between pulses. Top panel: A column plot of the mean current flowing in the circuit during each 1-sec. pulse. Addition of 50 μM Quercetin caused closure of the channels, i.e., a decrease of the mean current. A-D) Current records corresponding as indicated to the plot segments identified by bars and letters in the top panel. Immediately after the addition of quercetin the patch still exhibited a residual activity characterized by a fast gating ("noisy" trace) which eventually disappeared leaving only the leak current (D). Sampling: 1 KHz. Filter: 200 Hz.
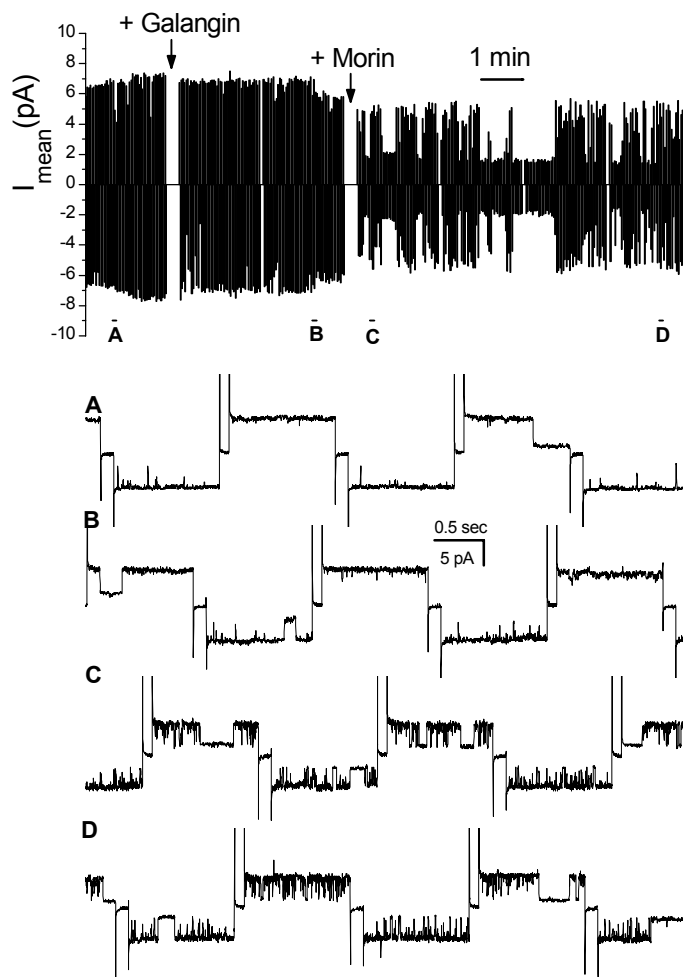
Figure 2. Morin, but not Galangin, induces a decrease of the open probability and fast gating of the MPTP. An experiment analogous to that of Fig. 1, on an HCT116 mitoplast. In this experiment the membrane patch contained only one active MPTP channel. A-D) Current records corresponding as indicated to the histogram segments identified by bars and letters in the top plot. Sampling: 1 KHz. Filter: 200 Hz.
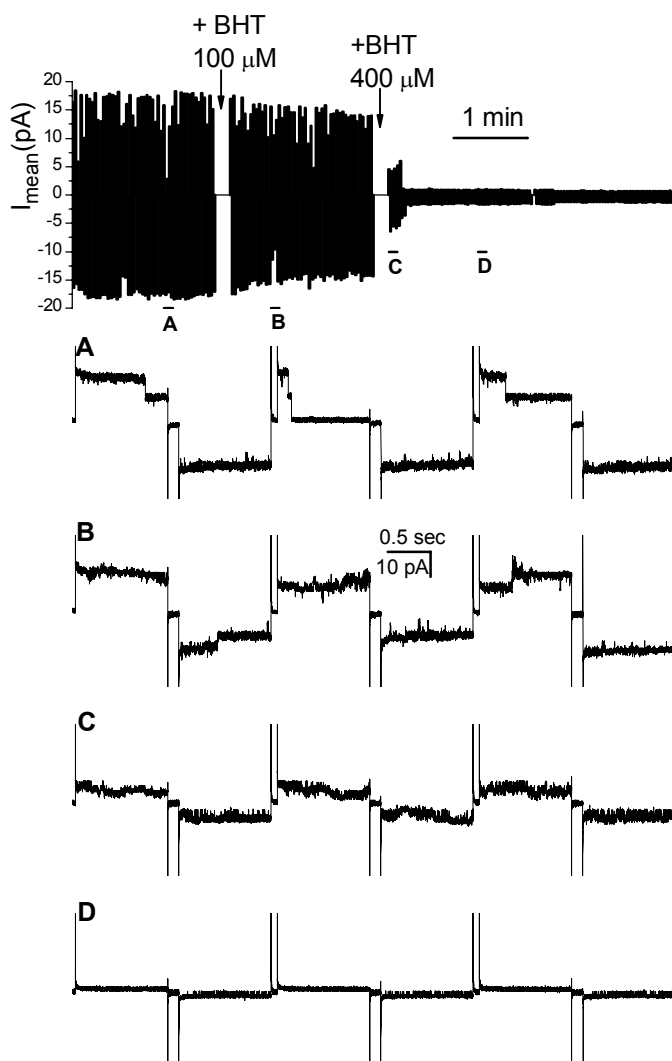
Figure 3. BHT, a widely used reducing agent, induces "noisy" MPTP behavior and pore closure much like quercetin. A representative experiment analogous to the ones in Fig.s 1 and 2.

In these experiments, a computer-driven routine applied trains of 100 1-second, 20 mV "square" voltage pulses of alternating sign, separated by 100-ms "resting" zero-voltage intervals, to the mitoplast membrane patch. This protocol is routinely used in our lab in order to distinguish drug-induced from voltage-induced channel closure. A channel may stochastically close during a pulse, but it will re-open during the resting interval unless inhibited by the compound being tested. The effect of the would-be inhibitor can then be appreciated at a glance by observing a graph plotting the mean current flowing in the circuit during each 1-second voltage pulse vs. time (top panel in Fig.s 1-3). Channel activity was monitored for a control period, after which the desired compound was added. Since the experiments were conducted in the mitoplast-attached configuration, the effect, if

any, took place after a variable lag time, inversely related to the concentration applied, needed for the diffusion from the bath to the membrane patch enclosed by the pipette rim. Activity was therefore generally monitored for at least two 100-pulse (110-second) trains after the addition before pronouncing the addition to have been ineffective.

The results are summarized in Table I. For this tabulation, an experiment was arbitrarily considered to have produced inhibition if the open probability (i.e, the mean current, leak subtracted) of the megachannels in the patch was eventually reduced by at least 70%. According to this criterion, among the polyphenols tested only quercetin (Fig. 1), myricetin and morin (Fig. 2) were able to inhibit the MPTP in a majority of experiments. BHT, a widely used reducing agent, radical scavanger and MPTP inhibitor (e.g. [17] and refs. therein) had an analogous effect (Fig. 3). Catechin and EpiGalloCatechin, which have hydroxylic substitution patterns in common with quercetin and myricetin, but are less readily oxidized, did not inhibit the pore (Table I).
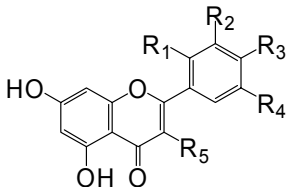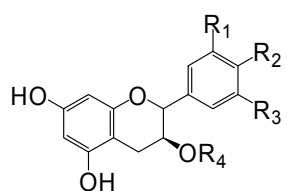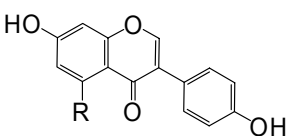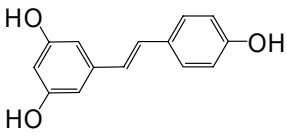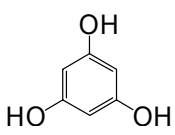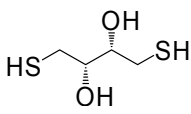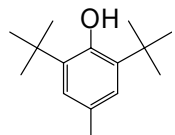
The current records reveal an interesting detail. In most experiments, the addition of an effective polyphenol initially caused only a partial inhibition of channel activity. The residual activity was characterized by a previously absent fast gating, which - especially if more than one active channel was present in the patch - generally appeared as irregular "open channel noise". This behaviour is clearly visible in Fig.s 1-3, and particularly in Fig. 2, an example chosen because morin induced a relatively slower and more regular gating pattern. The amplitude of this "flickering" generally decreased in time and in most experiments (quercetin: 19/24; myricetin: 1/3; morin: 1/3) a complete and permanent (i.e., lasting at least a few minutes) disappearance of activity eventually set in within the timeframe of the experiment.

Quercetin induces the permeability transition in mitochondrial suspensions. While patch-clamp allows one to follow the behaviour of the individual channels "in real time", the most popular technique used to study the mitochondrial permeability transition relies on MPT-dependent colloidosmotic swelling of isolated mitochondria. We therefore investigated the effects of quercetin, the most reliable inhibitor in electrophysiological experiments, using that approach.

The addition of quercetin to mitochondria suspended in a standard sucrose-based buffer containing phosphate and a respiratory substrate but no added Ca^{2+} was without detectable effect (not shown). MPT induction generally requires Ca^{2+}, at concentrations depending on conditions and on the individual mitochondrial preparation and its "age". We checked therefore whether quercetin would inhibit MPT induction by Ca^{2+}. This was not the case.

Table I

Effects of selected polyphenols on MPTP activity in patch-clamp experiments

Structure	Polyphenol	Concentration range (μM)	Inhibition (N)
	Myricetin R_1=H, R_2,R_3,R_4,R_5=OH	50	Yes(2)/No(1)
	Quercetin R_1,R_4=H, R_2,R_3,R_5=OH	5-50	Yes(24)/No(3)*
	Morin R_2,R_4=H, R_1,R_3,R_5=OH	50	Yes(3)/No(1)
	Kaempferol R_1,R_2,R_4=H, R_3,R_5=OH	50	No(3)
	Galangin R_1,R_2,R_3,R_4=H, R_5=OH	50	No(3)
	Apigenin R_1,R_2,R_4,R_5=H, R_3=OH	20	No(1)
	Catechin R_1,R_4=H, R_2,R_3=OH	50	No(2)
	EpiGalloCatechin (EGC) R_4=H, R_1,R_2,R_3=OH	50	No(2)
	EpiGalloCatechinGallate (EGCG) R_1,R_2,R_3=OH, R_4=Gallate	50	No(1)
	Genistein R=OH	50-100	No(2)
	Daidzein R=H	50	No(5)
	Resveratrol	50	No(2)
	1,3,5- trihydroxybenzene	50	No(2)
	DiThioThreitol (DTT)	2000	No(2)
	Di-t-butyl-p-hydroxytoluene	200-500	Yes(4)/No(1)

* In the 3 negative experiments [quercetin] was 10 μM in 2 cases, 45 μM in the third.

On the contrary, at concentrations > 10 μM the polyphenol acted synergically to induce swelling (Fig. 4), which could be inhibited by CSP (Fig. 4B), an acidic (5.5) pH, ADP, Mg^{2+} and DTT, well known inhibitors of the MPT (Suppl. Fig. 2 and not shown). Respiration was analogously stimulated, and CSP inhibited this stimulation (not shown).
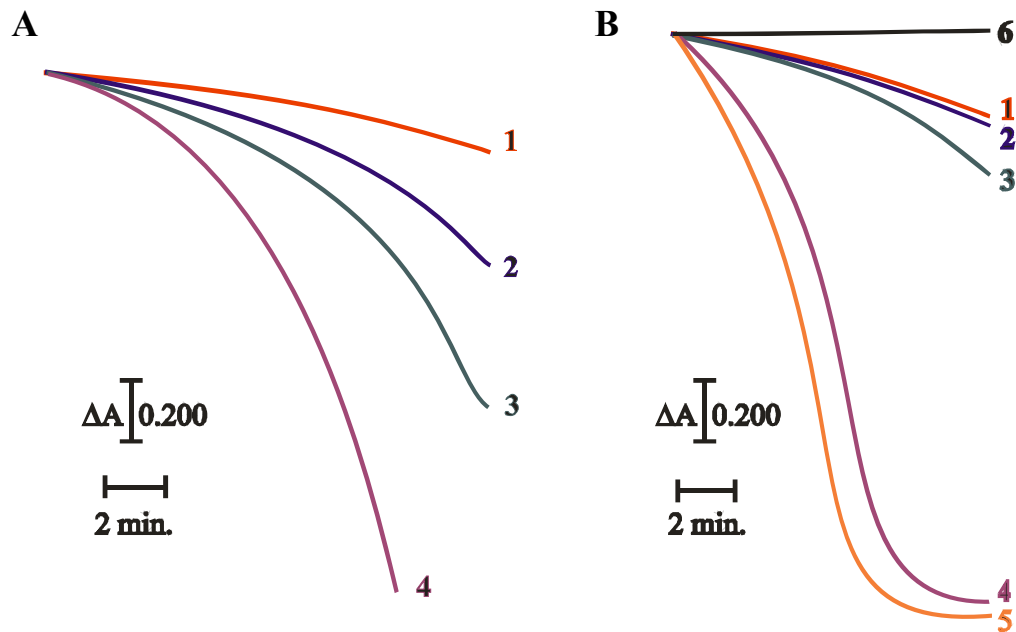
Figure 4. Ca^{2+} and Quercetin act synergically to induce swelling of mitochondria in suspension. Parallel light scattering experiments initiated by the addition of 1 mg.prot.ml^{-1} RLM to the cuvettes. **A**) Increasing concentrations of Quercetin (Q), at constant [Ca^{2+}], result in increasing rate of propagation of swelling in the mitochondrial suspension. The medium contained in all cases 40 μM $CaCl_2$ plus: trace 2: 25 μM Q; trace 3: 40 μM Q; trace 4: 50 μM Q.   **B**) Increasing concentrations of Ca^{2+}, at constant [Quercetin], result in accelerated swelling. Cyclosporin A blocks swelling. The medium contained: trace 1: 40 μM $CaCl_2$; trace 2: 20 μM $CaCl_2$ + 40 μM Q; trace 3: 30 μM $CaCl_2$ + 40 μM Q; trace 4: 40 μM $CaCl_2$ + 40 μM Q; trace 5: 50 μM $CaCl_2$ + 40 μM Q; trace 6: 40 μM $CaCl_2$ + 40 μM Q + 1 μM Cyclosporin A. The traces shown are representative of 6 (A) and 5 (B) analogous experiments.

Quercetin induces production of $O_2^{\cdot-}$. Since ROS are MPT inducers, and polyphenols can function as pro-oxidants, we checked whether the presence of quercetin was associated with an increased formation of superoxide anion in our system. Oxidation of dihydroethidine (HE), a "leuco dye" specifically sensitive to $O_2^{\cdot-}$ [80-82], was indeed greatly enhanced by 50 μM quercetin (compare traces A and C in Fig. 5). Note also that the extent and kinetics of "ethidium" fluorescence development are the same regardless of whether the suspension medium contained (trace B) or not (trace A) specific iron and copper ion chelators (see below and Discussion). These experiments are complicated by

the occurrence of the MPT: artefactual variations in the fluorescence signal are introduced by changes in light scattering due to swelling. To avoid this complication, observations of O_2^- generation by the isolated RLM were carried out in the presence of CSP.
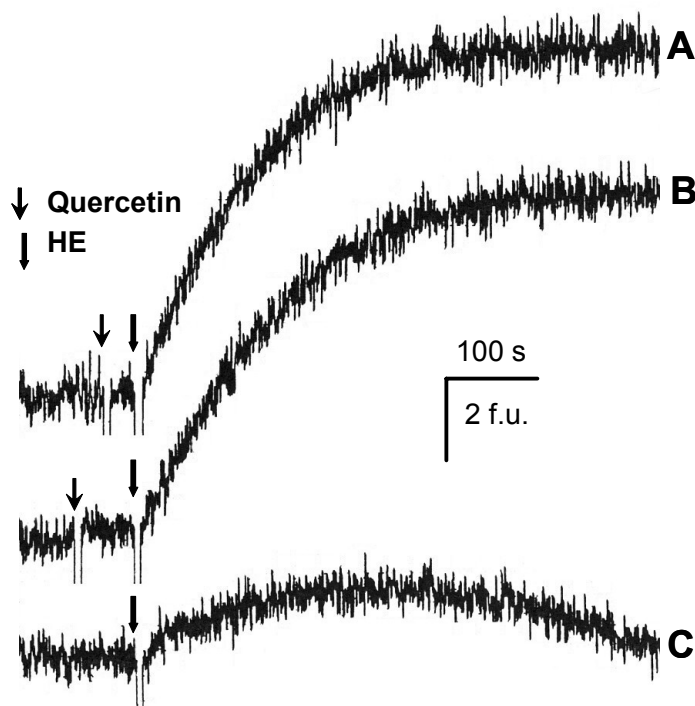

Figure 5. Quercetin enhances superoxide generation by suspensions of mitochondria. 50 µM Quercetin and 6.34 µM HE were added to the stirred suspension of RLM as indicated. Consecutive (A → C) recordings using the same preparation of RLM. For trace B the medium contained 10 µM Bathophenanthroline and Bathocuproine. For trace C (control), no Quercetin was added. The traces shown are representative of 4 analogous experiments.

A very slow fluorescence increase took place in cultured HCT116 cells loaded with MitoSOX Red®, a hydroethidine derivative targeted to mitochondria by a phosphonium ion moiety [83,84], and exposed to quercetin (Fig. 6). Although slow, this increase was reproducible, and reproducibly absent in controls (N = 5 and 3 respectively). As expected, it was markedly enhanced by the mitochondrial electron transport chain blocker Antimycin A, known as an inducer of superoxide production (e.g. [83]). These observations suggest a mild pro-oxidant effect of quercetin also within cells in culture.

MPT induction is due to Fe- and Cu-catalysed ROS production. Superoxide anion is well known to be rapidly converted to H_2O_2 by SOD. Hydrogen peroxide can in turn give rise to very reactive hydroxy and peroxy radicals via metal-catalyzed Fenton-type reactions.
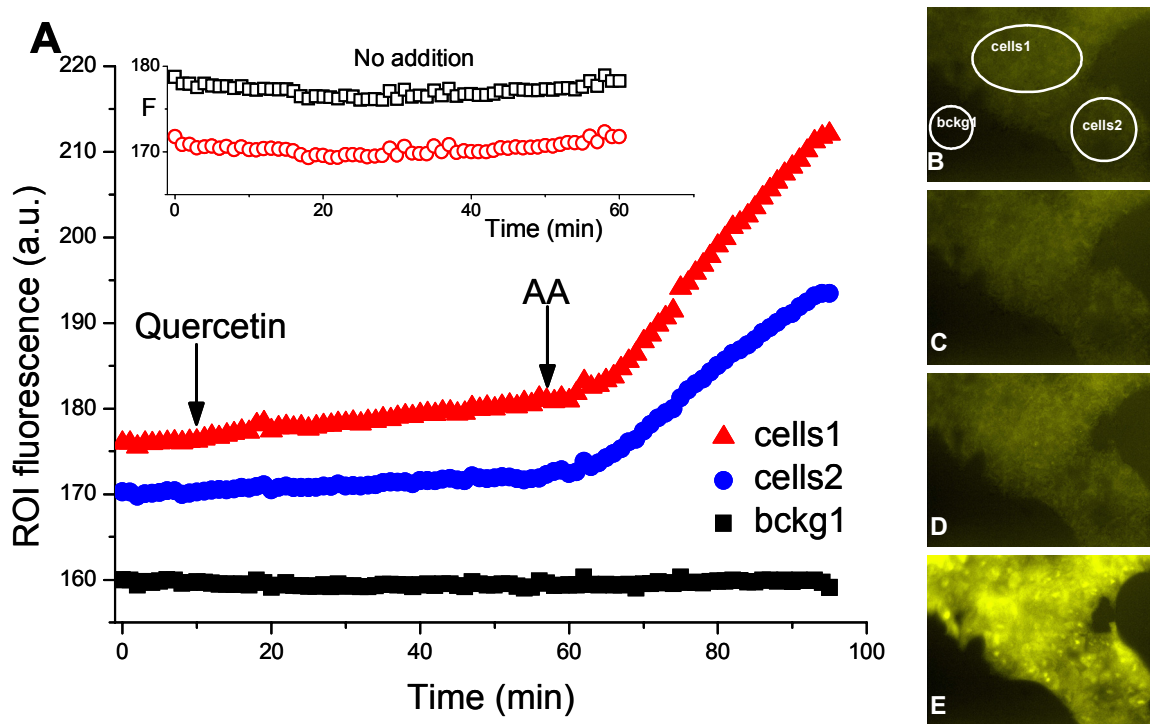
Figure 6. Superoxide production in cells exposed to Quercetin, monitored using MitoSOX Red®. A representative imaging experiment with MitoSOX Red-loaded HCT116 cells. See Materials and Methods for details. A) A computer-generated plot of the fluorescence emitted by the field areas (Regions Of Interest, ROI) identified in panel B. Images were acquired every 60 seconds. The difference in the fluorescence of the ROIs "cells1" and "cells2" is due to the difference in the area covered. 50 μM Quercetin and 2 μg/ml Antimycin A were added when indicated. The inset shows an analogous plot from two cell-filled ROIs in a control experiment in which quercetin was not added. B-E: Representative images taken from the sequence used. B) At time 0. C) After 10 min, no addition. Quercetin was added seconds after this image was acquired. D) After 46 min. in the presence of 50 μM Quercetin. Antimycin A was added seconds after this image was taken. E) After 40 min. in the presence of 2 μg/ml Antimycin A.

Whether O_2^- itself can act as an MPT inducer is unclear. A direct measurement of H_2O_2 production under our conditions was made difficult by the fact that flavonoids, including quercetin, inhibit the peroxidases used in most H_2O_2 assays (not shown; e.g.: [74,75,85]). A suspension of isolated mitochondria may be reasonably expected to contain a fair amount of reactive Fe and Cu species. We assessed the role of these metals by checking whether quercetin-enhanced swelling would be antagonized by specific chelators. Indeed bathophenanthrolin (BF; 4,7-diphenyl-1,10-phenanthroline; log K_{Fe}: 5.6; log K_{Cu}: 8.8) and

bathocuproin (BC; 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline; log K_{Cu}: 19.1; log K_{Fe}: ~0), specific chelators of iron and copper, with negligible affinity for Ca^{2+}, strongly reduced swelling and the attendant respiration increase (Fig. 7). Deferoxamine and penicillamine, two other iron and copper (respectively) chelating agents, had similar effects (not shown). As expected, the presence of added µM-range concentrations of Fe and Cu ions as well as of quercetin produced a rapid, Ca^{2+}-independent, CSP-insensitive pseudo-adsorbance decrease (not shown).
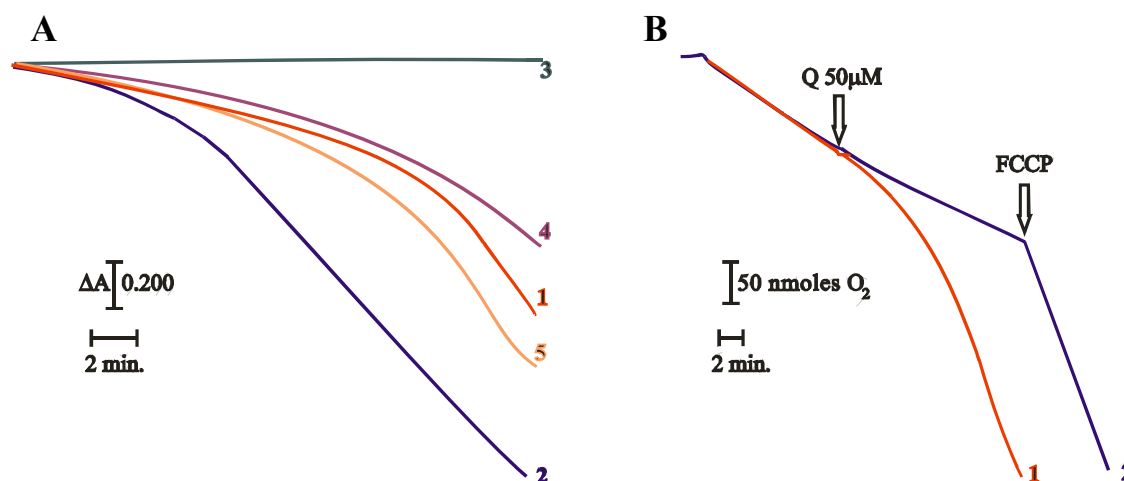
Figure 7. Induction of the Permeability Transition by Quercetin is mediated by Fe and Cu ions. **A**) Swelling. All traces: the medium contained 50 µM $CaCl_2$. Further additions (from the beginning): trace 2: 50 µM Q; trace 3: 50 µM Q + 1 µM CSA; trace 4: 50 µM Q + 10 µM BF and BC; trace 5: 50 µM Q + 10 µM BF. **B**) Respiration. When indicated, 50 µM Q was added in both runs, 1 µM FCCP only in 2. The medium of trial 2 contained 10 µM BF and BC. The data are representative of 6 analogous experiments.

Discussion

The results show that readily oxidizable polyphenols, quercetin in particolar, can both inhibit and activate the permeability transition pore, in a clear demonstration of the ambivalent nature of these redox-active compounds. Inhibition can be observed in patch-clamp experiments, conducted under well-controlled conditions, in the presence of very few mitoplasts (in a typical experiment only perhaps 100 remain in the 1-ml chamber after washing) bathed in a medium containing no transition metal ions. Activation is observed under different circumstances: a dense (typically 1 mg.prot.ml^{-1}) suspension of isolated organelles containing sequestrable iron and copper species capable of catalyzing quercetin oxidation with the production of $O_2^{\cdot-}$, H_2O_2 and other ROS which are the proximal inducing agents.

A priori, inhibition might be due to molecular interactions, i.e., phenomena not involving a chemical reaction, or to redox events. What is known about the MPTP points to this latter hypothesis as the more likely of the two. Electrochemical data on polyphenols are scarce and vary depending on the experimental conditions used. Nonetheless, available $E_{p/2}$ values [86-88] are sufficient to indicate the following ease-of-oxidation ranking: myricetin, quercetin, morin > kaempferol, catechin > resveratrol > galangin > genistein, daidzein > apigenin. These data are thus consistent with a redox mechanism for MPT inhibition. Compounds with structure and functional groups similar to those of quercetin, e.g. kaempferol or catechin, but less readily oxidizable, do not inhibit the channel, whereas BHT, in which the bulky t-butyl groups presumably impede strong interactions of the lone hydroxyl group with proteins, does.

The fast gating induced in a first phase of the inhibition process may thus be associated with redox events, i.e., it may reflect the electron transfers and conformational changes involved in the transition between (the) oxidized specie(s) constituting the open state of the MPTP and the reduced and closed channel, and viceversa. These redox events are believed to be reversible thiol/disulfide interconversions (see Introduction). The current variations thus may represent a record of the forward and backward steps of a chemical reaction: reduction of a disulfide group would cause channel closure, its re-oxidation would precipitate opening. The frequency of these events would be expected to be a function of the concentration of the reagent at the site of action - which in turn depends on the geometry of the pipette/membrane system and on the time elapsed since the addition - and of its propensity to cede electrons. In some cases, such as the one in Fig. 3, this would be low enough for the events to be well resolved. Alternatively, a reduction may alter the structure of the channel, destabilising both the open and the closed states (fast gating). In any case, a further, subsequent redox event would then block the protein in the closed-channel state (prolonged closure), explaining the eventual complete disappearence of any activity. The observed behaviour may thus be easily reconciled with the presence of multiple dithiol groups exerting control of MPTP activity [44-47].

It is relevant that in patch-clamp experiments quercetin nearly always induced channel inhibition. This implies that all the individual channels observed contained oxidized groups whose reduction caused channel disappearance, and thus that MPTP channel genesis always (or at least in the vast majority of cases) involves oxidation, presumably of thiols. This conclusion is in full agreement with the long-held views of, in particular, Vercesi and collaborators (see Introduction). Note that in the experiments reported here the

mitochondria had been made to swell by incubation for a few minutes, in the patch-clamp dish, with Ca^{2+} and Pi, i.e., under classical MPT-inducing conditions, without any added oxidizing agents.

Presumably MPTP-inhibiting reduction of disulfide groups by quercetin also takes place in suspension experiments. They do not result in any detectable inhibition of the propagation of swelling in the population. This lack of effect is not surprising, since any antioxidant action is presumably overwhelmed by the generation of ROS. Furthermore, for the disulfide moieties to be reduced, they must first of all form via oxidation of thiols. Their formation is thought to coincide with PTP opening, which leads to complete swelling in a matter of hundreds of milliseconds [31]. It is also possible that the relevant reaction may be thiol arylation by products arising from oxidation of the quercetin catechol moiety, as proposed in the case of mangiferin, another polyphenol that can induce the MPT [89]. Subsequent closure of the pore is not expected to lead, except under specific experimental conditions [90] to shrinkage. Furthermore, more than one MPTP may be open in any given mitochondrion at any given time, and there is no reason to expect individual pores to synchronize their opening or closing. Just one open pore would be sufficient to induce swelling of its mitochondrion.

The protective effect of Fe/Cu chelation (Fig. 7) implies that "secondary", Fenton reaction-derived ROS, rather than $O_2^{\cdot-}$ itself, are mostly involved in the activation of the MPTP. Indeed, BF and BC drastically slow down the propagation of swelling but have little or no effect on the $O_2^{\cdot-}$-mediated formation of fluorescent HE-derived species in RLM suspensions (compare traces A and B in fig. 5). Quercetin-promoted ROS production in cells (Fig. 6), although minor in comparison with a strong stimulus such as Antimycin, may well account at least in part for the antiproliferative/cytotoxic activity exhibited by readily oxidizable polyphenols at high concentrations [71,87,91,92].

Given that *in vitro* reactive polyphenols can act as either agonists or antagonists of the MPT depending on conditions as well as on their properties, it is difficult to predict how they would act *in vivo* [71]. Whether a flavonoid has a protective or cytotoxic action may well depend on its concentration [93]. This ambivalence is exemplified by the recent report that "mitoQ" [64], developed as an anti-oxidant agent, can also act as a pro-oxidant [94]. In electrophysiological experiments quercetin often succeeded in inhibiting the MPTP at low μM levels. MPT-inducing effects have been observed only at higher (> 10 μM) concentrations, in the presence of Ca^{2+}, and with dense suspensions of mitochondria. These conditions are not likely to occur *in vivo*. The bioavailability of polyphenols in foodstuffs

is low and metabolism by enterocytes and hepatocytes is very effective [95-97]. Metabolites make up the vast majority of polyphenol-derived molecules circulating in blood and lymph, and their total concentration does not exceed a few μM units even after a polyphenol-rich meal. Their redox properties and bioactivities have yet to be adequately studied.

On the other hand, other modes of administration may be envisioned in a therapeutic perspective, and pro-drugs are beginning to be considered as a strategy to enhance adsorption and to increase the levels of active species where needed (e.g.: [98]). Furthermore, *in vitro* experiments are necessarily carried out over a relatively short time, and the lack of effects does not rule out a significant action upon prolonged exposure, even at low levels. Indeed, this is the concept underlying current attempts to use chronic administration of mitochondria-targeted antioxidants intended to hinder processes associated with oxidative stress such as aging and neurodegeneration (rev. [63]). A variety of cancers endure oxidative stress and elevated levels of copper ions [99]. Our results suggest that these conditions may make them vulnerable to polyphenol-induced, MPT-mediated cell death if sufficient levels of oxidizable molecules are delivered. An anti-oxidant overall effect may also have oncological applications, even though the report that ROS generation by mutation-carrying mitochondria can increase metastatic aggressiveness [100] has been criticised for not adequately demonstrating the formation of the ROS themselves [6]. Some polyphenols have been found to inhibit cell shedding from primary tumours [7]. The results reported in this paper thus support a polyphenol-based approach to mitochondrial medicine, and stress the need for observations at the level of cellular and animal experimental systems.

Abbreviations

ANT: Adenine Nucleotide Translocator; BC: Bathocuproine; BF: Bathofenanthroline; BHT: 2,5-di-t-Butyl-p-HydroxyToluene; CL: CardioLipin; CSP: Cyclosporine A; FCCP: carbonyl cyanide p-trifluorometoxyphenyl hydrazone; HIF: Hypoxia-Inducible transcription Factor; IMM: Inner Mitochondrial membrane; HE: diHydroEthidine; HBSS: Hank's Balanced Salt Solution; I/R: Ischemia/Reperfusion; MPTP: Mitochondrial Permeability Transition Pore; PiC: Phosphate Carrier; RLM: Rat liver Mitochondria; ROI: Region of Interest; ROS: Reactive Oxygen Species; SOD: SuperOxideDismutase; VDAC: Voltage-Dependent Anion Channel (mitochondrial porin).

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126

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8. A mitochondriotropic derivative of quercetin: a strategy to increase the effectiveness of polyphenols

A Mitochondriotropic Derivative of Quercetin: A Strategy to Increase the Effectiveness of Polyphenols

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Mitochondria-targeted compounds are needed to act on a variety of processes that take place in these subcellular organelles and that have great pathophysiological relevance. In particular, redox-active molecules that are capable of homing in on mitochondria provide a tool to intervene on a major cellular source of reactive oxygen species and on the processes they induce, notably the mitochondrial permeability transition and cell death. We have linked the 3-OH of quercetin (3,3',4',5,7-pentahydroxy flavone), a model polyphenol, and the triphenylphosphonium moiety, a membrane-permeant cationic group, to produce proof-of-principle mitochondriotropic quercetin derivatives. The remaining hydroxyls were sometimes acetylated to hinder metabolism and improve solubility. The new compounds accumulate in mitochondria in a transmembrane potential-driven process and are only slowly metabolised by cultured human colon cells. They inhibit mitochondrial ATPase activity much as quercetin does, and are toxic for fast-growing cells.

Introduction

Polyphenols are a large family of natural compounds exhibiting, at least in vitro, a variety of biomedically important activities. A vast literature documents their potential relevance for such major healthcare endeavours as protection of the cardiovascular[1] and nervous[2] systems, the prevention and therapy of cancer,[1,3] counteracting senescence,[2,4] reducing chronic inflammation[4,5] and lengthening the lifespan of model organisms.[6–8]

These effects are attributed in part to direct interactions of polyphenols with proteins, and in part to their redox properties as reducing agents and ROS (reactive oxygen species) scavengers, that is, antioxidants.[9,10] This antioxidant character has been proposed to underlie the alleged antagonistic effects of polyphenols on ROS-inflicted damage and/or radical-mediated signalling, such as aging and neurodegeneration.[11,12] The level of reactive polyphenol attainable at the site of action is of obvious importance: cells normally maintain redox homeostasis with a mM-range pool of molecules such as glutathione. To have a measurable effect as general reducing agents, polyphenols should therefore reach concentrations that are on the same order of magnitude.

Mitochondria are the subcellular compartment in which most ROS are produced, and are the site of key events in both apoptosis and necrosis. Oxidative processes are of major importance in both cases.[13–15] In apoptosis, for example, oxidation of cardiolipin is needed for the release of cytochrome c.[15] The ROS-induced mitochondrial permeability transition (MPT)[16,17] is now believed to have a fundamental role in necrotic death, such as occurs upon reoxygenation following ischemia.[18,19] Enhanced ROS production is the common theme of mitochondrial dysfunction.[20,21]

A new sector of pharmacology targets mitochondria to prevent or induce, as the case may be, cell death.[22–25] The control

of mitochondrial redox processes is an attractive perspective in this context, and the development of drugs capable of accumulating specifically in mitochondria—"mitochondriotropic" compounds—is of obvious importance for such an effort. Important progress in this direction has been made by exploiting the matrix-negative voltage difference of about 180 mV that is maintained by energised mitochondria across their inner membrane. Compounds formed by a redox-active part, which is linked to a membrane-permeant permanent cation (usually triphenylphosphonium, TPP), accumulate in regions that are held at negative potential, that is, the cytoplasm and the mitochondrial matrix.[25,26] Importantly, no significant toxic effects of these compounds have been observed in vivo.[25]

We reasoned that polyphenol–TPP conjugates might act as antioxidants in vivo and be useful for counteracting "basal" ROS production and long-term effects such as chronic inflammation and neurodegeneration. Furthermore, they might find application against acute pathologies, for example those caused by ischemia. Antioxidants (and MPT inhibitors) would be expected to counteract this process, and indeed this seems

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to be the case.[14,24,27] In a relevant piece of work, the mito-chondriotropic antioxidant decylquinone–TPP (MitoQ$_{10}$) was administered to rats before explanting the hearts and subject-ing them to ischemia and reperfusion (I/R).[28] Treatment result-ed in a significant reduction of the necrotic area. A similar pro-tective effect was afforded by high doses of intravenous re-sveratrol in models of cerebral[29] and cardiac[30] ischemia.

Polyphenols can also induce a potentiation, rather than a re-duction, of oxidative and radical chain processes, that is, they might act as "pro-oxidants".[31–33] Which behaviour predominates depends on the abundance of metal ions capable of maintain-ing a redox cycle and/or of redox-active enzymes, such as tyrosinases ("polyphenol oxidas-es") and peroxidases, on the ion-chelating properties of the poly-phenols themselves, and on pH. Additional factors could in-clude the concentration of the polyphenol[33] and even the subcellular compartment involved.[34] Depending on such fac-tors and on the particular polyphenol involved, polyphenol–TPP conjugates might act as cytotoxic, apoptosis or necrosis-inducing pro-oxidants.

The pro-oxidant mode of action is as potentially useful as the antioxidant one, because it can be exploited to induce the death of unwanted, that is, cancerous, cells. Cancer cells live under oxidative stress,[35] which, in principle, makes them more vulnerable to ROS-mediated damage. Cell death and/or MPT induction by "redox-cycling" compounds, such as menadione or adriamycin, are well known, and antitumour, mitochondri-on-targeted, pro-oxidant-based chemotherapeutic approaches have been proposed.[36,37] In vivo it might be possible to focus the action on cancerous cells because in many tumour types, the mitochondria maintain a higher transmembrane potential than in normal tissue.[22,38,39] This approach has already been used in pioneering pharmacological work.[22,40,41] Mitochondrio-tropic polyphenols might provide significant oncological bene-fits also if they turn out to act as antioxidants. Recent studies have shown that mitochondrial ROS production is an impor-tant determinant of the metastatic potential of cancerous cells,[42] and that polyphenols are capable of reducing cell shedding from tumour masses.[43]

The sheer number and variety of properties of natural poly-phenols, their varied reactivity and the relevance of the patho-physiological processes for which they offer promise, suggest that mitochondriotropic polyphenol derivatives could find clin-ically relevant applications that are not necessarily limited to circumstances that involve redox processes.

Here we report the proof-of-principle synthesis, characterisa-tion and initial biological assessment of triphenylphosphoni-um-comprising derivatives of quercetin, a widely used model polyphenol.

Results

Quercetin (1) and the potentially useful target mitocondrio-tropic derivatives considered in this work are shown in Scheme 1.
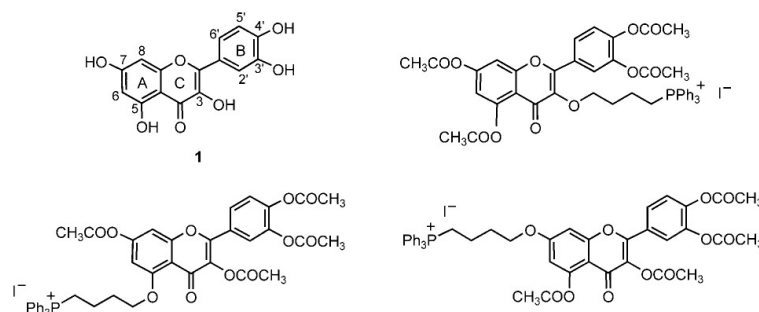

Scheme 1. Quercetin (1) and potentially useful target mitocondriotropic derivatives.

Such derivatives were identified on the basis of the follow-ing considerations: 1) the hydroxyls of quercetin provide con-venient sites to connect—through an ether bond—with a linker that bears the TPP group; 2) because the catecholic hy-droxyls determine to a large extent the redox properties of quercetin, they should be maintained as such in the target de-rivative. The connection to the TPP group ought therefore to involve one of the hydroxyls on the A or C rings; 3) to hinder metabolism and limit the formation of negative charges due to conjugation or ionisation, it was desirable to protect the remaining hydroxyls with groups expected to be rapidly re-moved by cellular enzymes, such as acetyl moieties. Moreover, hydroxyl groups are also in part responsible for the well-known tendency of polyphenols to form colloidal particles, and for unspecific interactions with proteins. The introduction of the TPP group and the protection of the hydroxyls were thus also expected to increase the low solubility of quercetin and possibly to counteract its notorious tendency to bind to proteins such as haemoglobin and albumin.[44,45] In conclusion, any of the three isomeric derivatives shown in Scheme 1, or even any mixture of them, were in principle useful candidates.

Synthesis

Following the strategy outlined in Scheme 2, we succeeded in synthesising the target derivative 7 with the TPP-bearing linker on the C ring of quercetin. Briefly, the sequence involves pro-tection of the catecholic hydroxyls, selective O-alkylation to introduce a chloroalkyl group, unblocking of the catecholic hydroxyls, acetylation and introduction of the TPP group by nucleophilic substitution on the chloroalkyl linker. The nonace-tylated derivative 9 was prepared in a similar manner from 4.

Ketal 2 was readily obtained by following the procedure of Bouktaib et al.[46] with only small modifications. The reaction of 2 with 1.2 equiv of 1-bromo-4-chlorobutane in the presence of

136
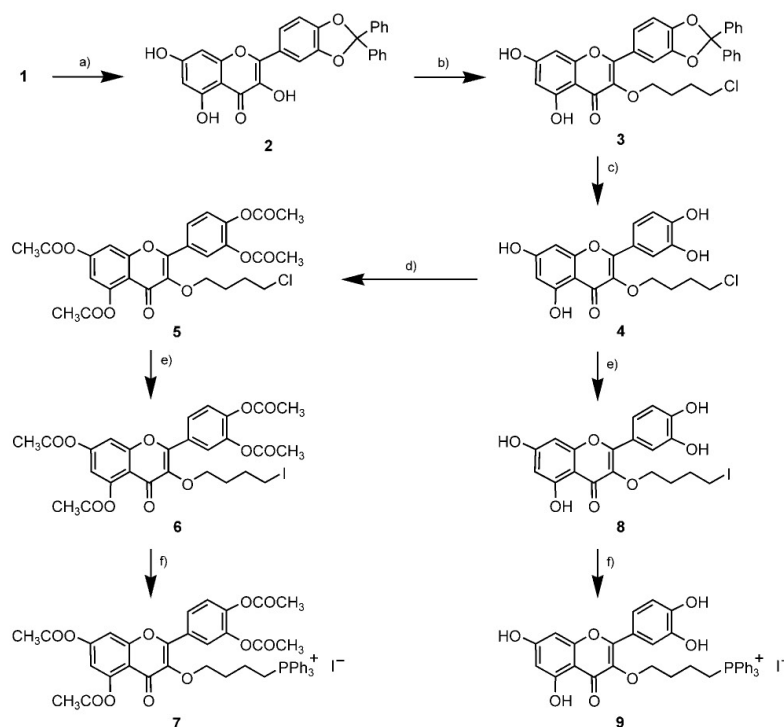
Scheme 2. Synthesis of mitochondriotropic derivatives **7** and **9**. a) Ph_2CCl_2 (3 equiv), 180 °C, 10 min; b) 1-bromo-4-chlorobutane (1.2 equiv), K_2CO_3 (1.3 equiv), DMF, Ar, room temperature, 20 h; c) $AcOH/H_2O$ 8:2, reflux, 2 h; d) $CH_3C(=O)Cl$, pyridine, room temperature, 24 h; e) NaI, acetone, reflux, 20 h; f) PPh_3, 95 °C, 6 h.

K_2CO_3 yielded **3** in reasonably good yield (45% after purification). The assignment of the site of O-alkylation in **3** was based on NMR spectroscopic data. Specifically, the presence of a characteristically narrow NMR peak at approximately 12 ppm indicates the presence of the slowly exchanging proton of the hydroxyl at C5.[47,48] To distinguish the two remaining possibilities, that is, alkylation at C3 or at C7, either of which would have been a useful outcome in this study, we went on to unblock **3** and to compare the NMR chemical shifts of the ring protons of the resulting product, **4**, with those of **1**. We found that the presence of a $-O(CH_2)_4Cl$ group in place of an $-OH$ group does not affect the chemical shifts of H6 and H8, whereas a considerable difference is observed for H2' and H6'; this indicates that the substitution is at C3 (see Table S1 in the Supporting Information).

Deprotection of the catecholic hydroxyls (Scheme 2, step c) according to a literature procedure[46] gave the desired product **4** in 80% yield after purification. Careful control of the progress of the reaction was required to avoid hydrolysis of the ether linkage. Acetylation of the free hydroxyls in **4** (Scheme 2, step d), gave the chloro derivative **5**. NMR spectroscopic analysis of this intermediate provided additional support for the attribution of the O-alkylation site to C3: comparison of the NMR spectra of **5** and of penta-acetylquercetin showed significant

differences in the chemical shifts of ring protons H2' and H6'; this confirms that C3 was the site of O-alkylation (Table S2). A similar analysis was not possible for the final product **7** due to heavy spectral interferences by the TPP group.

Compound **5** was converted to **7** via the iodo derivative **6**. This indirect route avoided the high temperature necessary for direct reaction of triphenylphosphine with the primary chloro derivative, which caused some decomposition. Compound **4** was converted to **9** in a similar manner.

Solubility in water

The solubility of **7** in water was $(4.96 \pm 0.21) \times 10^{-4}$ mol L^{-1} as determined by spectrophotometric measurements (see the Experimental Section). This solubility is at least 200-fold higher than that of quercetin.[49] This derivative thus satisfies the requirement of increased solubility in aqueous media. The solubility of **9** turned out to be less than 2 μM, presumably because the free hydroxyls facilitate the formation of large aggregates. Because this concentration is too low for recording fluorescence spectra and performing metabolism studies, solutions for those experiments were prepared in HBSS (Hank's balanced saline solution) that contained 0.1% DMSO.

Stability in aqueous solution

Both **7** and **9** are stable for at least 24 h in deionised H_2O, as determined by spectrophotometric and HPLC analysis. Compound **9** is also stable in 90% HBSS, 10% CH_3CN (added to ensure solubility of the reaction products), while in this medium **7** undergoes very slow hydrolysis of the protective acetyl groups (Figure 1).

Metabolism

We assayed metabolism of **1**, **7** and **9** by HCT116 cells, that is, the cells that were used in the experiments concerning mitochondriotropic behaviour (see below). HPLC and LC–MS analysis of the culture medium and cell extracts (see the Experimental section) showed that **1** and **9** were metabolised only to a very limited extent by these cells over a period of 8 h. Modification consisted in the introduction of a methyl group. In anal-
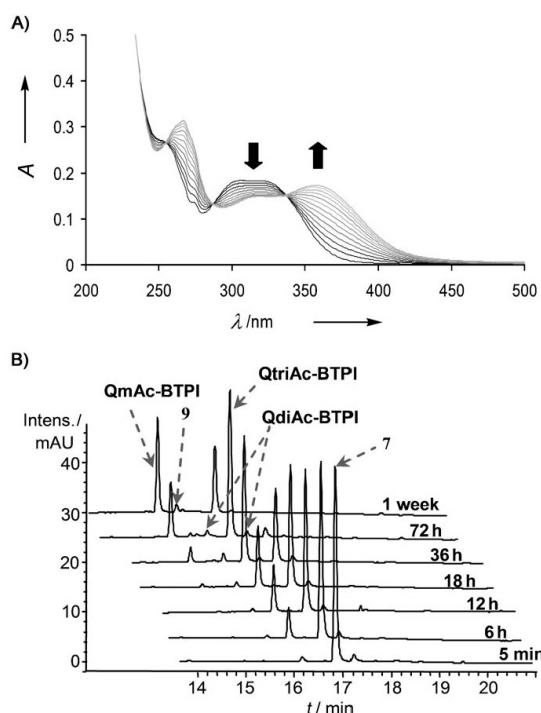
137

A)

B)

Figure 1. Hydrolysis of **7** in HBSS/CH₃CN (9:1). A) UV/Vis spectra were recorded every 6 h for 72 h. B) HPLC traces were recorded at 300 nm at different reaction times. Abbreviations: QmAc-BTPI: acetyl-3-(4-O-triphenylphosphoniumbutyl) quercetin iodide; QdiAc-BTPI: diacetyl-3-(4-O-triphenylphosphoniumbutyl) quercetin iodide; QtriAc-BTPI: triacetyl-3-(4-O-triphenylphosphoniumbutyl) quercetin iodide.
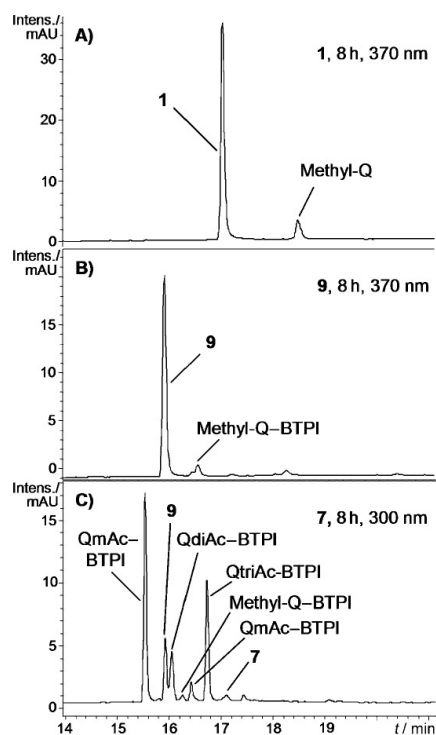
A)

B)

C)

Figure 2. HPLC chromatograms that were recorded at 300 or 370 nm, as indicated, for the extracts obtained after 8 h of incubation of: A) **1**, B) **9** and C) **7** with HCT116 cells. See the Experimental Section for details. Abbreviations as in Figure 1; methyl-Q: methylquercetin; methyl-Q-BTPI: methyl-3-(4-O-triphenylphosphoniumbutyl) quercetin iodide.

ogous experiments, **7** was progressively deacylated with the eventual transformation of most of it into the monoacylated compound, but it also underwent little conjugation. Figure 2 shows representative HPLC chromatograms.

Mitochondriotropic behaviour

We verified that compounds **7** and **9** indeed accumulate in mitochondria by two methods. In the first approach we monitored their uptake by isolated, respiring rat liver mitochondria using a TPP-sensitive electrode (Experimental Section).[50] A representative experiment with **7** is shown in Figure 3. The introduction of mitochondria causes a decrease (upward deflection of the trace) of the concentration of our compounds in the incubation medium because they become partly sequestered in the mitochondrial matrix. The subsequent addition of excess Ca²⁺ induces the mitochondrial permeability transition, with loss of mitochondrial transmembrane potential ($\Delta\psi_m$) and release of the TPP derivatives. Release is also induced by the addition of a $\Delta\psi_m$-dissipating protonophore (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, FCCP; Figure S1). The accumulation ratio, that is, the fraction of compound that is
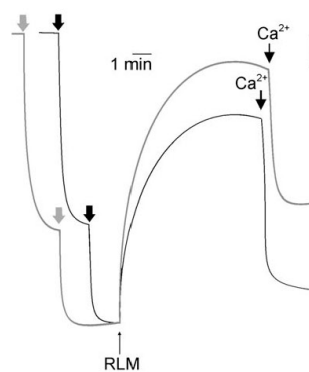

Figure 3. Accumulation of tetraphenylphosphonium (Ph₄P⁺; black trace) and **7** (grey trace) by rat liver mitochondria (RLM). Thick arrows indicate the addition of 0.16 μM Ph₄P⁺ I⁻ or **7** to the medium (200 mM sucrose, 10 mM HEPES/K⁺, 5 mM succinate/K⁺, 1 mM NaH₂PO₄, (1.25×10⁻³) mM rotenone, pH 7.4). Addition of RLM (1 mg protein mL⁻¹) and of CaCl₂ (40 μM) are also shown. The traces have been normalised to take into account the different response of the electrode in the two cases, which is quantified by the bars in the upper right corner.

138

taken up by the organelles, differs somewhat from one compound to the other. This can be attributed to different extents of binding to mitochondrial constituents (lipids, proteins, nucleic acids), which is well known to cause apparent deviations from Nernst's law. This interpretation is supported by the observation that "excess" **7** taken up is retained also after depolarisation. Analogous results were obtained with compound **9** (not shown).

In the second approach we exploited the fluorescence of **7** (Figure S5) to follow its accumulation in the mitochondria of cultured cells (Figure 4). The spectral properties of the compound, which are similar to those of quercetin itself (Figures S2–S5), allowed its fluorescence to be monitored upon excitation in the near-UV (380 nm; Experimental Section). Mitochondria can be easily recognised by their characteristic granulated/filamentous morphology and perinuclear distribution. After the addition of **7** to the medium, their weak autofluorescence due to pyridine nucleotides is progressively overwhelmed by the much more intense signal that is due to accumulation of the quercetin derivative (panels B–D). Addition of FCCP causes the rapid release of **7** (and partially deacylated derivatives) from the mitochondrial matrix (panels E–F). Some of it remains in the cytoplasm of the cells due to the presence of a cytoplasm-negative voltage difference, which is maintained by K$^+$ diffusion, across the plasma membrane. Complete series of images showing the uptake and release of the compound in an analogous experiment are available as Supporting Information. Compound **9** is expected to behave in the same manner, but its low fluorescence quantum yield (Figure S5) hindered the observation of its accumulation in mitochondria in situ.

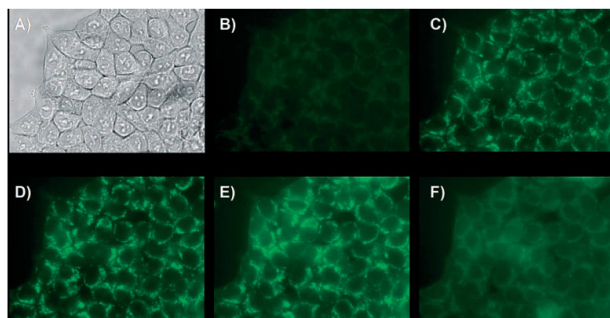We compared the inhibition by quercetin, **7** and **9** of the activity of the mitochondrial ATPase. The results were in good agreement with previous reports of an inhibition by quercetin with an IC$_{50}$ in the 50 μM range,[51] and showed that the introduction of the linker and TPP groups did not have a major effect on its activity (Figure 5).
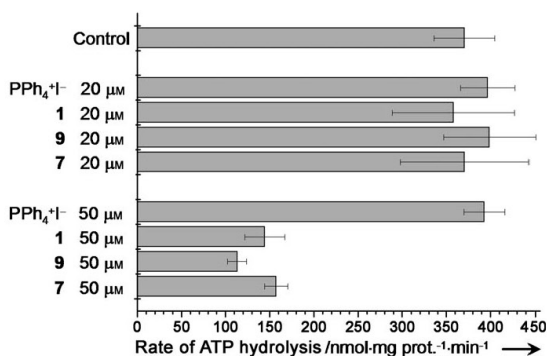

Figure 5. Effects of **7**, **9**, quercetin and tetraphenylphosphonium on the rate of ATP hydrolysis by permeabilised rat liver mitochondria. See the Experimental Section for details. Determinations were carried out in triplicate and averages ±s.d. are reported.

As a preliminary test of possible anticancer activity, we also verified the effects of these compounds, and, as controls, of the parent polyphenol quercetin, of two phosphonium salts and of quercetin plus these latter compounds on cultured cells. We used the murine colon cancer cell line C-26 and, as controls, fast- and slow-growing nontumour mouse embryonic fibroblast cell lines (MEF). Cell growth and viability was quantified by using the tetrazolium salt reduction (MTT) assay. As illustrated by Figure 6A, the various compounds had little effect on C-26 cell proliferation at 1 μM. At 5 μM, quercetin, with or without Ph$_4$P$^+$I or TPMP (triphenylmethylphosphonium chloride), significantly hindered cell growth, whereas the phosphonium salts by themselves remained innocuous. The mitochondriotropic quercetin derivatives displayed marked cytotoxicity. A similar pattern was followed with a rapidly growing line of cells of nontumour origin, that is, SV-40 immortalised mouse embryo fibroblasts (MEF; Figure 6B); none of the compounds tested had a significant effect, either at 1 or 5 μM, on a distinct, slower-growing MEF line (Figure 6C, and data not shown). Alternative protocols—in which the various compounds were provided only once at the beginning of the three-day period or were added every day (thus reaching formal final concentrations of 3 or 15 μM)—gave results that were in line with those of the substitution protocol (not reported).

Figure 4. Δψ$_m$-dependent accumulation of compound **7** in the mitochondria of cultured HCT116 cells. A) Phase contrast image taken shortly before the start of the sequential automatic acquisition of 20 fluorescence images that were taken 1 min apart. About 20 s after the first image was recorded, **7** was added to give a final concentration of 4 μM. B–D) Fluorescence images of the same field, taken approximately 0.7, 10 and 20 min, respectively, after the addition of **7**. The compound accumulates in perinuclear organelles that have mitochondrial morphology. E–F) Images recorded approximately 1 and 10 min, respectively, after the subsequent addition of 2 μM FCCP—a classic Δψ$_m$-dissipating protonophore (uncoupler). The fluorescence is released from the mitochondria and diffuses into the cytoplasm. Video clips showing the complete image sequences of fluorescence uptake and release in a similar experiment are available as Supporting Information.
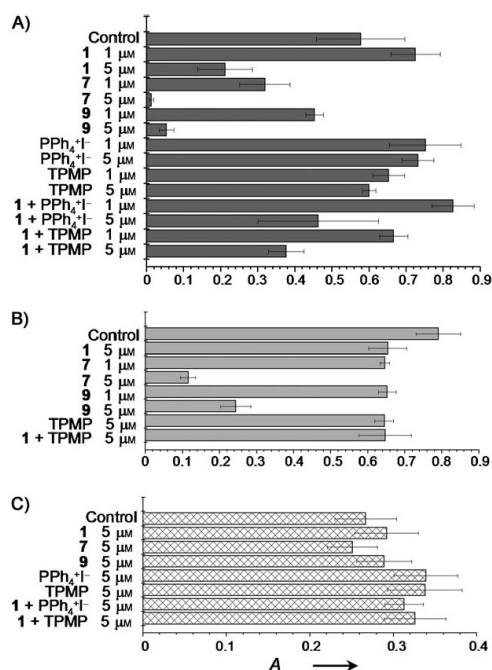
139

Figure 6. Effect of the mitochondriotropic quercetin derivatives and control compounds on the readout of tetrazolium reduction cell proliferation assays. Cells were allowed to grow for three days in the presence of the specified compounds (see the Experimental Section for details). The panels show the results of individual experiments that are representative of four (A, C) or three (B) similar ones. All measurements were performed in quadruplicate; averages ±s.d. are given. A) C-26 mouse colon tumour cells. B) Fast-growing mouse embryonic fibroblasts (MEF). C) Slow-growing MEF (note different scale).

Discussion

We have synthesised the target compound **7** as well as its non-acylated analogue **9** from the natural polyphenol quercetin (**1**). Both carry a TPP group at the end of a four-carbon, saturated linker connected through an ether bond at C3 of the quercetin skeleton. Both **7** and **9** exhibit the expected mitochondriotropic behaviour. Because a cytoplasm-negative voltage difference also exists across the plasma membrane, the charged compounds are also more concentrated in the cytoplasm than in the surrounding medium. This is best appreciated in fluorescence images taken after protonophore-induced release from the mitochondria, for example, in Figures 4E and F.

As an initial verification of whether the modifications introduced had altered the pharmacological properties of the quercetin ring system, we compared their activity as mitochondrial F_0F_1 ATPase inhibitors to that of quercetin. The latter is known to inhibit the enzyme,[51] presumably as a consequence of its binding to a site in the F_1 portion, which has been characterised by X-ray crystallography of the enzyme–polyphenol complex.[52] In our assays the mitochondrial membranes had been permeabilised with alamethicin. Therefore, no transmembrane

electrical potential could be maintained, no accumulation of mitochondriotropic compounds could occur, and inhibition was expected to take place with similar dose dependence unless the substituent(s) interfered. No such major interference was revealed (Figure 5). Compounds **7** and **9** behave similarly; this suggests that interactions with the aromatic core of the molecule are most relevant for binding and inhibition. ATP synthase inhibition is thus expected to take place upon accumulation of mitochondriotropic polyphenols in mitochondria, and it might well contribute to their overall effects on cells and organisms.

Compounds **7** and **9** behave as cytostatic/cytotoxic agents against fast-growing, but not against slow-growing cells in culture (Figure 6). This mode of action is characteristic of many chemotherapeutic agents. Inhibition of ATP synthesis might obviously be a component of this cytotoxic action. Another tentative mechanism might be that a fraction of the positively charged derivatives associates with mitochondrial DNA due to charge interactions. Quercetin itself is known to form covalent bonds to DNA.[53,54] This might result in a cytotoxic or cytostatic effect that may be more relevant in the case of fast-dividing cells, such as cancerous cells. It should at any rate be emphasised that the parent compound, quercetin, and phosphonium cations (PPh_4^+I and TPMP), alone or in combination, have much weaker toxic effects. Clearly the activity of quercetin–TPP conjugates in this case is not simply the sum of the activities of quercetin and PPh_4^+I.

These and related new compounds will be available for specific tests of their properties in vivo. Their production opens interesting perspectives because, in principle, they can either quench or promote radical oxidative processes. Thus, a class of natural compounds with useful properties can now be targeted to subcellular compartments where they ought to realise their biomedical potential in full.

Experimental Section

Materials and instrumentation: Starting materials and reagents were purchased from Aldrich, Fluka, Merck–Novabiochem, Riedel de Haen (Seelze, Germany), J. T. Baker (Deventer, The Netherlands), Cambridge Isotope Laboratories Inc. (Andover, MA, USA), Acros Organics (New Jersey, USA), Carlo Erba (Milano, Italy) and Prolabo (Fontenay sous Bois, France), and were used as received. The ^1H and ^{13}C NMR spectra were recorded with a Bruker AC 250F spectrometer operating at 250 MHz for ^1H NMR and 62.9 MHz for ^{13}C NMR. Chemical shifts (δ) are given in ppm, and the residual solvent signal was used as an internal standard. 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 ion trap detector and ESI source. Accurate mass measurements were obtained by using a Mariner ESI-TOF mass spectrometer (PerSeptive Biosystems). TLCs were run on silica gel that was supported on plastic (Macherey–Nagel Polygram®SIL G/UV$_{254}$, silica thickness 0.2 mm), or on silica gel that was supported on glass (Fluka; silica thickness 0.25 mm, granulometry 60 Å, medium porosity) and were 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 of analytical or synthetic

140

grade and were used without further purification. HPLC–UV analyses for assessing the purity of the compounds synthesised were performed by a Thermo Separation Products Inc. system with a P2000 Spectra System pump and a UV6000 LP diode array detector (190–500 nm). UV/Vis spectra were recorded at 25 °C with a Perkin–Elmer Lambda 5 spectrophotometer equipped with water-thermostated cell holders. Fluorescence spectra were recorded at 25 °C with a Perkin–Elmer LS-55 spectrofluorimeter equipped with a Hamamatsu R928 photomultiplier and thermostated cell holder. Quartz cells with an optical pathlength of 1 cm were used for measurements of both absorption and fluorescence spectra.

Synthesis procedures

3′,4′-O-Diphenylmethane quercetin (2): The protection of quercetin catechol ring was carried out by a slight modification of the procedure by Bouktaib et al.[46] Briefly, compound **1** (3.0 g, 8.9 mmol, 1 equiv) and dichlorodiphenylmethane (5.1 mL, 27 mmol, 3 equiv) were mixed and heated at 180 °C for 10 min. The residue was diluted in minimal CH_2Cl_2, sonicated and purified by flash chromatography by using CH_2Cl_2/EtOAc (95:5) as eluent to afford **2** in 67 % yield. ^{1}H NMR (250 MHz, [D$_6$]DMSO): $\delta = 6.22$ (d, $J = 2.0$ Hz, 1 H; Ar), 6.49 (d, $J = 2.0$ Hz, 1 H; Ar), 7.20 (d, $J = 8.0$ Hz, 1 H; H5′), 7.39–7.60 (m, 10 H; Ar), 7.78–7.86 (m, 2 H; H2′, H6′), 9.68 (br s, 1 H; OH), 10.87 (br s, 1 H; OH), 12.41 ppm (s, 5-OH); ESI-MS (ion trap): m/z: 466 [M+H]$^+$.

3′,4′-O-Diphenylmethane-3-(4-O-chlorobutyl) quercetin (3): K_2CO_3 (0.75 g, 5.4 mmol, 1.3 equiv) and 1-bromo-4-chlorobutane (0.86 g, 5.0 mmol, 1.2 equiv) were added under argon to a solution of **2** (1.94 g, 4.16 mmol) in DMF (10 mL). After stirring overnight, the mixture was diluted in CH_2Cl_2 (30 mL) and washed with distilled H_2O (3×50 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography by using EtOAc/petroleum ether (3:7) as eluent to afford **3** in 45 % yield. ^{1}H NMR (250 MHz, CDCl$_3$): $\delta = 1.84$ (m, 4 H; CH$_2$), 3.46 (t, 2 H; CH$_2$), 3.94 (t, 2 H; CH$_2$), 6.32 (d, $J = 2.0$ Hz, 1 H; Ar), 6.42 (d, $J = 2.0$ Hz, 1 H; Ar), 6.98 (d, $J = 8.25$ Hz, 1 H; H5′), 7.34–7.44 (m, 5 H; Ar), 7.55–7.69 (m, 7 H; Ar), 12.60 ppm (s, 5-OH); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): $\delta = 27.0$ (CH$_2$), 28.9 (CH$_2$), 45.2 (CH$_2$Cl), 71.5 (OCH$_2$), 94.1, 98.9, 104.5, 108.8, 109.1, 117.5, 124.2, 124.3, 126.0, 128.8, 129.8, 137.4, 139.4, 146.8, 148.7, 155.4, 156.6, 161.4, 164.5, 178.2 ppm (C4); ESI-MS (ion trap): m/z: 557, [M+H]$^+$; HRMS (ESI-TOF): m/z: calcd for $C_{32}H_{26}O_7Cl$: 557.1362 [M+H]$^+$; found: 557.1329.

3-(4-O-Chlorobutyl) quercetin (4): The catechol ring protection was removed according to the procedure employed by Bouktaib et al. for analogous quercetin derivatives.[46] Briefly: compound **3** (1.0 g, 1.80 mmol) was dissolved in a mixture of acetic acid/H$_2$O (4:1; 50 mL) and the solution was heated at reflux for 2 h. Then H$_2$O (100 mL) and EtOAc (50 mL) were added and the organic layer was collected, washed with sat. aq NaHCO$_3$ solution (100 mL) and dried over MgSO$_4$. After filtration, the solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography by using CHCl$_3$/acetone (8:2) as solvent to afford **4** in 80 % yield. ^{1}H NMR (250 MHz, [D$_6$]DMSO): $\delta = 1.82$ (m, 4 H; CH$_2$), 3.66 (t, 2 H; CH$_2$), 3.94 (t, 2 H; CH$_2$), 6.19 (d, $J = 1.75$ Hz, 1 H; Ar), 6.40 (d, $J = 1.75$ Hz, 1 H; Ar), 6.89 (d, $J = 8.25$ Hz, 1 H; H5′), 7.44 (dd, $J = 8.25$, 2.0 Hz, 1 H; H6′), 7.51 (d, $J = 2.0$ Hz, 1 H; H2′,), 12.72 ppm (s, 5-OH); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): $\delta = 27.0$ (CH$_2$), 28.9 (CH$_2$), 45.3 (CH$_2$Cl), 71.3 (OCH$_2$), 93.8, 98.7, 104.4, 115.7, 115.8, 120.9, 121.1, 136.8, 145.4, 148.8, 156.2, 156.6, 161.5, 164.3, 178.2 ppm (C4); ESI-MS (ion trap): m/z: 393, [M+H]$^+$; HRMS (ESI-TOF): m/z: calcd for $C_{19}H_{18}O_7Cl$: 393.0736 [M+H]$^+$; found: 393.0736.

3′,4′,5,7-Tetra-acetyl-3-(4-O-chlorobutyl) quercetin (5): Acetyl chloride (1.1 mL, 15 mmol, 20 equiv) was added dropwise and under continuous stirring to a mixture of **4** (300 mg, 0.76 mmol, 1 equiv) and anhydrous pyridine (0.85 mL, 7.6 mmol, 10 equiv), which was cooled in a bath of dry ice/acetone (−78 °C). A white precipitate (pyridinium chloride) formed immediately. The mixture was subsequently allowed to warm to room temperature and stirred overnight. Then CH$_2$Cl$_2$ (50 mL) was added, and the organic layer was washed with 0.5 N HCl (3×50 mL) and H$_2$O (2×30 mL). The organic solution was dried over MgSO$_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography by using CH$_2$Cl$_2$/petroleum ether/EtOAc (6:3:1) as eluent to afford the acetylated product **5** in 78 % yield. ^{1}H NMR (250 MHz, CDCl$_3$): $\delta = 1.85$ (m, 4 H; CH$_2$), 2.33 (m, 9 H; OAc), 2.45 (s, 3 H; OAc), 3.56 (t, 2 H; CH$_2$), 3.99 (t, 2 H; CH$_2$), 6.82 (d, $J = 2.25$ Hz, 1 H; Ar), 7.30 (d, $J = 2.25$ Hz, 1 H; Ar), 7.35 (d, $J = 8.5$ Hz, 1 H; H5′), 7.92 (d, $J = 2.0$ Hz, 1 H; H2′), 7.96 ppm (dd, $J = 8.5$, 2.0 Hz, 1 H; H6′); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): $\delta = 20.5$ (CH$_3$), 20.6 (CH$_3$), 21.1 (CH$_3$), 26.9 (CH$_2$), 28.8 (CH$_2$), 45.3 (CH$_2$Cl), 71.5 (OCH$_2$), 110.0, 114.2, 114.9, 124.0, 124.3, 127.1, 128.6, 140.4, 142.2, 144.1, 149.6, 153.1, 154.1, 156.3, 168.3 (C=O), 168.4 (C=O), 168.6 (C=O), 169.1 (C=O), 172.5 ppm (C4); ESI-MS (ion trap): m/z: 561, [M+H]$^+$; HRMS (ESI-TOF): m/z: calcd for $C_{27}H_{26}O_{11}Cl$: 561.1158 [M+H]$^+$; found: 561.1120.

3′,4′,5,7-Tetra-acetyl-3-(4-O-iodobutyl) quercetin (6): Compound **5** (100 mg, 0.18 mmol, 1 equiv) was added to a sat. solution of NaI in anhydrous acetone (10 mL) and heated at reflux for 20 h. After cooling, the resulting mixture was diluted in CHCl$_3$ (30 mL), filtered and washed with H$_2$O (3×30 mL). The organic layer was dried over MgSO$_4$ and filtered. The solvent was evaporated under reduced pressure to afford **6** in 86 % yield. ^{1}H NMR (250 MHz, CDCl$_3$): $\delta = 1.74$–2.00 (m, 4 H; CH$_2$), 2.35 (m, 9 H; OAc), 2.47 (m, 3 H; OAc), 3.22 (m, 2 H; CH$_2$), 3.99 (m, 2 H; CH$_2$), 6.83 (m, 1 H; Ar), 7.32 (m, 2 H; Ar), 7.95 ppm (m, 2 H; H2′, H6′); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): $\delta = 8.6$ (CH$_2$I), 20.6 (CH$_3$), 21.1 (CH$_3$), 29.7 (CH$_2$), 30.4 (CH$_2$), 71.1 (OCH$_2$), 110.0, 114.2, 114.9, 124.0, 124.3, 127.1, 128.6, 140.4, 142.2, 144.1, 149.6, 153.1, 154.1, 156.3, 168.3 (C=O), 168.4 (C=O), 168.6 (C=O), 169.0 (C=O), 172.5 ppm (C4); ESI-MS (ion trap): m/z: 653, [M+H]$^+$; HRMS (ESI-TOF): m/z: calcd for $C_{27}H_{26}O_{11}I$: 653.0514 [M+H]$^+$; found: 653.0521.

3′,4′,5,7-Tetra-acetyl-3-(4-O-triphenylphosphoniumbutyl) quercetin iodide (7): A mixture of **6** (100 mg, 0.15 mmol) and triphenylphosphine (200 mg, 0.76 mmol, 5 equiv) in toluene (10 mL) was heated at 95 °C under argon. After 6 h, the solvent was eliminated under reduced pressure and the resulting white solid was dissolved in the minimum volume of CH$_2$Cl$_2$ (1 mL) and precipitated with diethyl ether (50 mL). The solvent was decanted and the precipitation was repeated five times. Residual solvent was then removed under reduced pressure to afford compound **7** in 72 % yield and 96–98 % purity. The small amount of impurities consisted of a triacetyl derivative and of an isomer of **7**. ^{1}H NMR (250 MHz, [D$_6$]DMSO): $\delta = 1.62$–1.96 (m, 4 H; CH$_2$), 2.17 (s, 3 H; OAc), 3.32 (m, 9 H; OAc), 3.68 (t, 2 H; CH$_2$), 4.00 (t, 2 H; CH$_2$), 7.10 (d, $J = 2.25$ Hz, 1 H; Ar), 7.34 (d, $J = 9.25$ Hz, 1 H; H5′), 7.62 (d, $J = 2.25$ Hz, 1 H; Ar), 7.72–8.04 ppm (m, 17 H; H2′, H6′); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): $\delta = 18.3$ (CH$_2$), 19.6 (CH$_2$), 20.5 (CH$_3$), 20.6 (CH$_3$), 20.9 (CH$_3$), 21.1 (CH$_3$), 29.5 (CH$_2$), 70.3 (OCH$_2$), 110.1, 114.3, 114.8, 118.6 (Ph, $J(^{13}$C/^{31}P)=85.9 Hz), 123.8, 124.1, 127.1, 128.5, 130.5 (Ph, $J(^{13}$C/^{31}P)=12.4 Hz), 133.7 (Ph, $J(^{13}$C/^{31}P)=10.1 Hz), 135.1 (Ph, $J(^{13}$C/^{31}P)=2.8 Hz), 140.2, 142.2, 144.1, 149.5, 153.1, 154.1, 156.2, 168.2 (C=O), 168.4 (C=O), 168.6 (C=O), 168.9 (C=O), 172.7 ppm (C4); ESI-MS (ion trap): m/z: 787,

$[M]^+$; HRMS (ESI-TOF): m/z: calcd for $C_{45}H_{40}O_{11}P^+$ 787.2303 $[M]^+$; found: 787.2346

3-(4-O-Iodobutyl) quercetin (8): Compound **4** (100 mg, 0.25 mmol, 1 equiv) was added to a saturated solution of NaI in dry acetone (10 mL) and heated at reflux for 20 h. After cooling, the resulting mixture was diluted in $CHCl_3$ (30 mL), filtered and washed with H_2O (3×30 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure to afford the product in 87% yield. ^1H NMR (250 MHz, [D$_6$]DMSO): $\delta =$ 1.72 (quintet, 2H; CH$_2$), 1.88 (quintet, 2H; CH$_2$), 3.29 (t, 2H; CH$_2$), 3.92 (t, 2H; CH$_2$), 6.18 (d, $J = 1.95$ Hz, 1H; Ar,), 6.39 (d, $J = 1.95$ Hz, 1H; Ar), 6.88 (d, $J = 8.3$ Hz, 1H; H5'), 7.43 (dd, $J = 8.3$, 1.95 Hz, 1H; H6'), 7.51 ppm (d, $J = 2.0$ Hz, 1H; H2'); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): $\delta = 8.7$ (CH$_2$I), 29.8 (CH$_2$), 30.5 (CH$_2$), 70.9 (OCH$_2$), 93.8, 98.7, 104.4, 115.6, 115.8, 120.9, 121.0, 136.8, 145.4, 148.8, 156.2, 156.6, 161.5, 164.3, 178.2 ppm (C4); ESI-MS (ion trap): m/z: 485, $[M+H]^+$; HRMS (ESI-TOF): m/z: calcd for $C_{19}H_{18}O_7I$: 485.0092 $[M+H]^+$; found: 485.0060.

3-(4-O-Triphenylphosphoniumbutyl) quercetin iodide (9): A mixture of **8** (100 mg, 0.21 mmol) and triphenylphosphine (275 mg, 1.05 mmol, 5 equiv) in toluene (15 mL) was heated at 95 °C under argon. After 6 h, the solvent was evaporated at reduced pressure, and the resulting yellow solid was dissolved in a minimal volume of CH_2Cl_2 (1 mL) and precipitated with diethyl ether (5×50 mL). The solvents were decanted after each precipitation. Residual solvent was then removed under reduced pressure to afford compound **9** in 73% yield. ^1H NMR (250 MHz, [D$_6$]DMSO): $\delta = 1.65$ (quintet, 2H; CH$_2$), 1.84 (quintet, 2H; CH$_2$), 3.63 (t, 2H; CH$_2$), 3.96 (t, 2H; CH$_2$), 6.18 (d, $J = 2.0$ Hz, 1H; Ar), 6.39 (d, $J = 2.0$ Hz, 1H; Ar), 6.74 (d, $J = 8.4$ Hz, 1H; H5'), 7.33 (dd, $J = 8.4$, 2.0 Hz, 1H; H6'), 7.44 (d, $J = 2.0$ Hz, 1H; H2'), 7.50–7.93 ppm (m, 15H; Ar); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): $\delta = 18.6$ (CH$_2$), 19.6 (CH$_2$), 29.7 (CH$_2$), 70.4 (OCH$_2$), 93.8, 98.8, 104.3, 115.5, 115.7, 118.6 (Ph, $J(^{13}C/^{31}P) = 85.8$ Hz), 120.9, 121.0, 130.4 (Ph, $J(^{13}C/^{31}P) = 12.4$ Hz), 133.7 (Ph, $J(^{13}C/^{31}P) =$ 10.1 Hz), 135.1 (Ph, $J(^{13}C/^{31}P) = 2.8$ Hz), 136.6, 145.4, 148.8, 156.1, 156.5, 161.4, 164.4, 178.1 ppm (C4); ESI-MS (ion trap): m/z: 619 $[M]^+$; HRMS (ESI-TOF): m/z: calcd for $C_{37}H_{32}O_7P^+$ 619.1882 $[M]^+$; found: 619.1893.

Solubility in water: Seven standard solutions of **7** within the concentration range $2–6×10^{-5}$ M were prepared by diluting a 10^{-3} M mother solution, which was in H_2O/CH_3CN (9:1), with water. The absorbance of each solution was measured at 300 nm, which corresponds to a plateau region in the UV absorption spectrum of **7**, and the spectra were plotted against concentration. Linear regression analysis of the data points yielded a slope of $(1.244 \pm 0.009) \times 10^4$. This curve (Figure S6) was used to interpolate the concentration of an aqueous saturated solution of **7**, which was prepared by vigorously stirring **7** (2 mg) in H_2O (1 mL). After sedimentation, the clear supernatant (200 μL) was added to H_2O (3 mL) and the absorbance of the resulting solution was measured at 300 nm. Three repetitions of this experiment yielded an average value of $A = 0.379 \pm 0.011$, from which a concentration of $(3.23 \pm 0.06) \times 10^{-5}$ M was interpolated by using the calibration curve. By correcting for the dilution factor, a solubility of $(4.96 \pm 0.21) \times 10^{-4}$ mol L^{-1} was obtained.

Chemical stability studies: The chemical stability of compounds **7** and **9** in H_2O and in HBSS buffer at 25 °C was tested by following changes in the UV/Vis spectra between 190 and 500 nm and by HPLC analysis of samples that were withdrawn at different reaction times. The reaction was initiated by adding a freshly prepared CH_3CN solution of the compound of interest (100 μL) to HBSS/

CH_3CN (9:1; 3 mL) to give a final concentration in the μM range. The composition of HBSS was: NaCl (136.9 mM), KCl (5.36 mM), CaCl$_2$ (1.26 mM), MgSO$_4$ (0.81 mM), KH$_2$PO$_4$ (0.44 mM), Na$_2$HPO$_4$ (0.34 mM), glucose (5.55 mM), pH 7.4 (adjusted with NaOH). Spectral changes were followed with a Perkin–Elmer Lambda 5 spectrophotometer (PerkinElmer) equipped with water-thermostated cell holders. Quartz cells with an optical path of 1 cm were used for all measurements. HPLC analyses were performed with the Thermo Separation Products Inc. system by using a reversed-phase column (Gemini C18, 3 μm, 150×4.6 mm i.d.; Phenomenex, (Utrecht, The Netherlands). Solvents A and B were H_2O that contained 0.1% HCOOH and CH_3CN, respectively. The gradient for B was as follows: 10% for 5 min, then from 10 to 100% in 20 min; the flow rate was 0.7 mL min^{-1}. The eluate was preferentially monitored at 300 nm.

Mitochondria: Rat liver mitochondria were isolated by conventional differential centrifugation procedures[55] from fasted male albino Wistar rats that weighed approximately 300 g and had been raised in the local facilities. The standard isolation medium was sucrose (250 mM), HEPES (5 mM, pH 7.4) and EGTA (1 mM); EGTA was omitted in the final resuspension step. The protein content was measured by the biuret method with bovine serum albumin as a standard.[56] 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. No accreditation registry for experimentation on vertebrates exists in Italy.

TPP-selective electrode: The setup used to monitor the concentration of TPP-bearing compounds in solution was built in-house following published procedures.[57,58] A calomel electrode was used as reference and the potentiometric output was directed to a strip chart recorder. The experiments illustrated by Figures 3 and S1 were conducted in a water-jacketed cell at 20 °C. The suspension medium contained sucrose (200 mM), HEPES (10 mM), succinate (5 mM), phosphate (1 mM), rotenone (1.25 μM), pH 7.4 (adjusted with KOH).

Cells: Human colon tumour (HCT116) cells,[59] which were kindly provided by B. Vogelstein, as well as fast- and slow-growing SV-40 immortalised mouse embryo fibroblast (MEF) cells (kindly provided by L. Scorrano and W. J. Craigen, respectively) and mouse colon cancer C-26 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) that contained HEPES buffer (10 mM), foetal calf serum (10%, v/v; Invitrogen), penicillin G (100 U mL^{-1}, Sigma), streptomycin (0.1 mg mL^{-1}, Sigma), glutamine (2 mM, GIBCO) and nonessential amino acids (1%, 100× solution; GIBCO), and incubated in a humidified atmosphere of 5% CO$_2$ at 37 °C.

Metabolism studies: HCT116 cells were seeded onto a 12-well plate, and allowed to grow to about 80% of confluence. They were then washed with warm HBSS, and incubated for the specified periods with 1 mL per well of 20 μM solutions of **7**, **9** or quercetin. The compounds were used as aliquots from 20 mM freshly made stock solutions in DMSO, which were diluted in HBSS just prior to adding the resulting solution to the washed cells. Medium and cells were collected together after 1, 3, 6 and 8 h of incubation. Acetic acid (100 μL, 0.6 M) and fresh ascorbic acid (100 μL, 10 mM) solutions were added, and the samples were immediately stored at −20 °C until treatment and analysis. Treatment consisted of the addition of acetone (1 mL), followed by sonication (2 min), filtration through 0.45 μm PTFE syringe filters (Phenomenex) and concentration under N$_2$. HPLC analyses were performed with the Agilent Technologies system by using a diode array detector that

142

FULL PAPERS

operated from 190 to 500 nm (G1315B) and the ion trap mass spectrometer with ESI source. Mass spectra were acquired in positive-ion mode operating in full-scan from *m/z* 100 to 1500. The HPLC protocol was the same as that employed for the chemical stabilities studies.

Fluorescence microscopy: HCT116 cells were sown onto 24 mm round coverslips and allowed to grow for 48 h. The coverslips were then washed with HBSS, mounted into supports, covered with HBSS (1 mL) and placed onto the microscope stage. The imaging apparatus consisted of an Olympus IX71 microscope equipped with an MT20 light source and CellR® software. The excitation wavelength was 380 nm and fluorescence was collected in the 500–550 nm range in the images shown in Figure 4. Sequential images were automatically recorded by following a pre-established protocol. The acquisition and display parameters of all fluorescence images shown were the same, that is, fluorescence intensities can be compared.

ATP hydrolysis assays: The enzymatically coupled NADH oxidation assay was used.[60] Mitochondria (0.25 mg protein mL^{-1}) were incubated for about 1 min in sucrose (250 mM), Tris-HCl (10 mM), EGTA–Tris (20 μM), NaH$_2$PO$_4$ (1 mM), MgCl$_2$ (6 mM), rotenone (2 μM), pH 7.6, with phosphoenolpyruvate (PEP; 1 mM), NADH (0.1 mM), alamethicin (20 μM), pyruvate kinase (PK; 20 units), lactate dehydrogenase (LDH; 50 units), all of which were from Sigma, together with the desired compound in a thermostated (25 °C), magnetically stirred cuvette in an Aminco DW-2000 UV/Vis spectrophotometer operating in the dual-wavelength mode. Membrane-permeabilising alamethicin was used to measure ATPase activity without the potential kinetic complications associated with trans-membrane transport of the adenine nucleotides.[61] Differential absorbance at 340–372 nm was sampled every 0.6 s. The reaction was started by the addition of ATP (0.5 mM). In this assay, the ADP that was formed by ATP hydrolysis was rephosphorylated by PK to generate pyruvate as the other product. Pyruvate was reduced to lactate by LDH by using NADH, which was oxidised to NAD with a decrease in absorbance, which was the parameter that was monitored. Rates of hydrolysis were determined as the best linear fit of the data.

Cell growth/viability (MTT) assays: C-26 or MEF cells were seeded in standard 96-well plates and allowed to grow in DMEM (200 μL) for 24 h to ensure attachment. In the experiments shown in Figure 6 initial densities were 1000 (C-26, fast MEF) or 2000 (slow MEF) cells per well. The growth medium was then replaced with medium that contained the desired compound from a mother solution in DMSO. The DMSO final concentration was 0.1% in all cases (including controls). Four wells were used for each of the compounds to be tested. The solution was substituted by a fresh aliquot twice, at intervals of 24 h. At the end of the third 24 h period of incubation with the compounds, the medium was removed, cells were washed with PBS, and 100 μL of CellTiter 96® solution (Promega; for details see: http://www.promega.com/tbs) was added. After 1 h colour development at 37 °C, absorbance at 490 nm was measured by using a Packard Spectra Count 96-well plate reader.

Acknowledgements

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144

Supporting information

for

A mitochondriotropic derivative of quercetin: a strategy

to increase the effectiveness of polyphenols

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Spiridione Garbisa, Mario Zoratti*, Cristina Paradisi

Table S1. Chemical shifts (δ) and, enclosed in parenthesis, chemical shift changes (Δδ) of the aromatic protons of quercetin (**1**) and 3-(4-O-chlorobutyl) quercetin (**4**) in DMSO-d$_6$ for the assignment of the O-alkylation site.

Compound	Chemical shif s(δ) and chemical shifts changes (Δδ)				
	δ(H-6)	δ(H-8)	δ(H-5')	δ(H-6')	δ(H-2')
Quercetin (1)	6.194	6.405	6.887	6.544	7.683
Compound 4	6.189 (-0.005)	6.397 (-0.008)	6.893 (+0.006)	7.436 (+0.892)	7.514 (-0.169)

The data in Table S1 show that while the chemical shift of H-6, H-8, H-5' are very similar in **4** and in **1**, those of H-2' and H-6' differ significantly, thus suggesting that O-alkylation has occurred at C-3. Table S2 shows that similar trends are found for pentaacetylquercetin and 3',4',5,7-tetraacetyl-3-(4-O-chlorobutyl) quercetin (**5**) thus supporting the assignment of C-3 as the site of O-alkylation.

Table S2. Chemical shifts (δ) and, enclosed in parenthesis, chemical shift changes (Δδ) in CDCl$_3$ of the aromatic protons of pentaacetylquercetin and of compound **5**, for the assignment of the O-alkylation site.

Compound	Chemical shif s(δ) and chemical shifts changes (Δδ)				
	δ(H-6)	δ(H-8)	δ(H-5')	δ(H-6')	δ(H-2')
Pentaacetylquercetin	6.876	7.333	7.351	7.717	7.690
Compound 5	6.822 (-0.054)	7.297 (-0.036)	7.346 (-0.005)	7.975 (+0.258)	7.919 (+0.229)

Figure S1. Accumulation of tetraphenylphosphonium (Ph_4P^+) (black trace) and 3',4',5,7-tetraacetyl-3-(4-O-triphenylphosphoniumbutyl) quercetin iodide (**7**) (gray trace) by Rat Liver Mitochondria (RLM). Thick arrows indicate the addition of 0.067 μM $Ph_4P^+I^-$ or **7** to the medium (in mM: sucrose 200, HEPES/K^+ 10, succinate/K^+ 5, NaH_2PO_4 1, rotenone 1.25×10^{-3}; pH 7.4). Addition of RLM (1 mg prot.·mL^{-1}) causes upward deflection of the traces since respiring mitochondria develop a matrix-negative transmembrane potential, and thus take up the positively charged, permeant compounds, which also bind in part to mitochondrial components. Addition of FCCP (in this experiment 0.33 μM), a classic $\Delta\psi_m$-dissipating protonophore (uncoupler), causes extensive release of the compounds. The traces have been normalized to take into account the different response of the electrode in the two cases, which is quantified by the bars in the upper right corner.

147
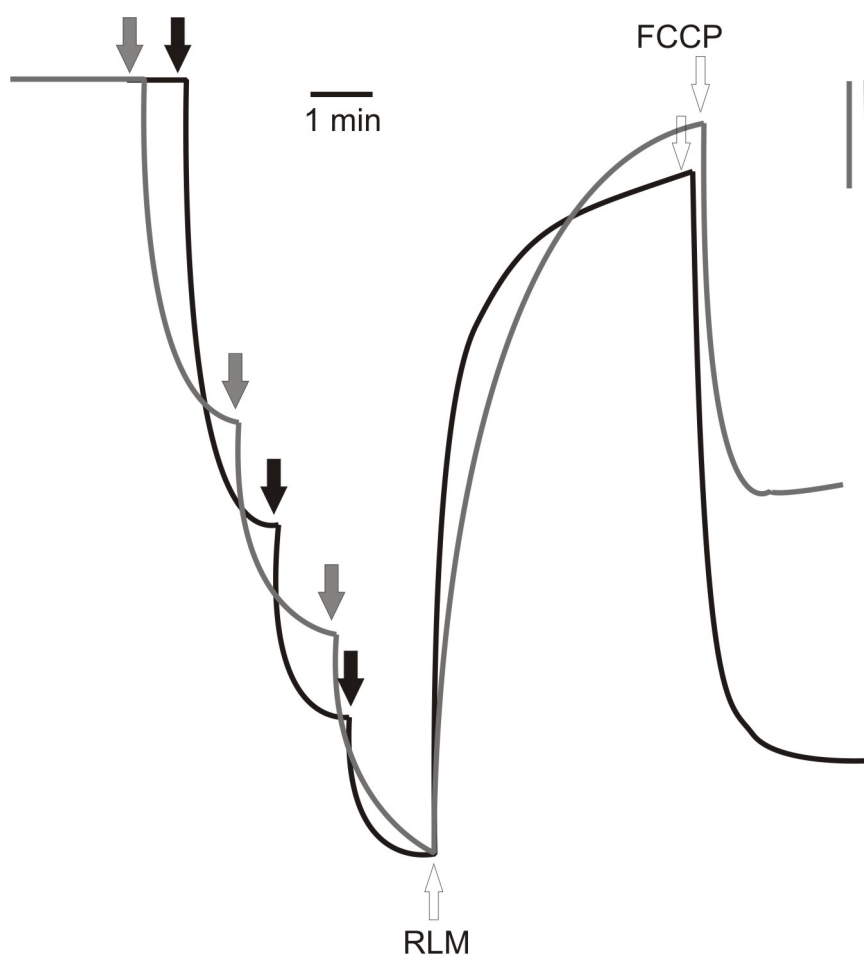
Figure S2. UV-Vis spectrum of **7** (20 μM) in HBSS:CH₃CN 9:1.
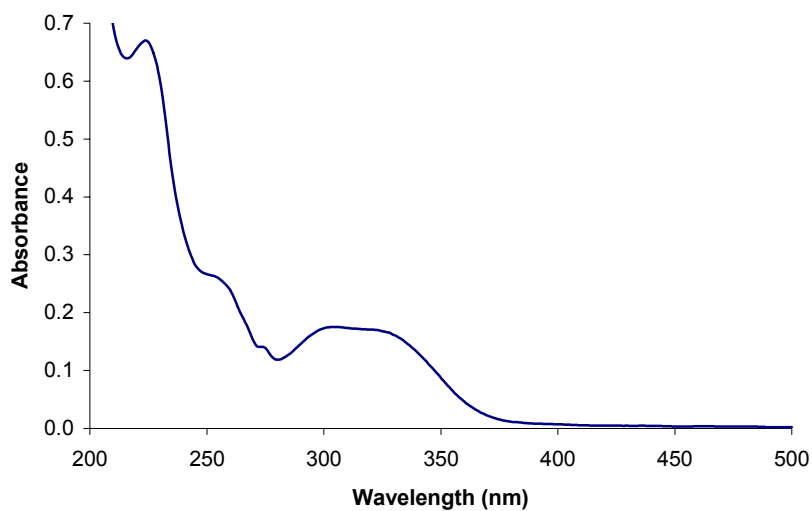
Figure S3. UV-Vis spectrum of 3-(4-O-triphenylphosphoniumbutyl) quercetin iodide (**9**) (10 μM) in HBSS:CH₃CN 9:1.
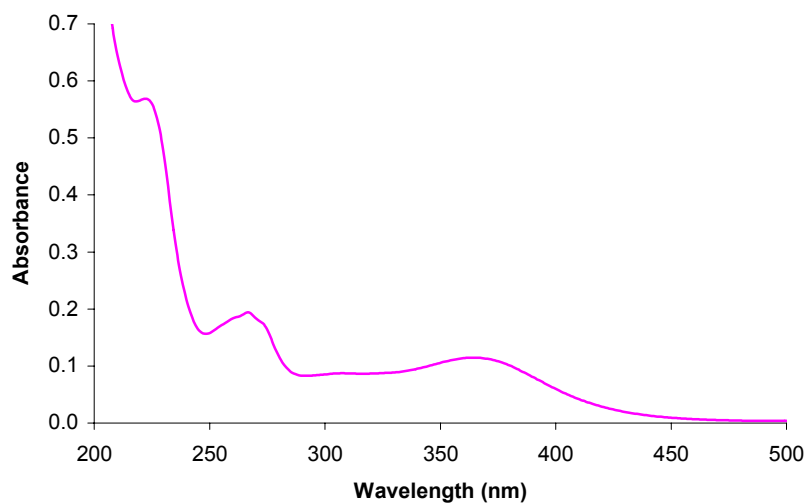
Figure S4. UV-Vis spectrum of quercetin (16 μM) in HBSS:CH₃CN 9:1.
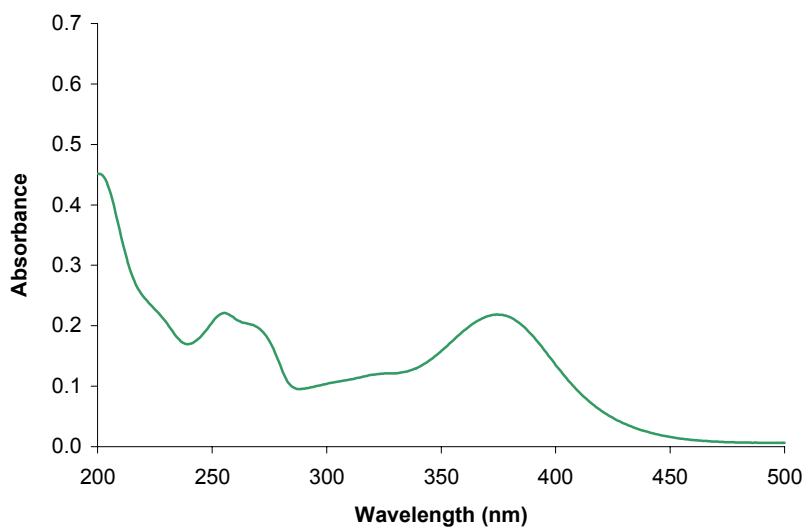
Figure S5. Fluorescence spectra of quercetin, **7** and **9** (2 μM in HBSS containing 0.1% DMSO) at 25°C. The spectra are shown in A and B with different ordinate scales. Compound **7** and quercetin were excited at 380 nm, the wavelength used in microscopy experiments (Figure 4). Compound **9** was excited at 360 nm (its absorption maximum) instead of 380 nm in order to remove a Raman scattering band.
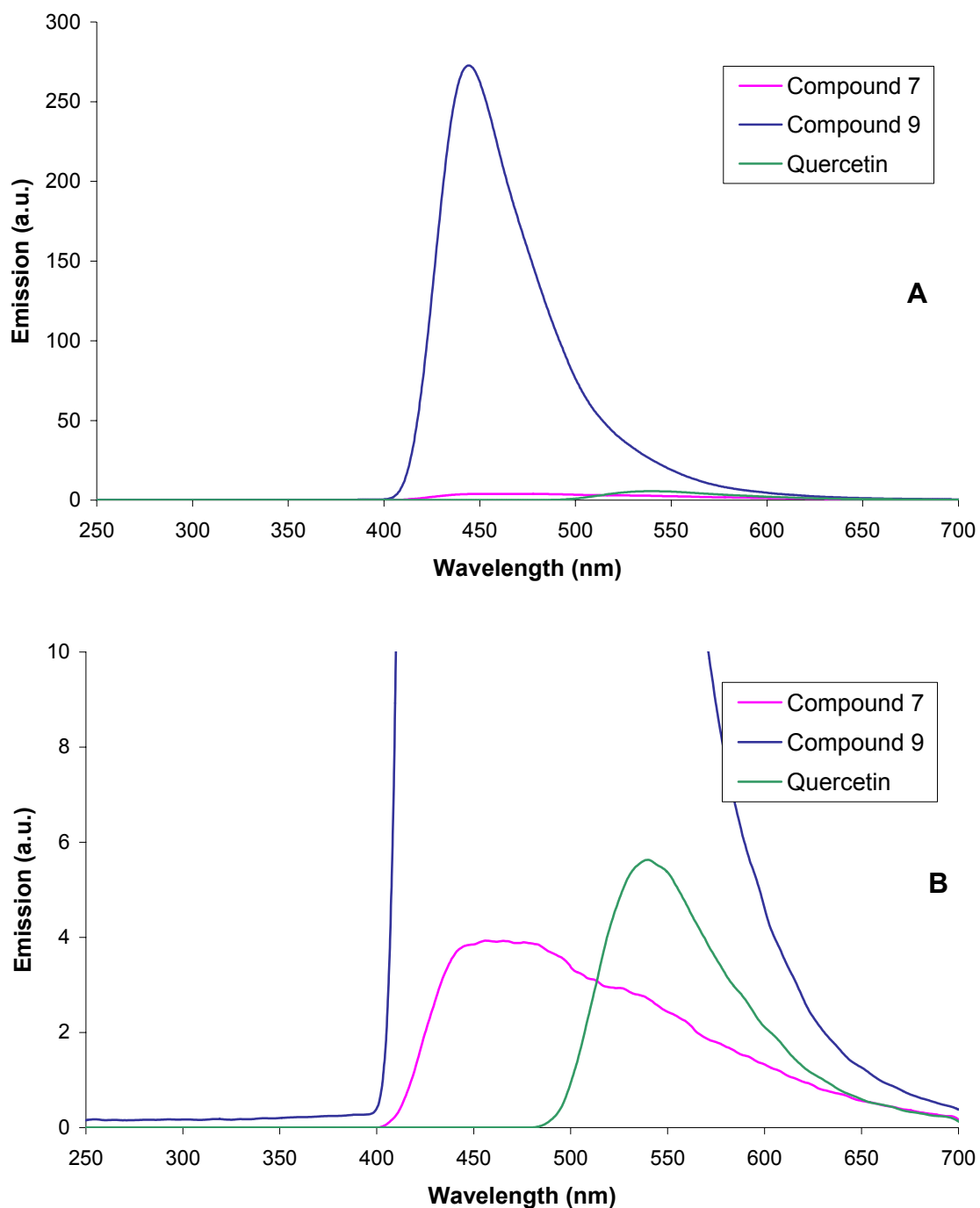
Figure S6. Calibration curve for the determination of the aqueous solubility of compound

7.
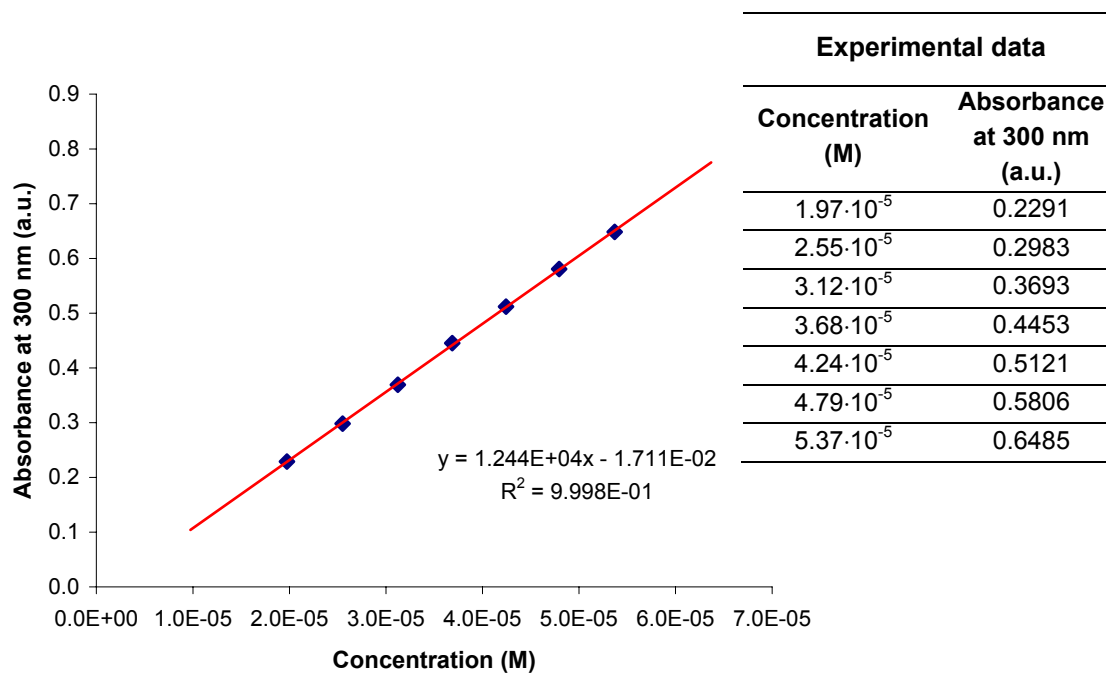
Experimental data	
Concentration (M)	Absorbance at 300 nm (a.u.)
$1.97 \cdot 10^{-5}$	0.2291
$2.55 \cdot 10^{-5}$	0.2983
$3.12 \cdot 10^{-5}$	0.3693
$3.68 \cdot 10^{-5}$	0.4453
$4.24 \cdot 10^{-5}$	0.5121
$4.79 \cdot 10^{-5}$	0.5806
$5.37 \cdot 10^{-5}$	0.6485

$y = 1.244E+04x - 1.711E-02$

$R^2 = 9.998E-01$

9. Development of mitochondria-targeted derivatives of resveratrol

Development of mitochondria-targeted derivatives of resveratrol

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ABSTRACT

To target natural polyphenols to the subcellular site where their redox properties might be exploited at best, that is, mitochondria, we have synthesised new proof-of-principle derivatives by linking resveratrol (3,4′,5-trihydroxy-$trans$-stilbene) to the membrane-permeable lipophilic triphenylphosphonium cation. The new compounds, (4-triphenylphosphoniumbutyl)-4′-O-resveratrol iodide and its bis-acetylated derivative, the latter intended to provide transient protection against metabolic conjugation, accumulate into energized mitochondria as expected and are cytotoxic for fast-growing but not for slower-growing cells. They provide a powerful potential tool to intervene on mitochondrial and cellular redox processes of pathophysiological relevance.

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Plant polyphenols are the object of intense interest because they display, at least in vitro, properties and effects of relevance for physiopathological conditions ranging from aging to cancer. These effects are ascribed to their redox properties and to interactions with signaling proteins. Polyphenols can act either as anti- or pro-oxidants, that is, inhibitors or enhancers of oxidative and radical chain processes.[1] Whether an anti- or a pro-oxidant effect predominates depends, besides the redox potential of the polyphenol, on the abundance of metal ions sustaining a redox cycle ($Fe^{2+/3+}$, $Cu^{+/2+}$) and/or of oxidizing enzymes, on the ion-chelating properties of the molecule, on pH, on the concentration of the polyphenol, and on the subcellular compartment. Either pro-oxidant or anti-oxidant activity may lead to useful oncological applications. Reactive Oxygen Species (ROS) are thought to be a major factor in cancerogenesis.[2] In particular, ROS production by mitochondria[3,4] is emerging as a key factor. The metastatic potential of cell lines has been convincingly related to this parameter.[5] Mitochondrial ROS are involved in the activation of Hypoxia Inducible Factor (HIF),[6,7] which influences angiogenesis and other aspects of tumor development.[7,8] Thus, an anti-oxidant action may limit metastasis and tumor growth. Indeed resveratrol, the representative polyphenol selected for this work, inhibits cell shedding from primary tumors.[9] On the other hand, ROS play fundamental roles in apoptosis[10] and can induce the Mitochondrial Permeability Transition

(MPT),[11] promoting in both cases cell death. Cancer cells are constitutively under oxidative stress[4] and an intensification of this stress may lead to their selective elimination. Resveratrol, in addition to other important activities,[12] reportedly exerts anti-proliferative and pro-apoptotic effects on various tumor-derived cells[13,14] and antagonizes growth of xenografts and mutagen-induced cancers.[15] It has been recently shown that resveratrol-induced death of cultured colorectal carcinoma cells involves generation of superoxide anion, that is, pro-oxidant action, at mitochondria.[14] The IC_{50} for death induction was found to be in the hundreds of μM range, a concentration which cannot be reached in vivo due to the poor bioavailability of polyphenols.[16]

We are interested in exploiting the potential of polyphenols through chemical modifications designed to serve specific purposes. Thus, an increase in solubility was achieved via esterification with aminoacids.[17] The present work aims at targeting polyphenols to the subcellular compartment where they are expected to best realize their anti-cancer potential (as well as other functions), that is, mitochondria. We report here the synthesis and properties of new mitochondriotropic derivatives of resveratrol obtained by coupling it to the membrane-permeable lipophilic cation triphenylphosphonium (TPP^+)[18] which drives accumulation in compartments held at negative relative voltage, such as the mitochondrial matrix, according to Nernst's law. Since the mitochondria of cancer cells maintain a higher-then-normal transmembrane potential,[19] mitochondria-targeted drugs may be cancer-selective.

The target derivative **4** was synthesised starting from resveratrol (**1**) in three steps as outlined in Scheme 1.[20] Briefly, O-alkyl-

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ation introduces a chlorobutyl group which is then converted to the desired TPP$^+$ derivative via two consecutive nucleophilic substitution steps: $-Cl \rightarrow -I \rightarrow - TPP^+I^-$. Direct substitution of chloride by triphenylphosphine was unsatisfactory because it required high temperatures which led to some decomposition. The assignment of the site of O-alkylation in **2** is based on ^{1}H NMR data: a unique signal is found for H-2 and H-6 indicating that these protons are equivalent. The acetylated derivative **6** was also prepared (Scheme 1), so as to compare it with **4** and assess the importance of the free hydroxyl groups for the behavior of these new mitochondriotropic molecules.

The solubility in water of the resveratrol derivatives is $(3.12 \pm 0.20) \times 10^{-5}$ mol L^{-1} and $(9.7 \pm 0.6) \times 10^{-5}$ mol L^{-1} for **4** and **6**, respectively. These solubilities are significantly higher (15- and 45-fold, respectively) than that of resveratrol. Both new compounds are essentially stable in aqueous media: **4** for at least one week both in deionised water and in Hank's Balanced Saline Solution (HBSS) with 10% CH$_3$CN (added to insure solubility of hypothetical reaction products); **6** for at least 24 h in deionised water, while in HBSS acetyl groups were slowly hydrolised (about 7% conversion to the monoacetylated derivative in 6 h). There were no detectable metabolic modifications of either **4** or **6** by cultured Human Colon Tumor (HCT) 116 cells[20] over 6 h (Fig. 1) or whole freshly drawn rat blood over 75 min (not shown), except for the hydrolysis of the acetyl ester groups of **6** in both cases.

Two methods were used to verify accumulation of the new compounds into mitochondria.[21] First, their uptake by isolated, respiring Rat Liver Mitochondria (RLM) was monitored using a TPP$^+$-sensitive electrode. A representative experiment with **6** is shown in Figure 2. The introduction of mitochondria causes a decrease (upward deflection of the signal) of **6** in the medium, due to uptake into the mitochondrial matrix. After addition of excess Ca^{2+}, which induces the MPT, or of uncouplers (not shown), **6** is partially released. The release is incomplete presumably due to binding of the resveratrol derivative to mitochondrial constituents. Analogous results were obtained with **4** (not shown).

In the second approach we exploited the spectral properties of **6** and **4**, similar to those of resveratrol itself (Supporting Data, Fig. S1 and S2), to follow their accumulation in the mitochondria of cul-
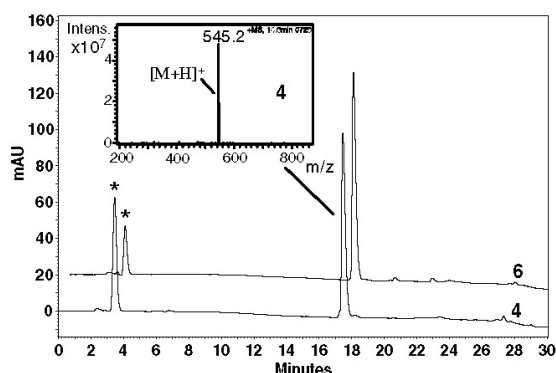

Figure 1. HPLC chromatograms recorded at 320 nm of the extracts obtained after incubation of **4** (lower trace) and **6** (upper trace) with HCT116 cells for 6 h. Inset: positive ESI-MS spectrum of **4**. For clarity, the upper trace was shifted slightly to the right along the time axis: the retention time and the mass spectrum of the major peak in this chromatogram match perfectly those of **4**. Peaks marked with ∗ are due to residual traces of acetone from the sample work-up.

tured cells by monitoring of their fluorescence upon excitation at 340 nm. Images from one such experiment are shown in Figure 3. After addition of **6** to the medium, intracellular structures become progressively fluorescent due to accumulation of the resveratrol derivative (panel B). Addition of a transmembrane potential ($\Delta\psi_m$)-dissipating protonophore (carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)) causes a loss of fluorescence due to efflux of the polyphenol (panel C). Some **6** remains in the cytoplasm of the cells due to the plasma membrane potential maintained by K$^+$ diffusion. Compound **4** behaved analogously (not shown).

As a first test of potential anti-cancer activity, we verified the effects of **4** and **6**, and of control compounds, on cultured cells (Fig. 4). Controls consisted of the parent polyphenol resveratrol, of the phosphonium salt TriPhenylMethylPhosphonium Iodide (TPMP) and of resveratrol plus this latter compound. We used
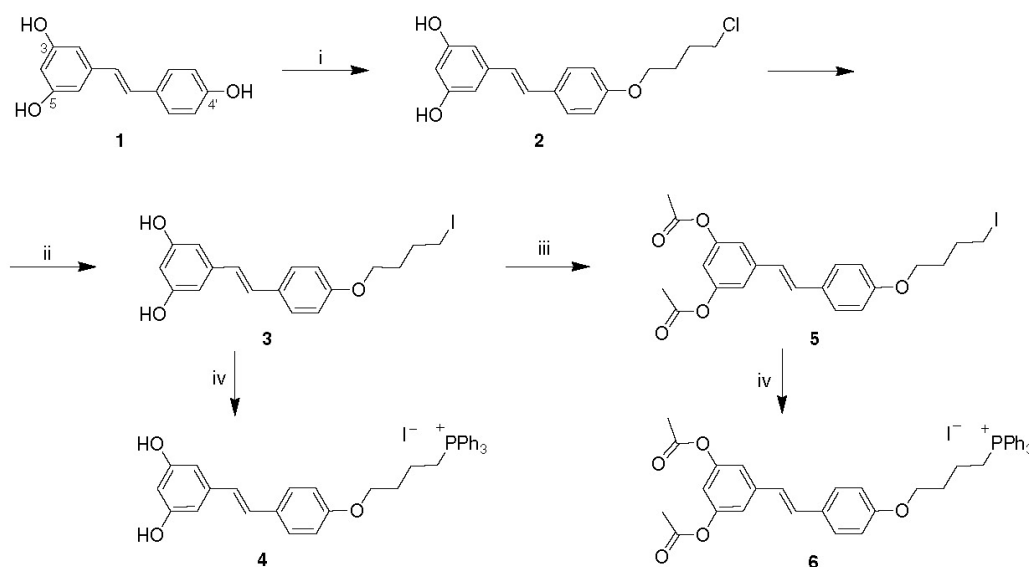
Scheme 1. Synthesis of mitochondriotropic derivatives **4** and **6**. Reagents and conditions: (i) 1-Bromo-4-chlorobutane (1.5 equiv), K$_2$CO$_3$ (1.1 equiv), DMF, Ar, rt, 20 h, yield 33%; (ii) NaI, acetone, reflux, 20 h, yield 89%; (iii) CH$_3$C(=O)Cl (20 equiv), pyr, CH$_2$Cl$_2$, yield 73%; (iv) PPh$_3$ (5 equiv), toluene, 100 °C, 6 h, yield 78%.
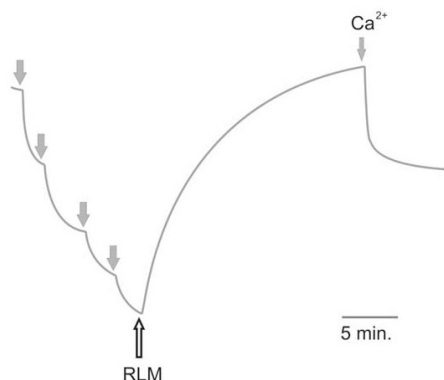
Figure 2. TPP⁺-selective electrode response to additions of **6** (thick gray arrows; 0.5 μM each), Rat Liver Mitochondria (RLM) (1 mg prot. mL⁻¹) and CaCl₂ (50 μM) at 20 °C.
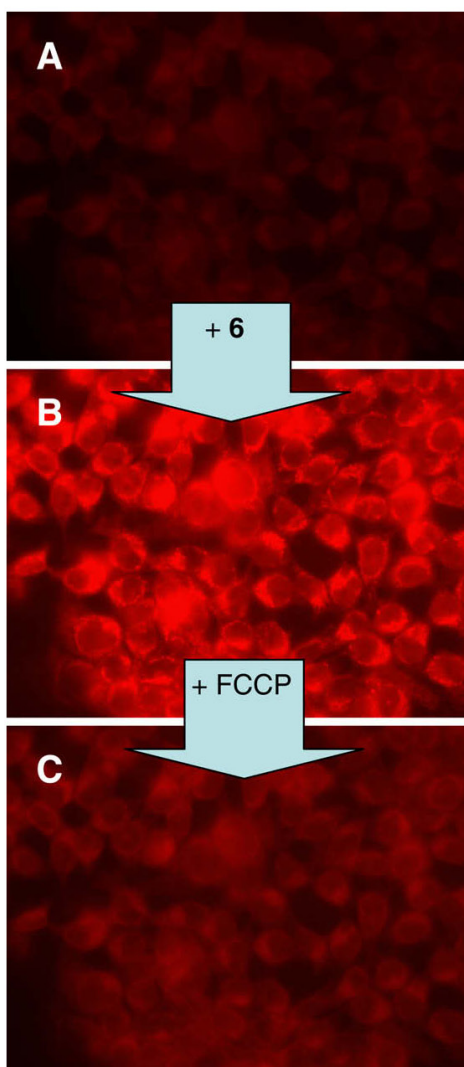

Figure 3. Fluorescence microscope images of cultured HCT116 cells: (A) just before the addition of 10 μM **6**, (B) 25 min after the addition of **6** and just before the addition of 2 μM FCCP, and (C) 10 min after the addition of FCCP.
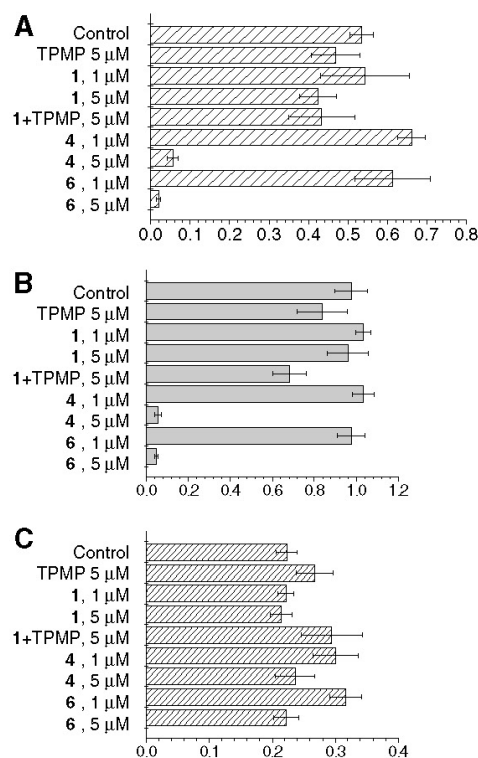

Figure 4. Effect of the mitochondriotropic resveratrol derivatives and control compounds on cell proliferation. Cells were allowed to grow for 3 days in the presence of the specified compounds and assayed using the tetrazolium salt reduction assay (see Supplementary data for details). All measurements were performed in quadruplicate. Averages ± s.d. are given. (A) C-26 mouse colon tumor cells. (B) Fast-growing Mouse Embryonic Fibroblasts (MEF). (C) Slow-growing MEF (note different scale).

the murine colon cancer cell line C-26 and, as controls, fast- and slow-growing non-tumoral mouse embryonic fibroblast (MEF) lines. Cell growth and viability was quantified using the tetrazolium salt reduction (MTT) assay.[21]

The various compounds had little effect on cell proliferation at the 1 μM level. At 5 μM, **4** and **6** displayed a marked cytotoxic effect on the two rapid-growth cell types, that is, C-26 (Fig. 4A) and non-tumoral 'fast' MEFs (Fig. 4B), but not on the slow-growth MEFs (Fig. 4C). Resveratrol, TPMP, and their combination had little effect also at 5 μM and with all three cell types, showing that the activity of the resveratrol-TPP conjugates is not just the sum of the activity of the two components. Selective cytotoxicity for fast-growing cells is characteristic of many chemotherapeutic drugs.

In conclusion we have produced mitochondriotropic resveratrol derivatives with good solubility and stability in aqueous media, which accumulate as expected in regions at negative potential. A class of natural compounds with useful properties can now be targeted to subcellular compartments where they ought to realize their biomedical potential in full. The results of initial cytotoxicity assessments encourage further experimentation in vivo to determine absorption, pharmacokinetics and possible anti-tumoral action.

Acknowledgments

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.08.100.

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20. Synthetic procedures and characterization.
    4′-(4-O-chlorobutyl) resveratrol (**2**). K_2CO_3 (1.33 g, 9.64 mmol, 1.1 equiv) and 1-bromo-4-chlorobutane (2.25 g, 13.14 mmol, 1.5 equiv) were added under argon to a solution of resveratrol (**1**) (2.00 g, 8.76 mmol) in DMF (10 mL). After stirring overnight, the mixture was diluted in EtOAc (100 mL) and washed with 1 N HCl (3 × 50 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2/EtOAc 85:15 as eluent to afford 0.930 g of **2** (33%). ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.76–1.98 (m, 4H, CH_2), 3.72 (t, 2H, CH_2), 4.02 (t, 2H, CH_2), 6.12 (t, 1H, H-4, J = 2.0 Hz), 6.40 (d, 2H, J = 2.0 Hz, H-2, H-6), 6.82–7.04 (m, 4H, =CH, H-2′, H-6′), 7.50 (d, 2H, H-3′, H-5′, J = 8.75 Hz), 9.21 (s, 2H, 3-OH, 5-OH); ^{13}C NMR (62.9 MHz, DMSO-d_6) δ (ppm): 26.14 (CH_2), 28.90 (CH_2), 45.19 (CH_2Cl), 66.72 (OCH_2), 104.38, 114.62, 126.62, 127.45, 127.76, 129.62, 139.07, 158.16, 158.49; ESI-MS (ion trap): *m/z*

319, [M+H]$^+$; HRMS (ESI-TOF): *m/z* 319.1092; Calcd for $C_{18}H_{19}ClO_3 \cdot H^+$ 319.1095. Anal.: Calcd for $C_{18}H_{19}ClO_3$ C 67.81, H 6.01; found: C 67.78, H 6.02.
4′-(4-O-iodobutyl) resveratrol (**3**). Compound **2** (500 mg, 1.57 mmol, 1 equiv) was added to a saturated solution of NaI in dry acetone (10 mL) and heated at reflux for 20 h. After cooling, the resulting mixture was diluted in EtOAc (100 mL), filtered and washed with water (3 × 30 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2/EtOAc 9:1 as eluent to afford 0.570 g of **3** (89%). ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.71–2.00 (m, 4H, CH_2), 3.35 (t, 2H, CH_2), 4.01 (t, 2H, CH_2), 6.12 (t, 1H, H-4, J = 2.0 Hz), 6.40 (d, 2H, H-2, H-6, J = 2.0 Hz), 6.82–7.04 (m, 4H, =CH, H-2′, H-6′), 7.50 (d, 2H, H-3′, H-5′, J = 8.75 Hz), 9.21 (s, 2H, 3-OH, 5-OH); ^{13}C NMR (62.9 MHz, DMSO-d_6) δ (ppm): 8.54 (CH_2I), 29.66 (CH_2), 29.76 (CH_2), 66.39 (OCH_2), 104.38, 114.62, 126.61, 127.45, 127.76, 129.62, 139.07, 158.16, 158.49; ESI-MS (ion trap): *m/z* 411, [M+H]$^+$. Anal.: Calcd for $C_{18}H_{19}IO_3$ C 52.70, H 4.66; found: C 52.75, H 4.66.
4′-(4-O-triphenylphosphoniumbutyl) resveratrol iodide (**4**). A mixture of **3** (500 mg, 1.22 mmol) and triphenylphosphine (1.60 g, 6.09 mmol, 5 equiv) in toluene (15 mL) was heated at 100 °C under argon. After 6 h, the solvent was eliminated at reduced pressure and the resulting white solid was dissolved in the minimum volume of acetone (3 mL) and precipitated with diethyl ether (100 mL). The solvents were decanted and the procedure repeated 4 more times. The precipitate was then filtered to afford 600 mg of **7** (73%). ^{1}H-NMR (250 MHz, DMSO-d_6) δ (ppm): 1.73 (quintet, 2H, CH_2), 1.92 (quintet, 2H, CH_2), 3.67 (t, 2H, CH_2), 4.06 (t, 2H, CH_2), 6.13 (t, 1H, H-4, J = 1.9 Hz), 6.40 (d, 2H, H-2, H-6, J = 1.75 Hz), 6.83–7.04 (m, 4H, =CH, H-2′, H-6′), 7.50 (d, 2H, H-3′, H-5′, J = 8.75 Hz), 7.71–7.95 (m, 15H, aromatic-H), 9.22 (s, 2H, 3-OH, 5-OH). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ (ppm): 18.48 (CH_2), 20.07 (CH_2), 29.15 (CH_2), 65.93 (OCH_2), 104.38, 114.66, 118.47 (Ph, J(^{13}C/^{31}P) = 85.6 Hz), 126.67, 127.41, 127.73, 129.72, 130.25 (Ph, J(^{13}C/^{31}P) = 12.4 Hz), 133.59 (Ph, J(^{13}C/^{31}P) = 10.1 Hz), 134.93 (Ph, J(^{13}C/^{31}P) = 2.9 Hz), 139.03, 158.00, 158.49; ESI-MS (ion trap): *m/z* 545, M$^+$; HRMS (ESI-TOF): *m/z* 545.2236; Calcd for $C_{36}H_{34}O_3P^+$ 545.2240.
3,5-diacetyl-4′-(4-O-iodobutyl) resveratrol (**5**). A solution of acetyl chloride (1.1 mL, 15 mmol, 20 equiv) in CH_2Cl_2 (20 mL) was added dropwise and under continuous stirring to a mixture of **3** (300 mg, 0.73 mmol, 1 equiv) and anhydrous pyridine (0.85 mL, 10.5 mmol, 15 equiv) in CH_2Cl_2 (20 mL) cooled in dry ice/acetone. The reaction mixture was then allowed to slowly warm up to room temperature. CH_2Cl_2 (50 mL) was added and the organic layer was washed with 1 N HCl (3 × 50 mL), dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2:n-hexane 4:1 as eluent to afford 280 mg of **5** (78%). ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.73–2.02 (m, 4H, CH_2), 2.29 (s, 6H, OAc), 3.38 (t, 2H, CH_2), 4.02 (t, 2H, CH_2), 6.86 (t, 1H, H-4, J = 1.9 Hz), 6.96 (d, 2H, H-2′, H-6′, J = 8.5 Hz), 7.08 (d, 1H, =CH, J = 16.5 Hz), 7.21–7.33 (m, 3H, H-2, H-6, =CH), 7.53 (d, 2H, H-3′, H-5′, J = 8.75 Hz); ^{13}C NMR (62.9 MHz, DMSO-d_6) δ (ppm): 8.49 (CH_2I), 20.83 (CH_3), 29.62 (CH_2), 29.75 (CH_2), 66.43 (OCH_2), 114.74, 116.79, 124.25, 128.08, 129.10, 130.09, 139.81, 151.15, 158.61, 168.99; ESI-MS (ion trap): *m/z* 495, [M+H]$^+$; HRMS (ESI-TOF): *m/z* 495.0664; Calcd for $C_{22}H_{23}O_5I \cdot H^+$ 495.0663.
3,5-diacetyl-4′-(4-O-triphenylphosphoniumbutyl) resveratrol iodide (**6**). A mixture of **5** (200 mg, 0.40 mmol) and triphenylphosphine (525 mg, 2.00 mmol, 5 equiv) in toluene (10 mL) was heated at 100 °C under argon. After 6 h, the solvent was eliminated under reduced pressure and the resulting white solid was dissolved in the minimum volume of acetone (3 mL) and precipitated with diethyl ether (100 mL) five times. The solvents were decanted after each precipitation. The precipitate was than filtered to afford 210 mg of **6** of 96–98% purity (69% yield). ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.73 (quintet, 2H, CH_2), 1.93 (quintet, 2H, CH_2), 2.29 (s, 6H, OAc), 3.67 (t, 2H, CH_2), 4.07 (t, 2H, CH_2), 6.86 (t, 1H, H-4, J = 2.2 Hz), 6.91 (d, 2H, H-2′, H-6′, J = 8.75 Hz), 7.08 (d, 1H, =CH, J = 16.25 Hz), 7.21–7.33 (m, 3H, H-2, H-6, =CH), 7.53 (d, 2H, H-3′, H-5′, J = 8.5 Hz), 7.72–7.96 (m, 15H, aromatic-H). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ (ppm): 19.25 (CH_2), 20.84 (CH_2), 29.25 (CH_2), 65.98 (OCH_2), 114.79, 116.81, 118.46 (Ph, J(^{13}C/^{31}P) = 85.9 Hz), 124.31, 128.06, 129.19, 130.05, 130.25 (Ph, J(^{13}C/^{31}P) = 12.4 Hz), 133.59 (Ph, J(^{13}C/^{31}P) = 10.1 Hz), 134.93 (Ph, J(^{13}C/^{31}P) = 2.9 Hz), 139.76, 151.16, 158.46, 169.01; ESI-MS (ion trap): *m/z* 629, M$^+$; HRMS (ESI-TOF): *m/z* 629.2453; Calcd for $C_{40}H_{38}O_5P^+$ 629.2451.
21. For materials, instrumentation, and experimental details please see Supporting Data.

Supporting data

for

Development of mitochondria-targeted derivatives of resveratrol

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Spiridione Garbisa, Mario Zoratti,* Cristina Paradisi

Materials and Methods

Materials and instrumentation. Chemicals 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 solvent signal (δ 2.49 ppm, DMSO-d_6). LC/MS analyses and mass spectra were performed with a 1100 Series Agilent Technologies system, equipped with MSD SL Trap mass spectrometer with ESI source. HPLC/UV analyses were performed with a Thermo Separation Products Inc. system with a P2000 Spectra System pump and a UV6000LP diode array detector (190-500 nm). Accurate mass measurements were obtained using a Mariner ESI-TOF mass spectrometer (PerSeptive Biosystems). UV-Vis spectra were recorded with a Perkin-Elmer Lambda 5 spectrophotometer. Fluorescence spectra were recorded with a Perkin–Elmer LS-55 spectrofluorimeter equipped with a Hamamatsu R928 photomultiplier. All absorption and fluorescence spectra were recorded at 25°C using thermostated quartz cells with an optical pathlength of 1 cm. Flash chromatographic separations were run on silica gel (Macherey-Nagel 60, 230-400 mesh) under air pressure. Elemental analyses were performed by the Microanalysis Laboratory of the Dept. of Chemical Sciences of the University of Padova.

Solubility of **4** *and* **6** *in water.* Calibration curves were built by plotting absorbance at 320 nm (a plateau in the UV absorption spectrum of both **4** or **6**) *vs* concentration for seven standard solutions

155

in the 10^{-6} - 10^{-4} M range, prepared from a 10^{-3} M mother solution in CH_3CN by dilution with water:CH_3CN/9:1. The concentration of aqueous saturated solutions of **4** and **6** was determined by interpolation (5 repetitions each).

Stability **4** *and* **6** *in water and in HBSS buffer.* At time zero, a 60 μL volume of a freshly prepared 10^{-3} M CH_3CN solution of either **4** or **6** was added to 3 mL of the medium of interest, i.e. water or HBSS:CH_3CN 9:1, at 25°C. The composition of HBSS (Hank's Balanced Saline Solution) was (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). Aliquots were withdrawn at desired times and analyzed by HPLC/UV (at 320 nm) and LC/MS on a reversed phase column (Gemini C18, 3 μm, 150 x 4.6 mm i.d.; Phenomenex). Solvents A and B were H_2O containing 0.1% HCOOH and CH_3CN, respectively. The gradient for B was as follows: 30% for 5 min, up to 60% in 15 min, up to 100% in 5 min; the flow rate was 0.7 mL·min^{-1}. For HPLC/UV analyses the 190 - 500 nm range was considered; in the LC/MS analyses mass spectra were acquired in positive ion mode operating in full-scan from 100 to 1500 m/z.

Cells. Human Colon Tumor (HCT116) cells (kindly provided by B. Vogelstein) as well as fast- and slow-growing SV-40 immortalized Mouse Embryo Fibroblast (MEF) cells (kindly provided by L. Scorrano and W.J. Craigen, respectively) and murine colon carcinoma C-26 cells were grown in Dulbecco's Modified Eagle Medium (DMEM), plus 10 mM HEPES buffer, 10% (v/v) fetal calf serum (Invitrogen), 100 U/mL penicillin G (Sigma), 0.1 mg/mL streptomycin (Sigma), 2 mM glutamine (GIBCO) and 1% nonessential amino acids (100X solution; GIBCO), in a humidified atmosphere of 5% CO_2 at 37 °C.

Metabolism studies. HCT116 cells were grown to ~90% of confluence in a 6-well plate, washed with warm HBSS, and incubated with 1 ml/well of 20 μM solution of **4**, **6** or resveratrol. Medium and cells were collected together after 6 hours of incubation. 100 μL of 0.6 M acetic acid and of fresh 10 mM ascorbic acid were added and the samples stored at −20°C until treatment and HPLC and LC/MS analysis. Treatment consisted in addition of acetone (1 mL), followed by sonication,

filtration through 0.45 μm Teflon® syringe filters (Chemtek Analytica) and concentration under N_2. The HPLC protocol was the same used for stability studies.

Mitochondria. Rat liver mitochondria were isolated by conventional differential centrifugation procedures from fasted male albino Wistar rats. The standard isolation medium was 250 mM sucrose, 5 mM HEPES (pH 7.4) and 1 mM EGTA. Protein content was measured by the biuret method with bovine serum albumin as standard.

TPP^+-selective electrode. The setup used to monitor the concentration of TPP^+-bearing compounds was built in-house following published procedures (Zoratti, M.; Favaron, M.; Pietrobon, D.; Petronilli, V. *Biochim. Biophys. Acta* **1984**, *767*, 231-239). A calomel electrode was used as reference. The suspension medium contained 200 mM sucrose, 10 mM Hepes, 5 mM succinate, 1 mM phosphate, 1.25 μM rotenone, pH 7.4 (with KOH).

Fluorescence microscopy. Cells were sown onto 24 mm round coverslips and allowed to grow for 48 hours. The coverslips were then washed with HBSS, mounted into supports, covered with 1 mL of HBSS and placed onto the microscope stage. The imaging apparatus consisted of an Olympus IX71 microscope equipped with an MT20 light source and Cell$^{R©}$ software. Excitation wavelength was 340 nm and fluorescence was collected in the 450-475 nm range. Sequential images were automatically recorded following a preordained protocol.

Cell growth/viability (MTT) assays. C-26 or MEF cells were seeded in standard 96-well plates and allowed to grow in DMEM (200 μL) for 24 hours to insure attachment. In the experiments of Figure 3 initial densities were 1000 (C-26, fast MEF) or 2500 (slow MEF) cells/well. The growth medium was then replaced with medium containing the desired compound from a mother solution in DMSO. DMSO final concentration was 0.1% in all cases (including controls). Four wells were used for each of the compounds to be tested. The solution was substituted by a fresh aliquot twice, at 24-hour intervals. At the end of the third 24-hour period of incubation with the drugs the medium was removed and substituted, after a wash with PBS, with 100 μL of CellTiter 96® solution (Promega; for details: www.promega.com/tbs). After a one-hour colour development period at 37°C absorbance at 490 nm was measured using a Packard Spectra Count 96-well plate reader.

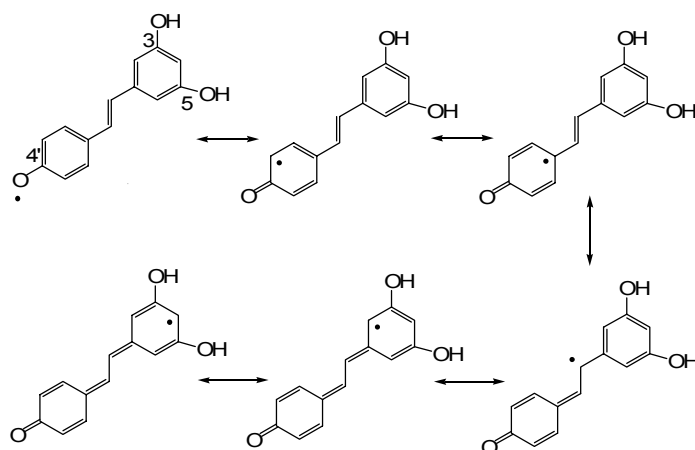# 10. Synthesis of two other mitochondriotropic quercetin and resveratrol isomers

Introduction

Working on the development of polyphenol derivatives homing in on mitochondria, we first synthesized 3-(4-O-triphenylphosphoniumbutyl)-quercetin (3-QBTPI), 4'-(4-O-triphenylphosphoniumbutyl)-resveratrol (4'-RBTPI) and their peracetylated analogs (3-QTABTPI and 4'-RDABTPI). These compounds proved to be stable, sufficiently water-soluble and capable of accumulating in mitochondria as expected (Chapt. 8, 9).

To exploit and potentiate the properties of the parent polyphenol through its accumulation in mitochondria, it is fundamental that the mitochondriotropic derivatives maintain the key characteristics of the parent compounds, so as to preserve the desired activity(ies). Redox characteristics and specific interactions with proteins are the key properties not to be significantly altered, or, if anything, to be improved in the derivatives. This consideration prompted us to avoid attachment of the mitochondria-targeting substituent to the catecholic group of quercetin, which largely determines the redox reactivity of the molecule. The 3-position of quercetin may also be relevant from this point of view, since it is involved in stabilizing the quercetil radical (Scheme 1); an analogous consideration can be made for the 4' position of resveratrol (Scheme 2).
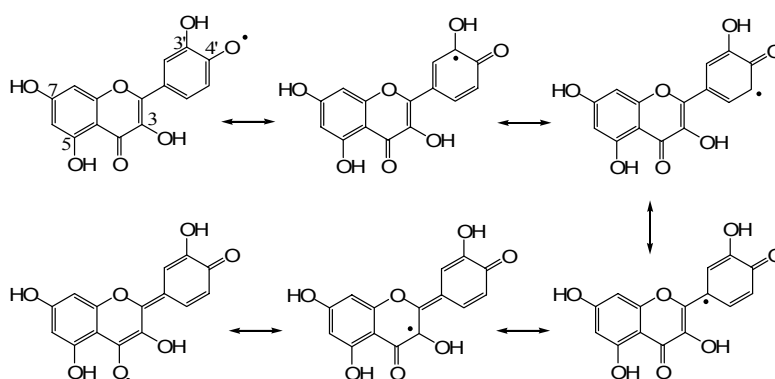
Scheme 1. Resonance structures of the quercetil radical.

Scheme 2. Resonance structures of the resveratrol radical

With the intent to better preserve the redox properties of the parent compound, it thus seemed opportune to synthesise also two other mitochondriotropic isomers, in which the attachment point of the linker was changed to position 7 for quercetin (7-QBTPI) and to position 3 for resveratrol (3-RBTPI). Their peracetylated analogs were also sinthesised (7-QTABTPI and 3-RDABTPI). All the derivatives are currently being assessed for their redox properties, as well as for their cytoprotective/cytotoxic action and their metabolism in cultured cells.

Materials and methods

Materials and instrumentation. Chemicals 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 solvent signal (δ 2.49 ppm, DMSO-d_6). Flash chromatographic separations were run on silica gel (Macherey-Nagel 60, 230-400 mesh) under air pressure.

Synthesis

3,3',4',5,7-Pentaacetylquercetin (QPA). QPA was synthesised as previously described (Chapt. 1).

3',4',5,7-tetraacetyl-7-(4-*O*-chlorobutyl) quercetin (7-QTABCl). K_2CO_3 (0.75 g, 5.4 mmol, 1.3 eq) and 1-bromo-4-chlorobutane (0.713 g, 4.16 mmol, 1 eq) were added under argon to a solution of QPA (2.13 g, 4.16 mmol) in DMF (10 mL). After stirring overnight, the mixture was diluted in EtOAc (100 mL) and then washed with 1M HCl (3x50 mL).

The organic layer was dried over MgSO₄ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2:Exane:EtOAc 8:1:1 as eluent to afford 7-QTABCl in 40% yield. ESI/MS (CH₃CN): m/z 561, [M+H]$^+$.

3',4',5,7-tetraacetyl-7-(4-*O*-iodobutyl) quercetin (7-QTABI). 7-QTABCl (100 mg, 0.18 mmol) was added to a saturated solution of NaI in anhydrous acetone (10 mL) and heated at reflux for 20 h. After cooling, the resulting mixture was diluted in EtOAc (50 mL), filtered and washed with water (3x30 mL). The organic layer was dried over MgSO₄ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2:Exane:EtOAc 8:1:1 as eluent to afford 7-QTABI in 86% yield. ESI/MS (CH₃CN): m/z 653, [M+H]$^+$.

3',4',5,7-tetraacetyl-7-(4-*O*-triphenylphosphoniumbutyl) quercetin iodide (7-QTABTPI). A mixture of 7-QTABI (100 mg, 0.15 mmol) and triphenylphosphine (200 mg, 0.76 mmol, 5 eq.) in toluene (10 mL) was heated at 95°C under argon. After 6 h, the solvent was eliminated under reduced pressure and the resulting white solid was dissolved in the minimum volume of dichloromethane (3 mL) and precipitated with diethyl ether (50 mL). The solvent was decanted and the precipitation was repeated for 5 times. Residual solvent was then removed under reduced pressure to afford 7-QTABTPI in 82% yield and 96-98% purity. The small amount of impurities consisted of a triacetyl derivative.

^{1}H-NMR (250 MHz, DMSO-d₆) δ (ppm): 1.62-1.83 (m, 2H, CH₂), 1.95 (t, 2H, CH₂), 2.23-2.45 (m, 12H, OAc), 3.58-3.75 (m, 2H, CH₂), 4.24 (t, 2H, CH₂), 6.80 (d, 1H, aromatic-H, J=2.25 Hz), 7.24 (d, 1H, J=2.5 Hz), 7.54 (d, 1H, J=9.25 Hz), 7.72-7.95 (m, 17H, aromatic-H). ESI/MS (CH₃CN): m/z 787, [M]$^+$.

7-(4-*O*-chlorobutyl) quercetin (7-QBCl). A solution of 7-QTABCl (1 g, 1,78 mmol) in 50 mL of MeOH was added to 20 mL of 5M HCl. The reaction mixture was then refluxed for 5 h under stirring. MeOH was then evaporated under reduced pressure and the residue in HCl solution was taken up in 3x50 mL of EtOAc. The organic layer was dried over MgSO₄ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CHCl₃:Acetone 7:3 as eluent to afford 7-QBCl in 91% yield. ESI/MS (CH₃CN): m/z 393, [M+H]$^+$.

7-(4-*O*-iodobutyl) quercetin (7-QBI). 7-QBCl (100 mg, 0.25 mmol) was added to a saturated solution of NaI in dry acetone (20 mL) and heated at reflux for 20 h. After cooling, the resulting mixture was diluted in EtOAc (30 mL), filtered and washed with water (3 × 30 mL). The organic layer was dried over MgSO₄ and filtered. The solvent was

then evaporated under reduced pressure and the residue was purified by flash chromatography using $CHCl_3$:Acetone 7:3 as eluent to afford 7-QBI in 86% yield. ESI/MS (CH_3CN): m/z 485, $[M+H]^+$.

7-(4-*O*-triphenylphosphoniumbutyl) quercetin iodide (7-QBTPI). A mixture of 7-QBI (100 mg, 0.21 mmol) and triphenylphosphine (275 mg, 1.05 mmol, 5 eq) in toluene (15 mL) was heated at 95°C under argon. After 6 h, the solvent was eliminated at reduced pressure and the resulting yellow solid was dissolved in the minimum volume of dichloromethane (3 mL) and precipitated with diethyl ether (5 × 50 mL). The solvents were decanted after each precipitation. Residual solvent was then removed under reduced pressure to afford 7-QBTPI in 73% yield. ^{1}H-NMR (250 MHz, DMSO-d_6) δ (ppm):1.65-1.85 (m, 2H, CH_2), 1.95 (t, 2H, CH_2), 3.63-3.75 (m, 2H, CH_2), 4.17 (t, 2H, CH_2), 6.28 (d, 1H, aromatic-H, J=2.0 Hz), 6.66 (d, 1H, aromatic-H, J=2.0 Hz), 6.89 (d, 1H, H-5', J=8.75 Hz), 7.55 (dd, 1H, H-6', J=8.25, 2.25 Hz), 7.69-7.95 (m, 16H, aromatic-H). ESI/MS (CH_3CN): m/z 619, $[M]^+$.
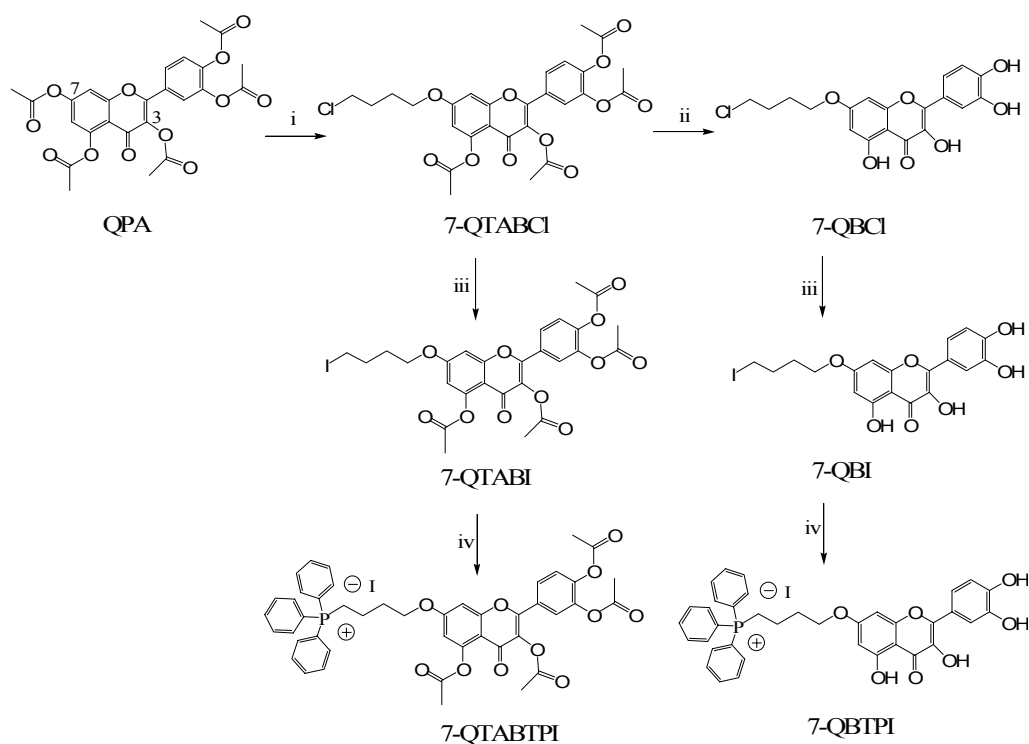
Scheme 2. Syntesis of 7-QBTPI and 7-QTABTPI. Reaction conditions were: i: K_2CO_3,1-bromo-4-chlorobutane, DMF, 14h; ii: MeOH, 5M HCl, 5 h; iii: NaI, anhydrous acetone, reflux, 20h; iv:.triphenylphosphine, toluene, 95°C.

3-RBTPI and 3-RDABTPI were obtained with the same synthetic procedures of 4'-RBTPI and 4'-RDABTPI (Chapt. 9). The alkylation reaction yields a mixture of 4'- and 3-substitution products. The minority 3-substituted 3-(4-O-chlorobutyl) resveratrol (3-RBCl) was isolated and served as starting material for the subsequent steps.

3-(4-O-chlorobutyl) resveratrol (3-RBCl). K_2CO_3 (1.33 g, 9.64 mmol, 1.1 eq.) and 1-bromo-4-chlorobutane (2.25 g, 13.14 mmol, 1.5 eq.) were added under argon to a solution of resveratrol (2.00 g, 8.76 mmol) in DMF (10 mL). After stirring overnight, the mixture was diluted in EtOAc (100 mL) and washed with 1M HCl (3x50 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2:EtOAc 85:15 as eluent to afford 0.930 g of the 3-isomer (33%). ^{1}H-NMR (250 MHz, DMSO-d_6) δ (ppm): 1.85 (m, 4H, CH_2), 3.71 (t, 2H, CH_2), 3.96 (t, 2H, CH_2), 6.20 (t, 1H, H-4), 6.50 (t, 1H, aromatic-H), 6.57 (t, 1H, aromatic-H), 6.75 (d, 2H, H-3', H-5', J=8.5 Hz), 6.94 (dd, AB system, H-7, H-8, J_{trans}=16.5 Hz), 7.39 (d, 2H, H-2', H-6', J=8.5 Hz). ESI/MS (CH_3CN): m/z 319, [M+H]$^+$.

3-(4-O-iodobutyl) resveratrol (3-RBI). 3-RBI (500 mg, 1.57 mmol) was added to a saturated solution of NaI in dry acetone (10 mL) and heated at reflux for 20 h. After cooling, the resulting mixture was diluted in EtOAc (100 mL), filtered and washed with water (3 × 30 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2:EtOAc 85:15 as eluent to afford 0.570 g of 3-RBI (88%). ESI/MS (CH_3CN): m/z 411, [M+H]$^+$.

3-(4-O-triphenylphosphoniumbutyl) resveratrol iodide (3-RBTPI): A mixture of 3-RBI (500 mg, 1.22 mmol) and triphenylphosphine (1.60 g, 6.09 mmol, 5 eq.) in toluene (15 mL) was heated at 100 °C under argon. After 6 h, the solvent was eliminated at reduced pressure and the resulting white solid was dissolved in the minimum volume of acetone (3 mL) and precipitated with diethyl ether (100 mL). The solvents were decanted and the procedure repeated 4 more times. The precipitate was then filtered to afford 600 mg of 3-RBTPI (90%). ^{1}H-NMR (300 MHz, DMSO-d_6) δ (ppm): 1.61-1.79 (m, 2H, CH_2), 1.89 (t, 2H, CH_2), 3.61-3.75 (m, 2H, CH_2), 4.00 (t, 2H, CH_2), 6.16 (t, 1H, H-4), 6.50 (m, 2H, H-2, H-6), 6.75 (d, 2H, H-3', H-5', J=9.9), 6.92 (dd, AB system, H-7, H-8, J_{trans}=19.8 Hz), 7.38 (d, 2H, H-2', H-6', J=10.6 Hz), 7.65-7.97 (m, 15H, PPh_3 aromatic-H). ESI/MS (CH_3CN): m/z 545, [M]$^+$.

4',5-diacetyl-3-(4-O-iodobutyl) resveratrol (3-RDABI). A solution of acetyl chloride (1.1 mL, 15 mmol, 20 eq) in CH_2Cl_2 (20 mL) was added dropwise and under continuous

stirring to a mixture of 3-RBI (300 mg, 0.73 mmol) and anhydrous pyridine (0.85 mL, 10.5 mmol, 15 eq) in CH_2Cl_2 (20 mL) cooled in dry ice/acetone. The reaction mixture was then allowed to slowly warm up to room temperature. CH_2Cl_2 (50 mL) was added and the organic layer was washed with 1M HCl (3x50 mL), dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2:n-hexane 4:1 as eluent to afford 280 mg of 3-RDABI (78%). ESI/MS (CH_3CN): m/z 495, $[M+H]^+$.

4',5-diacetyl-3-(4-*O*-triphenylphosphoniumbutyl) resveratrol iodide (3-RDABTPI). A mixture of 3-RDABI (200 mg, 0.40 mmol) and triphenylphosphine (525 mg, 2.00 mmol, 5 eq) in toluene (10 mL) was heated at 100°C under argon. After 6 h, the solvent was eliminated under reduced pressure and the resulting white solid was dissolved in the minimum volume of acetone (3 mL) and precipitated with diethyl ether (100 mL) five times. The solvents were decanted after each precipitation. The precipitate was than filtered to afford 210 mg of 3-RDABTPI of 96-98% purity (83.5% yield). ^{1}H-NMR (250 MHz, DMSO-d$_6$) δ (ppm): 1.65-1.87 (m, 2H, CH_2), 1.93 (t, 2H, CH_2), 2.28 (s, 6H, OAc), 3.59-3.75 (m, 2H, CH_2), 4.09 (t, 2H, CH_2), 6.60 (t, 1H, H-4), 6.97 (t, 1H, aromatic-H), 7.01 (t, 1H, aromatic-H), 7.13-7.35 (m, 4H, H-5', H-3', H-7, H-8), 7.63 (d, 2H, H-6', H-2', J=8.75 Hz), 7.70-7.95 (m, 15H, PPh$_3$ aromatic-H). ESI/MS (CH_3CN): m/z 629, $[M]^+$.
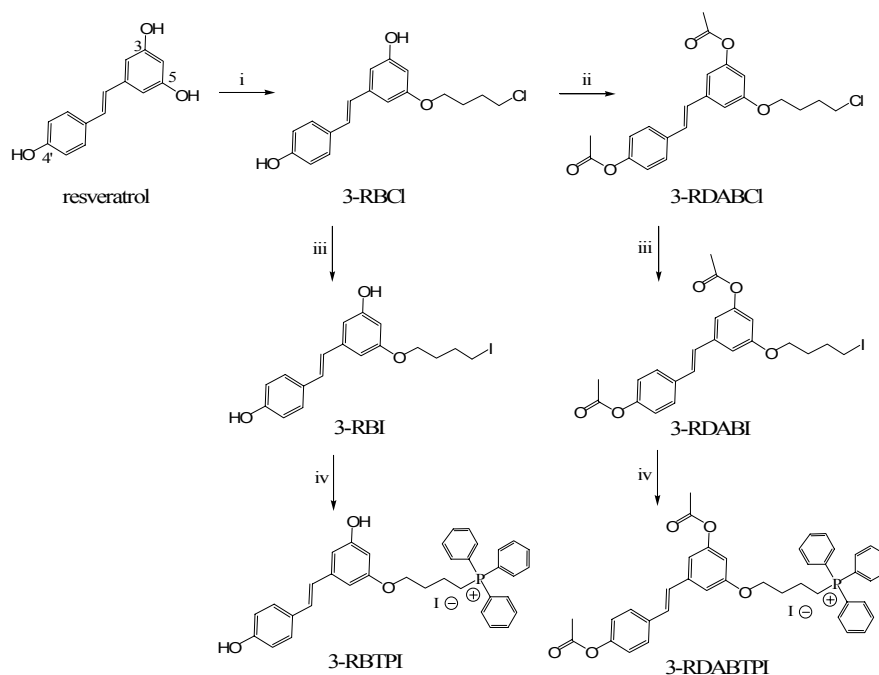
Scheme 3. Synthesis of 3-RBTPI and 3-RDABTPI. Reaction conditions were: i: K_2CO_3,1-bromo-4-chlorobutane, DMF, 14h; ii: acetyl chloride, CH_2Cl_2, anhydrous pyridine; iii: NaI, anhydrous acetone, reflux, 20h; iv:.triphenylphosphine, toluene, 100°C.

So far these new compounds have been utilized only in studies on the metabolism of mitochondriotropic compounds and on cytotoxicity. The Reader is referred to Chapters 11 and 13, respectively, for a summary of the observations.

Results

7-QBTPI was obtained through a different synthetic procedure than 3-QBTPI, exploiting the different reactivity of quercetin hydroxyls under different conditions. 3-OH is the most reactive position during O-alkyklation when only the catecholic hydroxyls are protected; anions deriving from deprotonation of C-5 and C-7 hydroxyls are stabilized through delocalization of the charge on the carbonyl oxygen, whereas the C-3 anion is not; the more localized charge confers a greater nucleophilicity to this position (Chapt. 8). However, when all hydroxyls are acetylated the reactivity changes (1). While free hydroxyls are alkylated in the order 3≈4'>7, acetylated ones react in the order 7>4'>3', giving the 7- derivative as the preferential/major product. The crucial step in the synthetic procedure is the initial alkylation. This presumably involves K_2CO_3 (a base)-catalysed hydrolysis of an acetyl group by trace water present in the solvent (DMF); the resulting anion proceeds to react rapidly with the alkylating agent. Thus selectivity of the overall reaction is determined by that of the initial hydrolysis, which takes place preferentially at position 7, as shown by *ad hoc* experiments performed by A. Mattarei.

3-RBTPI was obtained as the minor product with the same synthetic procedure of 4'-RBTPI; O-alkylation of resveratrol in fact afforded both mono-alkylated isomers, which were then separated by flash cromatography.

Perspectives

The ideal mitochondriotropic polyphenol ought to be redox-active; for this reason the redox potential of the derivatives and of their parent compounds are being determined through cyclic voltammetry and spectrophotometric radical quenching assays.

Since one of the hydroxyls has been changed to an ether linkage, the redox properties of the derivative might be significantly different from those of the parent polyphenol, and this may in turn alter the biological effects. If this is the case we shall turn to derivatives in which all hydroxyls are free; for example, for resveratrol the easiest approach might be that of producing the analogous derivative of piceatannol, i.e. 3,4,5,4'-tetrahydroxystilbene (resveratrol with an extra OH at position 4), a commercially available resveratrol metabolite. In this case alkylation is expected to occur preferentially at position 4; the

product would therefore be essentially resveratrol with an ether-linked TPP-carrying side chain in 4. Alternatively, a molecule containing an alkyl (C-C bond) substituent may be assembled from synthons following approaches already described (for example a Horner-Wadsworth-Emmons reaction (e.g. 2) or palladium-catalyzed decarbonylative coupling (e.g. 3)), essentially joining two intermediates containing only one opportunely substituted aromatic ring. All the isomers already available are being tested also for their possible cytoprotective or cytotoxic action on cell cultures (Chapt. 13), and data arising from these experimens will be correlated with the redox activity of the compounds.

The study of the metabolization profile of different mitochondriotropic isomers (initially on cell cultures) is also a useful tool to elucidate whether mitochondria provide a shelter from the action of conjugative/detoxifying enzymes, which they lack. Protection of both isomers against metabolization, in fact, will exclude the lack of conjugation because the position usually attached by conjugating enzymes is still functionalized (see Chapt. 11).

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11. Mitochondria as metabolic shelters: an idea being tested

Summary

Mitochondria-targeting groups offer a tool for specific pharmacological intervention on mitochondrial processes of pathophysiological relevance. They may find another potentially important application: directing the attached drug (in our proof-of-principle project quercetin and resveratrol) to a subcellular compartment in which Phase II conjugative enzymes are absent, except for a relatively weak methyltransferase activity. This chapter presents the ongoing work meant to verify this idea *in vitro*.

Introduction

As already mentioned elsewhere in this thesis, plant polyphenols offer much promise for biomedical applications. The bioactivities (and underlying mechanisms) of our two model compounds, quercetin and resveratrol, have been described in a number of recent reviews (quercetin: 1-3; resveratrol: 4-9).

The notoriously low bioavailability of these compounds is a major obstacle for their pharmacological exploitation. Since they are ready-made Phase II metabolism substrates, they are rapidly converted by sulfo- and glucuronosyl-transferases (SULTs and UGTs) in enterocytes into metabolites which are to a large extent re-exported to the intestinal lumen. Liver enzymes then intervene on the molecules which have entered the circulation, administering other rounds of Phase II metabolism (rev.: 10). SULTs (rev.: 11) are cytosolic, while UGTs (rev.: 12) are integral ER membrane proteins, with the catalytic site in the ER lumen. Methylation is used by cells for several purposes (e.g. epigenetic regulation) and methyltransferases are ubiquitous; catechol-O-methyl-transferases (COMTs) are particularly relevant in metabolizing polyphenols.

Many drugs are afflicted by bioavailability and metabolism problems just like polyphenols, 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". The development of prodrugs of polyphenols is indeed a major theme of our research project. A new strategy to oppose drug metabolization may derive from the consideration that the metabolic activities of SULTs and UGTs are not present in mitochondria. This consequently renders targeting of polyphenols to mitochondria not only a useful way to focus their activity, but also to hinder their metabolism. If mitochondrial targeting were

made reversible by linking the targeting group with a resistant, but not perennial, chemical bond, one may hope to develop a new type of slow-release, slow-metabolism drugs. This is indeed planned to be a major line of evolution for our project.

On the basis of these considerations, we have investigated the metabolism of the already sinthesized mitochondriotropic derivatives of quercetin and resveratrol (Chapt. 8, 9, 10). The metabolic conjugation pattern observed for the mitochondriotropic compounds was indeed strikingly different from that of the parent polyphenols. In principle this might have been due to an association of these compounds with membranes (out of reach of SULTs) rather than to accumulation in mitochondria. To check for this possibility, we have synthesised and begun to test the analogous n-pentyl (uncharged) derivatives. The length of the alkyl chain was chosen to approximately match that of the butyltriphenylphosphonium group in mitochondriotropic derivatives. This work is in progress, and we plan to produce and test compounds carrying other (shorter and longer) chains as well.

Metabolism experiments are being carried out imitating the route followed after oral administration: compound were incubated with an intestinal cell line (Caco2), an hepatic one (HepG2) and finally whole blood. The available data show protection of mitochondriotropic compounds against metabolism, which has instead an heavy impact on the parent compounds. The alkylated analogues lacking the ability to accumulate into mitochondria are susceptible to attack by membrane-associated methyl-transferases.

Experimental

Chemistry

Materials and instrumentation were as already described. Mitochondriotropic derivatives were synthesized as described (Chapt. 8, 9, 10); n-pentyl derivatives were obtained as follows.

3',4'-*O*-diphenylmethane quercetin (QDPM) was synthesised as previously described (Chapt. 8).

3',4'-*O*-diphenylmethan-3-(n-pentyl) quercetin: (QDPM-3P). K_2CO_3 (1 g, 7.24 mmol, 1.7 eq) and 1-bromo-n-pentane (0.75 g, 5.0 mmol, 1.2 eq) were added under argon to a solution of QDPM (1.94 g, 4.16 mmol) in DMF (20 mL). After stirring overnight, the mixture was diluted in EtOAc (100 mL) and washed with HCl 1 M (3x100 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated under

reduced pressure and the residue was purified by flash chromatography using CHCl$_3$:Acetone 8:2 as eluent to afford QDPM-3P in 45% yield.

ESI/MS (CH$_3$CN): m/z 537, [M+H]$^+$.

3-(n-pentyl) quercetin (3-QP). The cathecol ring protection was removed according to the procedure employed by Bouktaib et al. (13) for analogous quercetin derivatives. Briefly: QDPM-3P (1.0 g, 1.87 mmol) was dissolved into a mixture of acetic acid/water 8:2 (50 mL) and the solution was heated at reflux for 2 h. Then 100 mL of H$_2$O and 50 mL of EtOAc were added and the organic layer collected, washed with 100 mL of a NaHCO$_3$-saturated aqueous solution and dried over MgSO$_4$. After filtration the solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CHCl$_3$:acetone 8:2 as solvent to afford 3-QP in 83% yield. ^{1}H-NMR (250 MHz, DMSO-d$_6$) δ(ppm): 0.85 (t, 3H, CH$_3$), 1.19-1.26 (m, 4H, CH$_2$), 1.65 (q, 2H, CH$_2$), 3.92 (t, 2H, CH$_2$), 6.19 (d, 1H, H-6, J=2.00 Hz), 6.40 (d, 1H, H-8, J=1.75 Hz), 6.89 (d, 1H, H-5', J=8.50 Hz), 7.45 (dd, 1H, H-6', J=8.25, 2.0 Hz), 7.53 (d, 1H, H-2', J=2.0 Hz). ESI/MS (CH$_3$CN): m/z 373, [M+H]$^+$.
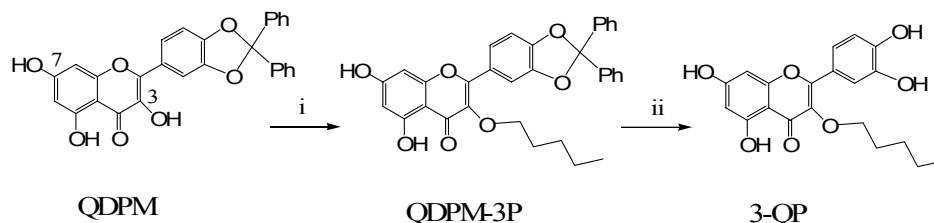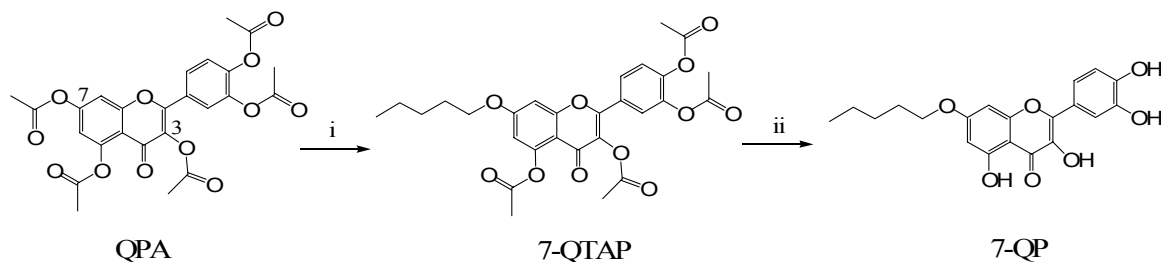

Scheme 1. Synthesis of 3-QP. Reaction conditions: i: K$_2$CO$_3$, 1-bromo-n-pentane, DMF, 16h; ii: acetic acid/water 8:2, reflux, 2 h.

3',4',5,7-tetraacetyl-7-(n-pentyl) quercetin (7-QTAP). K$_2$CO$_3$ (1 g, 7.24 mmol, 1.7 eq) and 1-bromo-4-n-pentane (0.55 g, 3.64 mmol, 1.42 eq) were added under argon to a solution of QPA (1.5 g, 2.93 mmol) in DMF (10 mL). After stirring overnight, the mixture was diluted in EtOAc and washed with 1 M HCl (3x50 mL). The organic layer was dried over MgSO$_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH$_2$Cl$_2$:petroleum ether:EtOAc 8:1:1 as eluent to afford QTA-7P in 35% yield. ESI/MS (CH$_3$CN): m/z 541, [M+H]$^+$.

7-(n-pentyl) quercetin (7-QP). 7-QTAP (1 g, 1.67 mmol) was dissolved 50 mL of MeOH; 5M HCl was then added (20 mL), and the solution was stirred 5 h at reflux. Methanol was then removed under reduced pressure, and the residual aqueous solution washed with

EtOAc (3 × 100 mL). The organic phase was evaporated under reduced pressure, and the residue was purified by flash chromatography using CHCl$_3$:acetone 7:3 as eluent to afford 7-QP in 78% yield. ^{1}H-NMR (250 MHz, DMSO-d$_6$) δ(ppm): 0.89 (t, 3H, CH$_3$), 1.25-1.45 (m, 4H, CH$_2$), 1.72 (q, 2H, CH$_2$), 4.06 (t, 2H, CH$_2$), 6.31 (d, 1H, H-6, J=2.00 Hz), 6.68 (d, 1H, H-8, J=1.75 Hz), 6.89 (d, 1H, H-5', J=8.50 Hz), 7.56 (dd, 1H, H-6', J=8.25, 2.0 Hz), 7.73 (d, 1H, H-2', J=2.0 Hz). ESI/MS (CH$_3$CN): m/z 373, [M+H]$^+$.

Scheme 2. Synthesis of 7-QP. Reaction conditions: i: K$_2$CO$_3$, 1-bromo-n-pentane, DMF, 16h; ii: MeOH, 5M HCl, reflux, 5h.

4'-(n-pentyl) resveratrol and 3-(n-pentyl) resveratrol (4'-RP, 3-RP). K$_2$CO$_3$ (2.85 g, 20.6 mmol, 2 eq) and 1-bromo-n-pentane (2.16 ml, 20.6 mmol, 2 eq) were added under argon to a solution of resveratrol (2.35 g, 10.3 mmol) in DMF (10 mL). After stirring overnight, the mixture was diluted in EtOAc (100 mL) and washed with 1 N HCl (3 × 50 mL). The organic layer was dried over MgSO$_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH$_2$Cl$_2$:EtOAc 90:10 as eluent to afford 0.65 and 0.22 g of 4'-RP (19.8%) and 3-RP (6.7%), respectively.

4'-RP. ^{1}H-NMR (250 MHz, DMSO-d$_6$) δ (ppm): 0.89 (t, 3H, CH$_3$), 1.25-1.45 (m, 4H, CH$_2$), 1.71 (q, 2H, CH$_2$), 3.96 (t, 2H, CH$_2$), 6.12 (t, 1H, H-4), 6. 39 (d, 2H, H-2, H-6, J= 2.0 Hz), 6.83-7.05 (m, 4H, H-5', H-3', H-7, H-8), 7.49 (d, 2H, H-6', H-2', J= 8.50 Hz). ESI/MS (CH$_3$CN): m/z 299, [M+H]$^+$.

3-RP. ^{1}H-NMR (250 MHz, DMSO-d$_6$) δ (ppm): 0.90 (t, 3H, CH$_3$), 1.25-1.45 (m, 4H, CH$_2$), 1.70 (q, 2H, CH$_2$), 3.92 (t, 2H, CH$_2$), 6.19 (t, 1H, H-4), 6.49 (t, 1H, aromatic-H), 6.56 (t, 1H, aromatic-H), 6.75 (d, 2H, H-3', H-5', J= 8.5 Hz), 6.95 (dd, AB system, H-7, H-8, J$_{trans}$=16.25 Hz), 7.40 (d, 2H, H-2', H-6', J= 8.50 Hz). ESI/MS (CH$_3$CN): m/z 299, [M+H]$^+$.
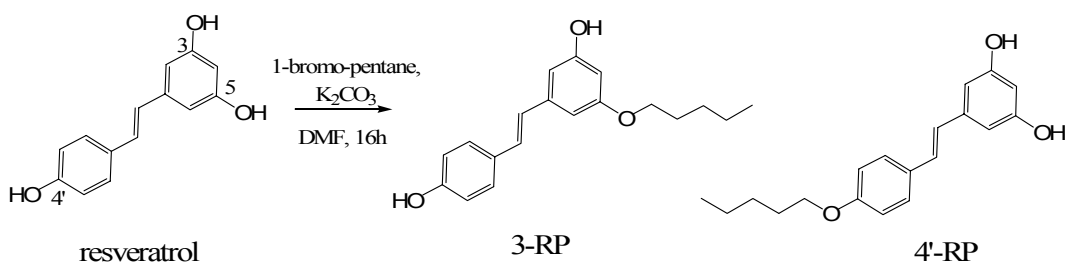
Scheme 3. Synthesis of 4'-RP and 3-RP

Metabolic assays

Materials and instrumentation. 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). Caco-2 and HepG2 cells were kindly provided by the groups of E. Papini and P. Bernardi (Dept. of Biomedical Sciences University of Padova) respectively.

Cells. Human epithelial colorectal adenocarcinoma (Caco-2) and hepatocarcinoma (HepG2) 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).

Metabolism studies with Caco-2 and HepG2 cells. 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 for Caco-2; 2 days for HepG2). They were then washed with warm HBSS, and incubated for 8 h with 1 mL/well of a 20 μM freshly prepared solution of the tested compound (dilution of a 1000× stock solution in DMSO; 0.1% final DMSO) in DMEM without phenol red (to avoid interference in HPLC analyses). Tested compounds were resveratrol, quercetin, 3-RBTPI, 4'-RBTPI, 3-RP, 4'-RP, 3-QBTPI, 7-QBTPI, 3-QP and 7-QP. 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 (Chemtek Analytica) and concentration

under N_2 or by using a rotational vacuum concentrator (Christ, RVC 2-25). HPLC analyses were finally performed as described below.

The incubation period was chosen to ensure conspicuous metabolism of both quercetin and resveratrol; the metabolic pattern of these "reference" compounds was then compared with that obtained with the derivatives.

To avoid heterogeneity in the expression of Phase II conjugating enzymes, metabolism experiments were performed after a 6 days period in which cells were grown in culture medium supplemented with 1 µM quercetin or resveratrol (depending on the derivatives to be tested) (see Chapter 2 - for details).

Metabolism in blood. Rats were killed and blood 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 5 mM stock solutions in DMSO, 0.1% final DMSO), incubated at 37°C for 1 hour and then treated as described in Chapters 3 and 14. Cleared blood samples were finally subjected to HPLC-UV analysis (see below).

HPLC-UV and LC-ESI/MS analyses. 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 (Gemini C18, 3 µm, 150 x 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 gradient for B was as follows: 30% (5 min), 60% in 15 min, 100% in 5 min (3 min); the flow rate was 0.7 mL/min. The eluate was preferentially monitored at 320 nm for resveratrol and its derivatives, and 370 nm for quercetin and its derivatives.

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 mass spectra.

Results and discussion

Synthesis of n-pentyl-derivatives. Resveratrol and quercetin were mono-alkylated with an n-pentyl moiety; the functionalized positions were the same as in the mitochondriotropic derivatives. Synthetic procedures were adapted from the protocols used to obtain the

chlorobutyl derivatives during the syntheses of 3-QBTPI, 7-QBTPI, 4'-RBTPI and 3-RBTPI.

Metabolism with Caco-2 cells. Both 3-QBPTI and 7-QBPTI were conjugated to a much lesser extent than quercetin itself, indicating this was not due to the transferase-preferred OH (position 3) being blocked. To check whether the pattern might be due to an association of these compounds with membranes, out of reach of SULTs, we studied the metabolism of the analogous 3- and 7-n-pentyl derivatives. The hydrophobic hydrocarbon chains in these compounds are expected to insert into membranes, anchoring the polyphenol "head". Since they are uncharged, they do not act as mitochondria-targeting tags. Their metabolic pattern was heavily biased towards methylation, and was quite different from that of the mitochondriotropic compounds. A straightforward control experiment, i.e. the assessment of the metabolism of mitochondriotropic compounds by cells with depolarized mitochondria, cannot be carried out because metabolism was severely depressed under these circumstances (not shown). Results from a representative experiment with quercetin derivatives and Caco-2 cells are shown in Fig. 1.
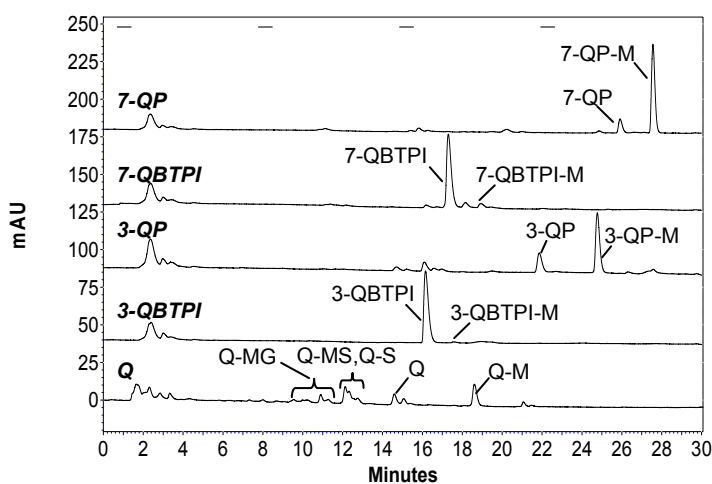
Fig. 1. HPLC chromatograms (370 nm) of the metabolite mix produced by Caco-2 cells exposed to the indicated compounds (20 μM, in DMEM without Phenol Red) for 8h hours. –M: methylated; -S: sulfate; -G: glucuronide; -MS: methyl-sulfate; -MG: methyl-glucuronide.

Experiments with the resveratrol derivatives yield fully coherent results. 3-RBTPI and 4'-RBTPI metabolism proved to be absent or strongly reduced with respect to resveratrol, which on the contrary was converted to the 3-monosulfate conjugate. The regioselectivity of this enzymatic conjugation explains the near-absence of any metabolites for 3-substituted resveratrol derivatives. 4'-n-pentyl resveratrol has a free 3-position, but underwent only a minor amount of sulfation; this compound is unable to accumulate into mitochondria, but it presumably associates with cell membranes, where SULTs are not present (Fig.2).
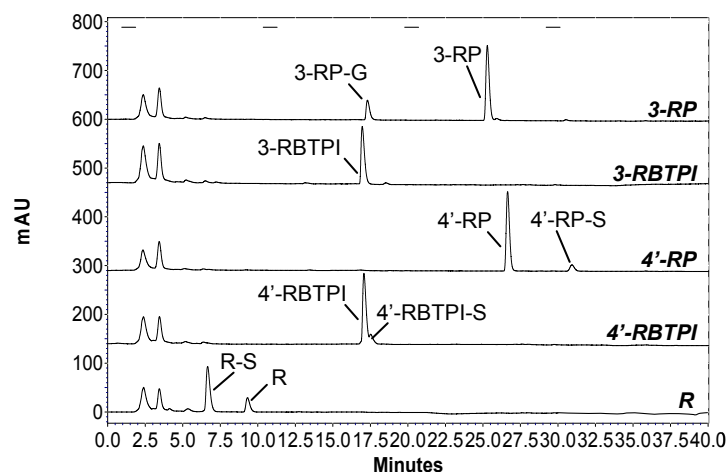
Fig. 2. HPLC chromatograms (320 nm) of the metabolite mix produced by Caco-2 cells exposed to the indicated compounds (20 μM, in DMEM without Phenol Red) for 8h hours. –M: methylated; -S: sulfate; -G: glucuronide.

Metabolism with HepG2 cells. At present, metabolism experiments with this cell line – intended to model hepatic metabolism - have been carried out only with quercetin and derivatives. In this case incubations with the various compounds were carried out for 16 hours, since after 8 hours conversion to metabolites was not extensive. Analyses showed a pattern analogous to that observed with Caco-2 cells (not shown).

Metabolism in blood. Incubation of the various compound in rat blood revealed only methyl transferase activity, while glucuronidation/sulfation were not observed. No information could therefore be obtained for resveratrol derivatives, since resveratrol itself does not undergo methylation. In agreement with data obtained with cell cultures, quercetin was conspicuously methylated over 1 hour, while 3-QBTPI and 7-QBTPI were significantly protected (not shown). Experiments with the n-pentyl and other derivatives are pending.

Conclusions and perspectives

Metabolism studies employing mitochondriotropic compounds, their membrane-associating analogs and their parent polyphenols on cultured cells and freshly drawn rat blood have provided data supporting an hypothesis we had made *a priori*: the rapid accumulation of these compounds into mitochondria seems to offer protection against at least glucuronidation and sulfation. While further verification is needed, the available results suggest that it might be possible to exploit this behaviour to decrease the impact of Phase II metabolism on drug disposition.

The data indicating that mitochondria can function as "safe havens" offering protection from conjugative Phase II metabolism in *in vitro* systems will be completed, confirmed and extended. To make sure that the observed variations in the metabolites produced can be attributed to sojourn in a "sanctuary" rather than being a consequence of the substitution pattern, we plan to determine and compare the metabolic patterns afforded by 3-methoxy-3',4',5,7-tetrahydroxy-flavone and 7-methoxy-3,5,3',4'-tetrahydroxy-flavone (rhamnetin, commercially available), i.e., quercetin derivatives bearing a methyl (rather than a 4-triphenylphosphonium-n-butyl or an n-pentyl) group at the 3 or 7 position. In the case of resveratrol, the corresponding compounds to be employed are trans-3,5-dihydroxy-4'-methoxystilbene (4'-O-methylresveratrol), trans-3,4'-dihydroxy-5-methoxystilbene (5-O-methylresveratrol). Analogous compounds with alkyl chains of more than 4 methylene units may also be produced. Metabolism of these compounds will be tested as we are doing with the parent polyphenols and the already available derivatives.

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12. Impact of mitochondriotropic quercetin derivatives on the performance of isolated rat liver mitochondria

Abstract

Polyphenols are more than just anti-oxidants, and the interaction of mitochondria-targeted derivatives with the organelles may be expected to be complex. To obtain a preview of what might happen *in vivo* we have studied the effects of 3-QBTPI and 3-QTABTPI on isolated rat liver mitochondria. As expected on the basis of the observations with quercetin itself, at high concentrations these compounds turned out to act as co-inducers of the mitochondrial permeability transition, and as inhibitors of the respiratory chain and of the mitochondrial ATP synthase. Furthermore, 3-QBTPI produced effects we interpret as due to its action as a protonophoric uncoupler. By definition, these compounds are designed to reach relatively high concentrations in mitochondria, so these observation may have significance also for *in vivo* circumstances. While these effects are to a large extent specific for quercetin, and the behaviour we observed can by no means be considered as a general feature of mitochondriotropic polyphenol derivatives, the observations emphasize the need for a careful assessment of the various facets of the interaction of these molecules with their subcellular targets.

Introduction

The development of mitochondriotropic polyphenol derivatives (Chapt. 8, 9, 10) implies the obvious question of what activities they may display once they reach the organelles, and of effects these compounds might have on the mitochondria themselves. Mitochondria-targeted "antioxidants" have been developed in the belief that they may antagonize undesirable ROS-mediated processes, but this may be just one side of the coin. At high concentrations, in experiments with suspensions of isolated mitochondria, quercetin induces the mitochondrial permeability transition in a process catalysed by Fe and Cu ions provided by the mitochondria themselves (Chapt. 7). Mitochondriotropic derivatives are designed to reach high concentrations in mitochondria. Will they act as pro-oxidant MPT inducers? Quercetin can also inhibit the mitochondrial respiratory chain (1) and (along with other polyphenols including resveratrol) the mitochondrial F_0F_1 ATPase, to which they bind at precisely defined sites in the F_1 portion (2-4). Will mitochondriotropic derivatives behave in the same way? The activity displayed by these molecules *in vivo* is

expected to depend on a number of parameters, the most important ones being the actual "concentration" reached in mitochondria (which may vary from one body district to the other) and the abundance of metal ions at the site. Will anti-oxidant effects or potentially mitochondria-damaging effects predominate? Before embarking in a more complex project to obtain this type of information from experiments with live animals it is certainly opportune to examine the interaction of these compounds with isolated mitochondria. This we are doing, using RLM and the biochemical approaches already adopted to study the action of quercetin (Chapt. 7).

Experimental

Swelling and respiration assays. Preparation of Rat Liver Mitochondria and swelling and respiration assays were carried out as described in De Marchi et al., submitted (Chapt. 7). The standard medium used in swelling and respiration assays contained 250 mM sucrose, 10 mM Hepes/K^+, 5 mM succinate/K^+, 1.25 μM rotenone, 1 mM P_i/K^+, pH 7.4, supplemented with the desired concentration of $CaCl_2$. RLM were used at a concentration of 1 or 0.5 mg.prot.mL^{-1}. Work has been to some extent hindered by a technical problem concerning the determination of respiration rates by isolated mitochondria: 3-QBTPI diffuses through the semi-permeable Teflon membrane covering the working surface of the Clark electrode (a cathode at which approximately -0.7 V are applied), and reacts there forming black polymeric products which rapidly gum up the surface making the electrode unserviceable. For this reason only a limited amount of respiration rate data could be obtained.

ATP hydrolysis assays were performed as described in Chapter 10.

Results and discussion

Experiments have so far been carried out with 3-QBTPI and 3-QTABTPI, the first isomers to be produced. A comparison with the 7-substituted isomers may well be worthwhile. The two compounds are dealt with separately since they turned out to have somewhat different effects.

3-QBTPI and mitochondria. Fig. 1 illustrates a representative swelling experiment monitoring the effects of increasing concentrations of 3-QBTPI in the presence of a fixed concentration of Ca^{2+}. By itself, Ca^{2+} (in the presence of 1 mM P_i in the medium) only induced a relatively slow pseudo absorbance decrease, i.e. swelling (trace 1). As expected, quercetin (20 μM) increases the rate of this phenomenon (trace 2). 3-QTABTPI produces a

stronger effect (trace 3), due to MPT onset, as further illustrated below. The presence of 3-QBTPI produces an unexpected result: swelling begins, but at a slower rate than with quercetin, and is interrupted after a period inversely related to the concentration of 3-QBTPI by an abrupt inversion with a recovery of pseudo-absorbance of the suspension (traces 4-6).
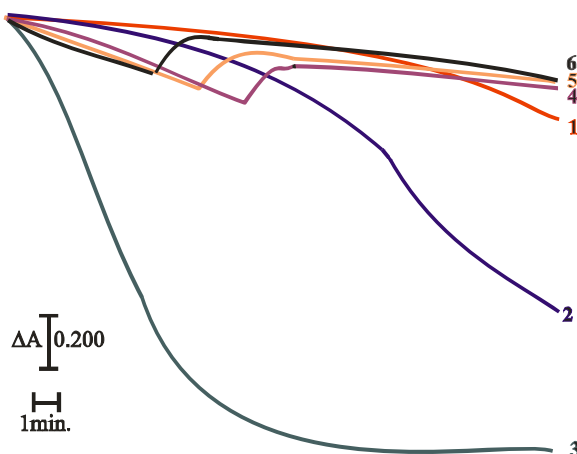

Fig. 1. 3-QBTPI induces only a slowly developing swelling of an RLM suspension under conditions favouring the onset of the MPT. Pseudo-absorbance was monitored at 540 nm. All cuvettes contained 40 μM $CaCl_2$, plus (from the start): trace 2: 20 μM quercetin; trace 3: 20 μM 3-QTABTPI; traces 4, 5, 6: respectively 10, 20, 40 μM 3-QBPTI. The experiment was started by the addition of RLM. pH was 7.4.

This abrupt and puzzling phenomenon is probably due to the exhaustion of oxygen in the spectrophotometer cuvettes, which lack magnetic stirring in the multiple-cuvette instrument used. If the contents of the cuvettes are stirred manually with a spatula at some point during the initial period, the inversion is delayed or it does not take place.

We deduced from this behaviour that mitochondria incubated in the presence of 3-QBTPI swell in a process at least partly distinct from that caused by the MPT. This process is presumably energy-dependent (at variance from the passive colloidosmotic swelling associated with the MPT), since it terminates and reverts when oxygen is exhausted. 3-QBTPI also causes oxygen to be consumed at an extremely high rate. A comparison with the behaviour induced by quercetin and by 3-QTABTPI indicates that these phenomena are associated with the simultaneous presence of the permeant cationic group (TPP^+), since quercetin does not cause them, and of free hydroxyls, since 3-QTABTPI induces a different behaviour.

Fig. 2 presents other observations. The phenomenon is to a large extent unrelated to the MPT: in the experiment of Fig. 2, 40 μM Ca^{2+} induce MPT and swelling (curve 1); 20 μM

3-QBTPI also induce swelling, but with different kinetics and amplitude of the absorbance signal. Most importantly, it is largely insensitive to Cyclosporin A (i.e., only a fraction of the swelling can be attributed to the MPT) (trace 3). FCCP, a mitochondrial uncoupler which dissipates the transmembrane potential, abolishes the effect of 3-QBTPI, which may therefore be linked to the uptake of this compound into mitochondria. Indeed, it seems likely that swelling is due to the electrophoretic import of 3-QBTPI, which is osmotically active, while the reversal upon oxygen exhaustion can be attributed to its release upon collapse of the transmembrane potential.
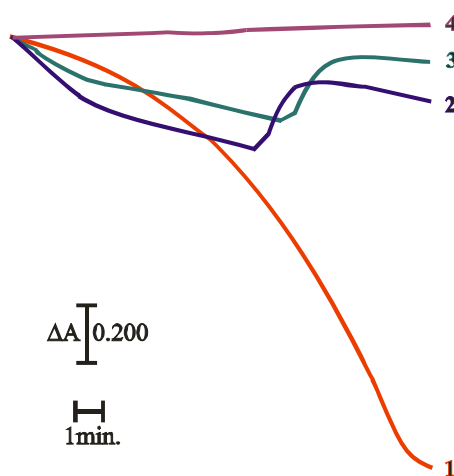
Fig. 2. The phenomena induced by 3-QBTPI are largely unrelated to the Mitochondrial Permeability Transition and involve an energy-dependent process. Pseudo-absorbance was monitored at 540 nm. All cuvettes contained 40 μM $CaCl_2$, plus (from the start): trace 2: 20 μM 3-QBTPI; trace 3: 20 μM 3-QTABTPI and 1 μM Cyclosporin A; trace 4: 20 μM 3-QTABTPI and 1 μM FCCP. pH was 7.4.

Since free OH groups are relevant, and since polyphenol hydroxyls are rather acidic (pKa_1 of quercetin is ~7 (5,6)), we investigated the effect of pH. Fig. 3 shows a representative experiment. In this case the chosen $[Ca^{2+}]$ is 20 μM, sufficient to induce the MPT under standard conditions (curve 1). 20 μM 3-QBTPI induce a pH-dependent effect. At pH 7.0, pseudo-absorbance decrease (curve 2) develops in a manner reminescent of that produced by 3-QTABPTI (Fig. 1, curve 3), and is substantially abolished by cyclosporin A (curve 6). It can therefore be largely attributed to onset and propagation of the MPT. The curve obtained in the presence of CSP shows a modest swelling and, after a comparatively long period, the characteristic inversion we attribute to oxygen exhaustion. As pH increases, the behaviour shifts to that shown in Fig.s 1 and 2. The "classic" behaviour is best followed precisely at pH 7.4. At pH 7.6 the rate of swelling is lower.
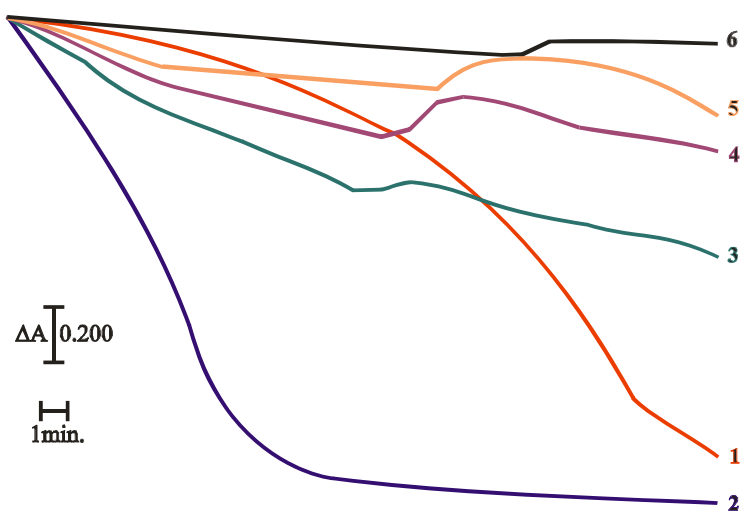
Fig. 3. pH variations in a narrow range around physiological values profoundly influence the phenomena induced by 3-QBTPI. $[Ca^{2+}]$ was 20 µM in all cuvettes. Cuvettes 2-6 also contained 20 µM 3-QBTPI (from the start). pH of the incubation medium was 7.4 for cuvettes n. 1 and 4, 7.0 for n. 2 and 6, 7.2 for n. 3, 7.6 for n. 5. CSP 1 µM was also present in cuvette n. 6.

We tentatively rationalize this complex behaviour in terms of two concomitant and alternative processes: MPT induction, unsurprising given the observations with quercetin (see Chapter 7) and uncoupling of the mitochondria by 3-QBTPI acting as a protonophore. Uncoupling is illustrated in Fig. 4.
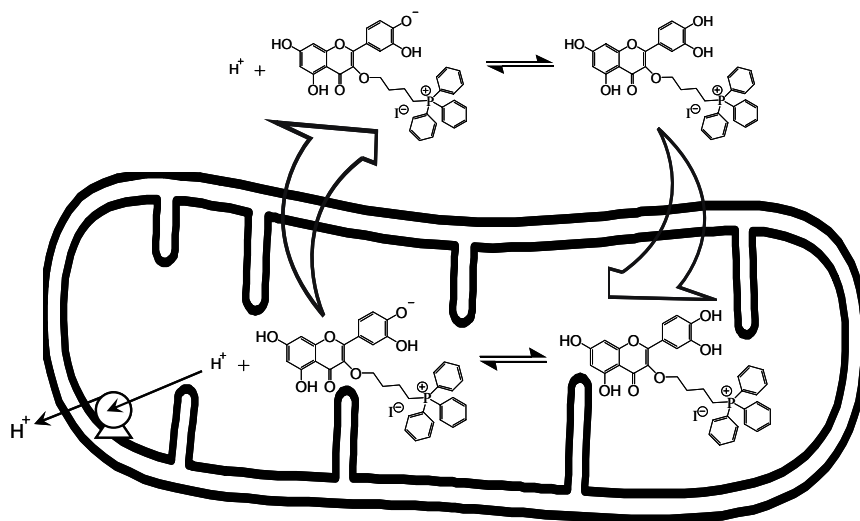
Fig. 4. An illustration of how 3-QBTPI may act as a protonophore and uncoupler. See text for details.

3-QBTPI exists in solution as a mixture of fully protonated (i.e., positively charged due to the TPP$^+$ group) and deprotonated species. Active import of the positive, permeant molecule explains energy-dependent swelling, whose rate, in fact, decreases as pH is

increased. Inside the mitochondrion, 3-QBTPI is obviously also engaged in a protonation equilibrium. If the zwitterionic specie generated by the first dissociation can permeate the mitochondrial membrane (which is plausible), the chemical gradient between the matrix and the suspension medium will drive its efflux. These are the elements of a classic protonophoric futile cycle: transmembrane potential-decreasing, energy-driven and -dissipating import followed by dissociation in the matrix with liberation of protons which are exported by the stimulated proton pumps (hence the increase in respiration) and by efflux of the uncharged specie which will engage in more rounds of proton transport. A textbook example of a protonophoric cycle, e.g. that formed by dinitrophenol, comprises the same elements, except that the species carrying the proton to the matrix is in that case neutral, while the permeant specie is negative and is expelled from the matrix in a potential-decreasing, energy-dissipating process.

This model explains the observed pH dependence if one assumes, plausibly, that pKa_1 of 3-QBTPI is close to 7.4. At higher pH values the zwitteironic neutral specie predominates, import is slowed down, and cycling is correspondly slower and uncoupling less severe. At lower pH values, the positive specie predominates, import is faster, efflux slower, a higher concentration is reached in the matrix, and this favours induction of the permeability transition. A corollary of this view is that the redox events presumably responsible for the opening of the MPT (Chapt. 7) are likely to take place on the matrix side of the IMM, since MPT induction is favoured at lower pH values which correspond to a greater accumulation.

3-QTABTPI and mitochondria. No doubt because hydroxyls are disguised as acetyls, the interaction of 3-QTABTPI with mitochondria is less complex, and it can be summarized by stating that this compound can induce the permeability transition in the experimental system we employed. This is illustrated in Fig. 5. Fig. 5A shows a comparison of the effectiveness of 3-QTABTPI and quercetin itself. The former is more effective, but the difference is not striking. This is understandable if one considers that on the one hand 3-QTABTPI is accumulated inside mitochondria, but on the other it is expected to be less effective as a reducing agent because its hydroxyls (hydroxyls are the groups being oxidised) are blocked and cannot be easily transformed into quinone carboxyls. Cyclic voltammetry experiments by A. Mattarei in our group show that 3-QTABTPI is oxidised at potentials close to those necessary to oxidise quercetin, while 3-QTABTPI requires much higher ones. Another confirmation of this difference in redox properties is that while 3-QBTPI reacts at the Clark electrode (see Experimental), 3-QTABTPI does not. It actually is surprising that it can act as an MPT inducer at all. This is probably explained however

by the observation that 3-QTABTPI undergoes hydrolysis of the acetyl groups upon incubation with the suspension of RLM. A first acetyl group is lost almost completely in 5 minutes, and at 15 minutes the major specie is the triacetylated molecule, with some diacetyl and a small amount of monacetyl derivatives (not shown). The "freed" hydroxyls are probably responsible for MPT induction. That the MPT is taking place is confirmed by the sensitivity of swelling to CSP. That 3-QTABTPI is anyhow a better inducer than quercetin confirms that MPT induction takes place inside the mitochondrion, since induction is potentiated by accumulation in the matrix (while the external concentration must decrease to some extent). This was not a foregone conclusion. The question now arises of whether protective chelation of Fe and Cu species by bathophenanthrolin and bathocuproin (Chapt. 7) takes place outside or inside mitochondria.

Note also that in the presence of this MPT inhibitor there still is some swelling when 3-QTABPTI is provided (trace 4 in Fig. 5A), while no swelling is observed with quercetin (trace 7). This is consistent with the notion that this relatively low-amplitude swelling is due to uptake of the cationic molecule itself. In Fig. 5B note how in the presence of CSP the addition of 3-QTABTPI results in a net inhibition of respiration (trace 2) (see below), while in the absence of the inhibitor respiration is progressively stimulated, coherently with induction of the MPT (trace 1).
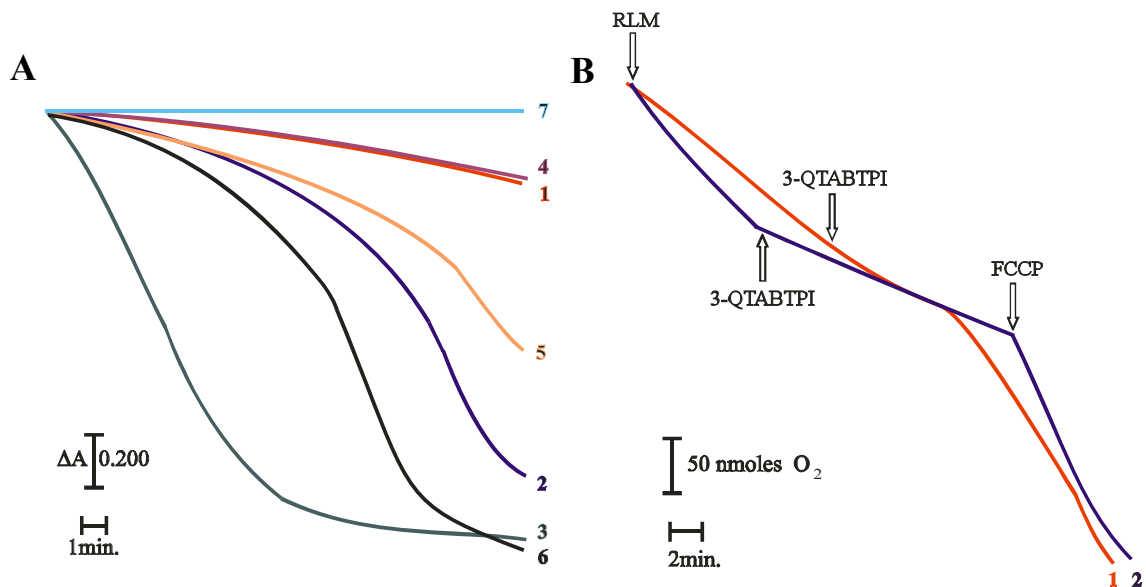

Fig. 5. Induction of the Mitochondrial Permeability Transition in suspensions of RLM by 3-QTABTPI. A) 3-QTABTPI is somewhat more effective than quercetin. Swelling experiments. All cuvettes contained 40 µM Ca^{2+} plus: trace 2: 5 µM 3-QTABTPI; trace 3: 20 µM 3-QTABTPI; trace 4: 20 µM 3-QTABTPI + 1 µM CSP; trace 5: 5 µM quercetin; trace 6: 20 µM quercetin; trace 7: 20 µM quercetin + 1 µM CSP. B) Respiration. 20 µM 3-QTABTPI or 1 µM FCCP were added when indicated. Trace 1: 1 µM CSP was present from the beginning.

3-QTABTPI inhibits mitochondrial respiration. Dorta et al. (1) observed an inhibition of respiration by isolated mitochondria by high concentrations of quercetin. We have therefore verified this aspect, of obvious relevance for possible applications. To avoid complications due to MPT induction, experiments have been performed without added Ca^{2+} and with quercetin and 3-QTABTPI, but not 3-QBTPI given its uncoupling effects (see above) and the technical problems already mentioned. Data collection has not yet been completed, but one can definitely conclude that 3-QTABTPI indeed exhibits a significant inhibitory effect, much heavier than that of quercetin, on mitochondrial respiration. These observations are illustrated by representative Clark electrode traces in Fig. 6.
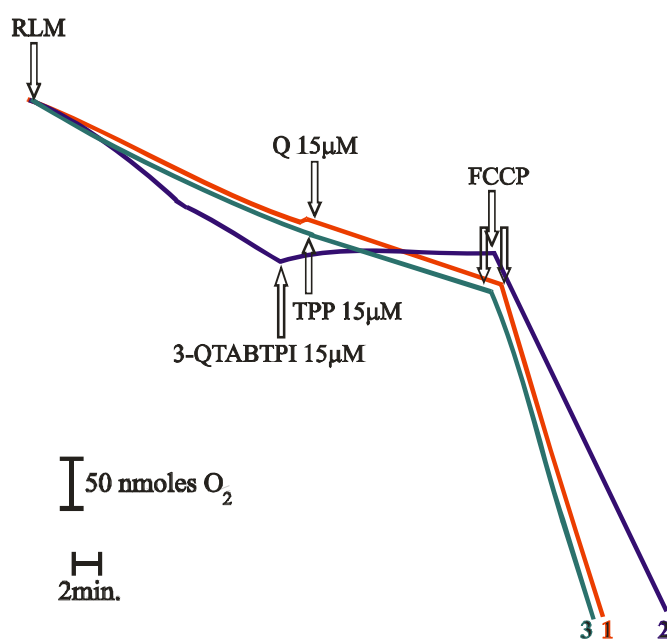

Fig. 6. 3-QTABTPI inhibits succinate-sustained respiration of isolated RLM. Additions were as indicated in the figure. FCCP was 1 µM. TPP: tetraphenylphosphonium chloride.

The addition of 15 µM 3-QTABTPI induces what appears to be a complete arrest of State 4 respiration. The apparent minor "production of oxygen" in the system (upward deflection of the trace upon addition) may be due to some interference with the electrode by this compound as well, but since the expected strong acceleration of oxygen consumption can be observed following the subsequent addition of FCCP, what is observed is unlikely to be mostly an artefact. Quercetin and tetraphenylphosphonium chloride (TPP, a control) had only a minimal effect at this concentration.

Inhibition is particularly evident when resting oxygen consumption is considered and is much milder, in comparison, on uncoupled respiration (Fig. 6 and not shown). This might

seem counterintuitive, since the respiratory chain functions at top speed in uncoupled mitochondria, but can be readily understood if one considers that uncoupling entails loss of the transmembrane potential and thus release of the inhibitor itself from the mitochondria. This reasoning implies that mitochondria can maintain a significant transmembrane potential despite a marked inhibition of the respiratory chain. A steady state must be reached at which inhibition of respiration, decrease in transmembrane potential, and concentration of the inhibitor in the matrix match. The data suggest, qualitatively, that this match is reached at low rates of respiration and relatively high values of transmembrane potential. The conclusion is in agreement with a number of bioenergetics studies carried out in the '80s by G.F. Azzone's group in Padova, in which correlations were established between rate of proton "pumping" by IMM respiratory chain and ATPase complexes and size of the transmembrane electrochemical proton gradient maintained (7-9).

3-QBTPI and 3-QTABTPI inhibit mitochondrial F_0F_1 ATPase. As reported in Chapter 8, 3-QBTPI and 3-QTABTPI also turned out to be inhibitors of the mitochondrial F_0F_1 ATPase. Fig. 7 here shows, as an example, a set of traces recorded in experiments of this type.
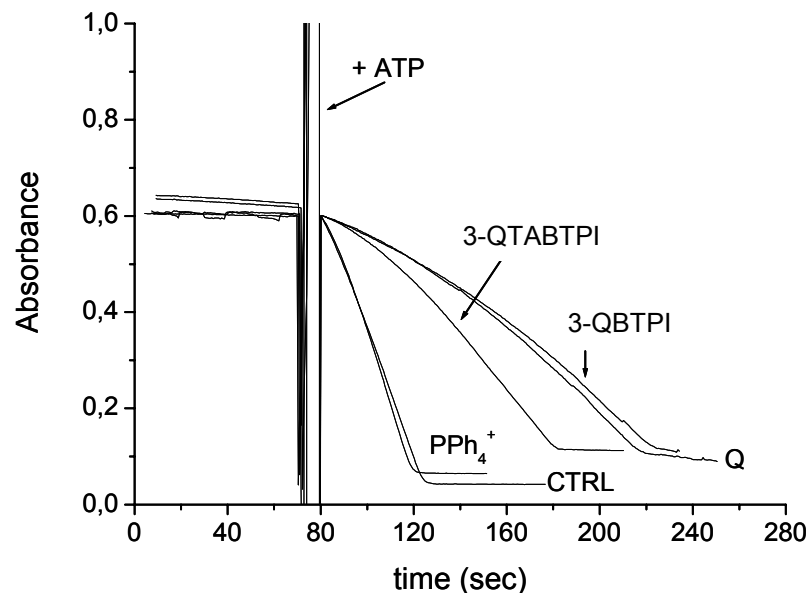

Fig. 7. Inhibition of RLM ATPase by 3-QBTPI and 3-QTABTPI. The readout in these experiments is NADH absorbance decrease (see Chapter 8). Representative recordings; the cuvette contains all reagents except ATP, and mitochondria. The reaction was started by the addition of 1 mM ATP (a 10-fold excess with respect to NADH). Absorbance was measured automatically every 0.6 sec. For illustration purposes, traces have been slightly shifted horizontally and/or vertically so that the first experimental point after ATP addition coincides for all traces. Rates of ATPhydrolysis have been mneasured as best fit of the linear (lower) portion of the curves.

Conclusions

In the experiments reported here and in Chapter 7 admittedly high concentrations of quercetin and of its mitochondriotropic derivatives were used in order to make any effects evident and easily observable. It is doubtful that concentrations in the order of 20 μM quercetin can ever be achieved *in vivo*. On the other hand, it might be possible to accumulate permeant and positively charged compounds up to μM levels in the cytoplasm of cells, which is at a negative potential (roughly -60 mV) with respect to the extracellular space. In any case, it seems reasonable to assume that there is no sharp threshold for the activities of these compounds: the effects observed using unrealistically high concentrations are likely to represent an exasperation of what may well happen, on a smaller scale, also at lower levels.

The results we have obtained pertain to quercetin derivatives. Other mitochondriotropic polyphenols are expected to behave differently, depending on their physico-chemical properties. Thus, the uncoupling effect described above for 3-QBTPI is accounted for by the acidity of quercetin: the fact that its first ionization occurs at physiological pHs is the key feature allowing it to act as a protonophore. The analogous resveratrol derivatives, for example, are expected to be much weaker uncouplers, since the pKa_1 of resveratrol is 8.1-8.3 (10,11). Furthermore, mild uncoupling of mitochondria is proposed to have potentially beneficial effects, such as mimicking life-extending caloric restriction (e.g.: 12,13) and protecting neurons against excitotoxicity (e.g.: 14; but see 15).

Mitochondriotropic polyphenols must in any case be "handled with care". It is not clear that they would behave *only* as beneficial antioxidants and ROS quenchers *in vivo*. On the contrary, they seem more likely to find application as cytotoxic chemotherapeutic agents than as a remedy for infarct damage. They may act as inducers of the MPT (or as inhibitors of it), as uncouplers, as pro-oxidant generators of ROS (this remains to be investigated), as inhibitors of the mitochondrial respiratory chain and of the mitochondrial ATPase. These effects may well contribute to their cytotoxic effects described in Chapter 13.

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13. Cytotoxic action of polyphenol derivatives

Abstract

Polyphenols are widely considered to be oncopreventive and/or potential chemotherapic agents. Investigating the possible role of our derivatives as anticancer agents seems therefore worthwhile. As a preliminary test, the already available mitochondriotropic derivatives, as well as their n-pentylated, non-acetylated analogs, were tested for their possible cytostatic/cytotoxic action on both tumoral and non-tumoral cell lines. Mitochondriotropic derivatives behaved as many chemotherapeutic agents, displaying cytotoxicity on tumoral or fast growing cells, but no significant effects on slow-growing, non-tumoral cells; the intensity of this action depended on the derivative, with 7-QBTPI being the most active one. Among n-pentil derivatives, 7-O-pentilquercetin (7-QP) showed to be a powerful cytotoxic agent for rapidly dividing cells; its action was the strongest among the tested derivatives, and opens new perspectives in developing membrane-anchored, polyphenol-based anticancer agents.
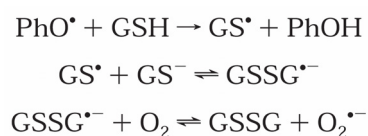
Introduction

Polyphenols, along with other easily oxidisable compounds such as ascorbic acid or vitamin E, are generally seen as anti-oxidants, useful to keep noxious radical species at bay, and as such they are marketed in dietary supplements. The scientific community is however aware that, depending on circumstances, they may act instead as pro-oxidants (e.g.: 1-6) (see also the introduction of Chapter 7). The enhancement – rather than quenching – of radicalic / oxidative processes is promoted by conditions such as an alkaline pH (because oxidation of ionised hydroxyls, especially catechols, is favoured) and the presence of transition metals forming ions of different charge but similar free energy levels, in particular $Fe^{2+/3+}$ and $Cu^{+/2+}$. Indeed, some of the data presented in De Marchi et al. (chpt. X) illustrate precisely this type of effect. The reactions involved may be schematized as shown in Scheme 1 (from ref. 3):
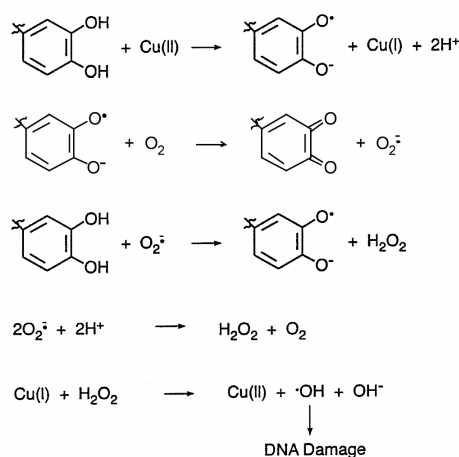
Scheme 1. Copper-mediated oxidation of a catecholic group

Production of ROS may be sufficiently sustained to result in a "redox catastrophe", leading to oxidation of the GSH pool and to oxidative damage of DNA, proteins and membrane lipids, and eventually to cell death. This can happen if the concentration of oxidizable species is sufficiently high, or if catalytic processes take hold. For example Galati et al. (1) have proposed the following scheme to explain the massive oxidation of GSH observed in the presence of apigenin or naringenin and of horseradish peroxidase:

$$PhO^{\bullet} + GSH \rightarrow GS^{\bullet} + PhOH$$
$$GS^{\bullet} + GS^{-} \rightleftharpoons GSSG^{\bullet -}$$
$$GSSG^{\bullet -} + O_2 \rightleftharpoons GSSG + O_2^{\bullet -}$$

Scheme 2. The core of a catalytic cycle leading to GSH depletion

The catalytic cycle is completed by SOD-catalyzed dismutation of superoxide to produce H_2O_2 and by the reduction of H_2O_2 to water, with the simultaneous oxidation of the phenol by peroxidase to produce again the phenoxy radical. The net result is reduction of oxygen to water with the oxidation of GSH to GSSG, without the intervention of free metal ions and with a polyphenol in the role of catalyst.

Thus, redox effects may be at least partly responsible for the cytotoxic effects of polyphenols on cultured cells, observed in a number of studies (e.g.: 7-9; rev.: 10). Indeed, Moridani et al. (11) have found a significant correlation between cytotoxicity and the $E_{p/2}$ parameter (a measure of the oxidation potential) of polyphenols. Mitochondria, as the major producers of ROS in the cell, may be considered as the most relevant site for either an anti-oxidant or a pro-oxidant effect, and this consideration prompted our work on the development of mitochondria-targeted polyhenol derivatives. Anti-tumoral,

mitochondrion-targeted, pro-oxidant-based chemotherapeutic approaches have been proposed (e.g.: 12-14). Conversely, the pro-oxidant, pro-death action of polyphenols has been reported to involve mitochondria (e.g.: 15-18).

Pro-oxidant effects may occur via other mechanisms. Some polyphenols, including flavonols, inhibit peroxidases (e.g.: 19-22), and may thus alter the "redox poise" of cells by acting on enzymes like the mitochondrial thioredoxin-peroxiredoxin system (reviewed in: 23-25) and mitochondrial phospholipid hydroperoxide glutathione peroxidase (reviewed in 26).

Cytotoxicity can have other, perhaps more relevant, origins as well. As already mentioned elsewhere, polyphenols interact, directly or indirectly, with a number of pivotal cell proteins. Cytotoxicity has been attributed for example to interference with topoisomerase II (27) or accumulation of tumor suppressor p53 (28). As we have confirmed (Chapt. 12) high concentrations of flavonoids can inhibit the mitochondrial respiratory chain (29) and F_0F_1 ATPase (30). The most pronounced effects may however be expected to be caused by polyphenol inhibition of kinases. Many polyphenols, in particular flavonoids and isoflavonoids like genistein, are well-known, powerful and not very specific (given the similarities among kinases) inhibitors of these all-important enzymes, to which they bind blocking the ATP binding site (see Fig. 1) (e.g.: 31,32).
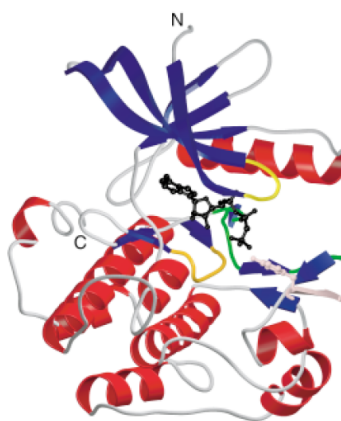

Fig. 1. The tyrosine kinase domain of the insulin receptor (from Hubbard and Till, 2000 (33))

Focusing on quercetin, which is relevant for this work even though it's not the best-known kinase inhibitor among polyphenols (genistein probably is): the kinases known to be inhibited by this flavonol include membrane-associated PI(3)K (IC_{50}: 180 nM) (34,35), Akt/PKB (36,37), Src kinases (38), Raf1 (39), FAK (40), receptor tyrosine kinases such as EGFR and Erb2 (41), as well as (e.g.) CK2 (31), MEK1 (39), cyclin-dependent kinases

(42,43) and so forth. Although the affinity of these interactions is not always very high, and in several cases the effect is actually indirect (cf., e.g., 44,45), the possibility clearly exists that kinase inhibition may account for much of the cytotoxicity exhibited by flavonoids *in vitro*. The importance of the –OH at position 3 for this inhibition has been recognized (31,32). Inhibition of pro-life kinases and of kinases involved in cell cycle progression (several flavonoids induce cell-cycle block; at what point depends on details of the hydroxylation pattern, which determine the mechanism of the process (44)) immediately suggest anti-cancer applications. Indeed, the development of selective kinase inhibitors is a frontier of oncology (e.g.: 46-59). Quoting Kandaswami et al. (32): "Certain dietary flavonols and flavones targeting cell surface signal transduction enzymes, such as protein tyrosine and focal adhesion kinases… appear to be promising candidates as anticancer agents."

Materials and methods

Materials and instrumentation. Quercetin was purchased from Sigma, resveratrol from Waseta Int. Trading Co. (Shangai, P.R.China). 3-QBTPI, 7-QBTPI, 4'-RBTPI, 3-RBTPI, 3-QP, 7-QP, 4'-RP and 3-RP were synthesised as decribed in Chapters 8, 9, 10, 11. All chemicals for buffer preparations were of laboratory grade, obtained from J. T. Baker, Merck, or Sigma.

Cells. Fast- (doubling time approx. 16 hours) and slow (doubling time approx. 3 days) - growing SV-40 immortalized Mouse Embryo Fibroblast (MEF) cells and mouse colon cancer C-26 cells (doubling time approx. 24 hours) were grown in Dulbecco's Modified Eagle Medium (DMEM) plus 10 mM HEPES buffer, 10% (v/v) fetal calf serum (Invitrogen), 100 U/mL penicillin G (Sigma), 0.1 mg/mL streptomycin (Sigma), 2 mM glutamine (GIBCO) and 1% nonessential amino acids (100× solution; GIBCO), in a humidified atmosphere of 5% CO_2 at 37 °C.

Cell growth/viability assays. C-26 or MEF cells were seeded in standard 96-well plates and allowed to grow in DMEM (200 μL) for 16-18 hours to ensure attachment. Initial densities were 1000 (C-26, fast-growing MEF) or 2500 (slow-growing MEF) cells/well in experiments with a 72 h incubation period, 3000 (C-26, fast-growing MEF) or 4000 (slow-growing MEF) cells/well in experiments involving both 24-hour and 72-hour growth periods. The different densities were calculated to ensure adequate cell growth (and consequently an adequate signal from the tetrazolium salt reduction (MTT) assay) in controls at the end of the experiment.

The growth medium was then replaced with medium containing the desired compound from a mother solution in DMSO. Three different concentrations of compounds have been used: 1, 3 and 5 μM; DMSO final concentration was 0.1% in all cases (including controls). Experiments were performed in quadruplicate, i.e., four wells were used for each of the compounds to be tested. Cells were incubated with the compounds for 24 or 72 hours; in this latter case, the solution was substituted by a fresh aliquot twice, at 24-hour intervals. At the end of the incubation period, the medium was removed and substituted, after a wash with PBS, with 100 μL of CellTiter 96[®] solution (Promega; for details: www.promega.com/tbs). After a one-hour colour development period at 37°C absorbance at 490 nm was measured using a Packard Spectra Count 96-well plate reader.

Results and discussion

Mitochondriotropic compounds (3-QBTPI, 3-QTABTPI, 7-QBTPI, 7-QTABTPI, 4'-RBTPI, 4'-RDABTPI, 3-RBTPI, 3-RDABTPI) were tested for their possible cytotoxic/cytostatic action on cultured cells. To study the role of both conjugation position and accumulation into mitochondria in determining the overall effect, we used also their n-pentylated, non-acetylated analogs (3-QP, 7-QP, 4'-RP, 3-RP). As controls, we checked also the action of parent polyphenols (quercetin and resveratrol), a phosphonium salt (TPMP) and of parent polyphenols plus these latter compounds. Cells chosen for this study were a cancer cell line (C-26), a resilient line which is a candidate for future studies in the mouse, and, as controls, fast- and slow-growing non-tumoural cells (MEF). The cytotoxic/cytostatic action of the compounds was evaluated by quantification of cell growth and viability through the tetrazolium salt reduction (MTT) assay.

Since many of the tested compounds may accumulate in mitochondria and/or interact with cell membranes, quantitatively their effect depends on the number of cells exposed to them (the greater the number of cells in the well, the weaker the final effect, because the same amount of compound must distribute among more cells, reaching a lower concentration in each of them). For this reason, the evaluation of the cytotoxic action of different compounds on the same cell line was based on the comparison of their effect within the same experiment, in which the number of viable cells at the beginning of the incubation period was approximately the same. For the same reason, illustrative experiments are shown, within which data can be safely compared. The results are representative of those obtained in (so far) 16 experiments comparing various compounds and cell lines within each experiment.

As illustrated in Fig.s 1, 2, 3 and 4, after 72 h of incubation all the mitochondriotropic derivatives, at 5 μM, displayed cytotoxicity on both C-26 and fast-growing MEF cells; no significant effects were observed using the 1 μM concentration. 7-QBTPI reproducibly proved somewhat more cytotoxic than the 3- isomer, while mitochondriotropic resveratrol derivatives were both hardly cytotoxic (Fig. 2). Among the pentyl derivatives, both 4'- and 3-RP did not diplay any cytotoxic action; 7-QP on the contrary was the only compound decreasing cell viability also at 1 μM, while 3-QP was markedly less effective.

No cytotoxic effects were observed for any derivative with slow-growing MEF (Fig. 1C and 2C). This mode of action is characteristic of many chemotherapeutic agents.
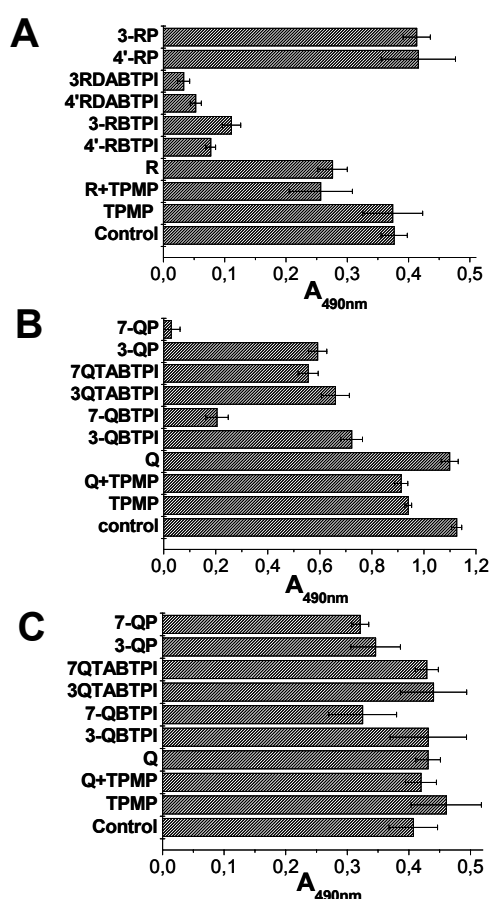

Fig. 1. Effect of quercetin derivatives and control compounds on the readout of tetrazolium reduction cell proliferation assays. Cells were allowed to grow for 3 days in the presence of 5 μM of the specified compounds. The panels show the results of individual representative experiments run in parallel with the three cell lines. All measurements were performed in quadruplicate. Averages ± s.d. are given. A) C-26 mouse colon tumour cells. B) Fast-growing Mouse Embryonic Fibroblasts (MEF). C) Slow-growing MEF. Abbreviations: TPMP: methyltriphenylphosphonium; BTPI: n-(4-triphenylphosphonium)butyl iodide; P: pentyl; Q: quercetin.
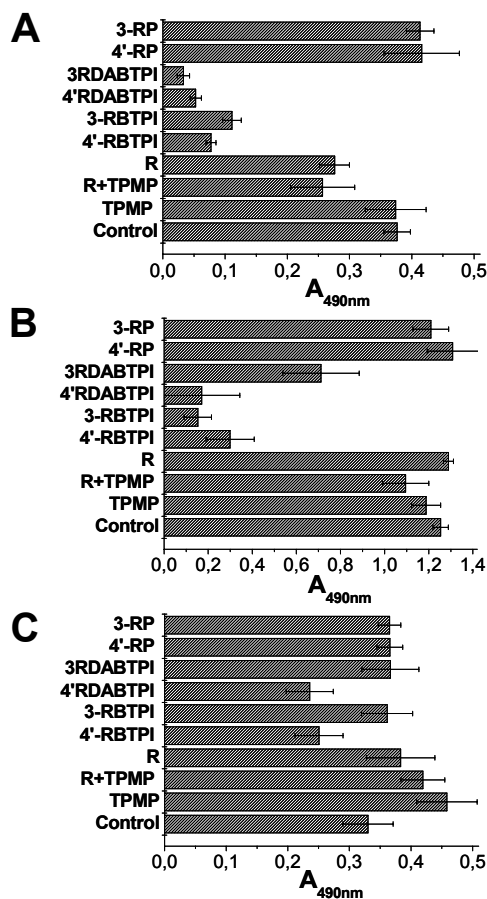
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Fig. 2. Effect of resveratrol derivatives and control compounds on the readout of tetrazolium reduction cell proliferation assays. Cells were allowed to grow for 3 days in the presence of 5 μM of the specified compounds. The panels show the results of individual representative experiments. All measurements were performed in quadruplicate. Averages ± s.d. are given. A) C-26 mouse colon tumour cells. B) Fast-growing MEF. C) Slow-growing MEF. Abbreviations: TPMP: methyltriphenylphosphonium; BTPI: n-(4-triphenylphosphonium)butyl iodide; P: pentyl; R: resveratrol.
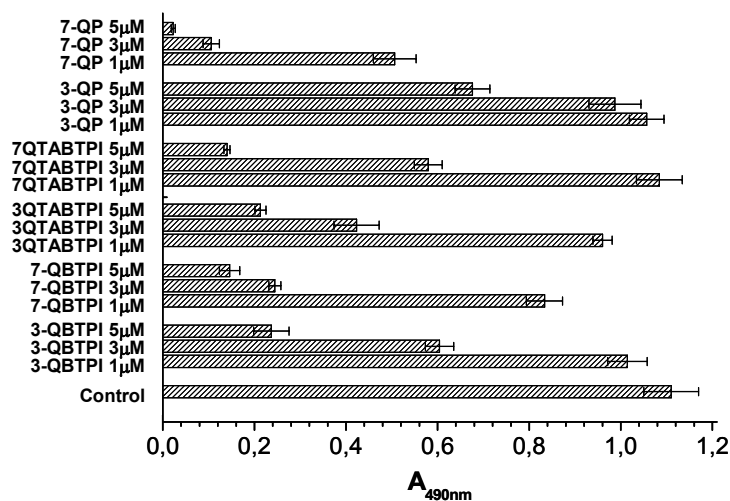

Fig. 3 Concentration dependence of the cytotoxic action of Q-derivatives on C-26 cells, 72 h incubation.
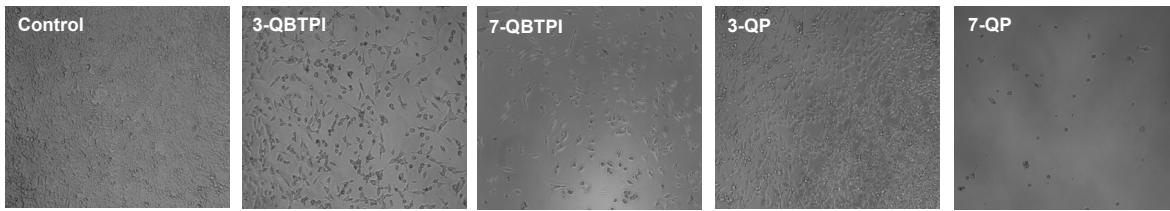
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Fig.4. C-26 cell culture images after 72 h incubation with 5 μM of the indicated compounds.

The drastic cytotoxic effects observed with 7-QP moved us to perform new experiments including a shorter incubation period (24 h). The same cell suspension was seeded in two 96 well plates, and after 16-18 h (for cell attachment) cells were exposed to compounds. The MTT assay was then performed 24 or 72 h after starting the incubation.

Mitochondriotropic compounds showed little effect on C-26 cells within 24 h, even when the higher concentration was used (5 μM); 7-QP was the only compound to greatly decrease cell viability also at these shorter incubation time (Fig. 5). As observed in 72-hour experiments, similar results were obtained for C-26 and rapidly growing MEF, while slow-growing MEF were not subject to any relevant cytotoxic effect.
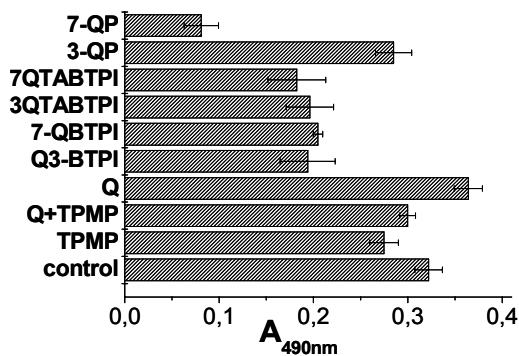

Fig.5. Effect of quercetin derivatives and control compounds on the readout of tetrazolium reduction cell proliferation/viability assays. C-26 cells were allowed to grow for 24 hours in the presence of the specified compounds. The panels show the results of an individual representative experiment. All measurements were performed in quadruplicate. Averages ± s.d. are given.

In another set of experiments, only 7-QP, 3-QP and quercetin (as control) were tested, and the MTT assay was performed 8, 16 and 24 h after starting the incubation. 7-QP (5 μM) was the only compound capable of blocking cell growth and decreasing cell viability (Fig. 6).
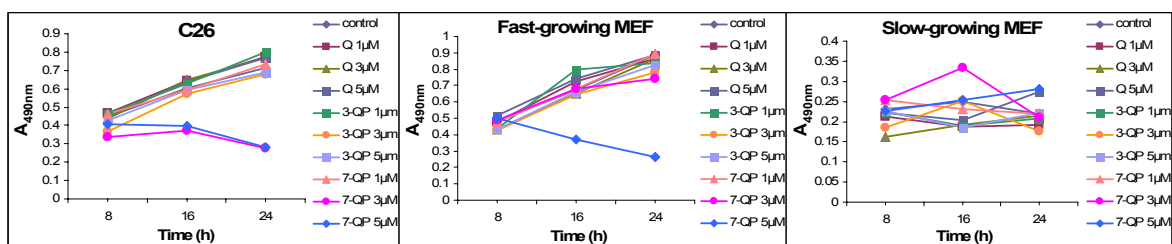
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Fig. 6. Effect of 7-QP, 3-QP and quercetin on the readout of tetrazolium reduction cell proliferation assays. Cells were allowed to grow for 8, 16 or 24 hours in the presence of the specified compounds. The panels show the results of an individual representative experiment. All measurements were performed in quadruplicate. Averages ± s.d. are given.

Conclusions

Mitochondriotropic derivatives were shown to exert a cytotoxic/cytostatic action on tumoral and fast-growing cells. The controls involving exposure to both quercetin or resveratrol and phosphonium salts showed that their cytotoxic action is greater than that exerted by the combination of their "building blocks" (see also Chapters 8 and 9). Accumulation in mitochondria thus plays a central role in determining their action. Please note, on this point, that 3-QP is reproducibly less toxic than 3-QBTPI. This is indicative of effects at the mitochondrial level, presumably induction of the MPT and/or uncoupling (Chapt. 12).

Pentylated analogs, which are conjugated at the same position but unable to accumulate in mitochondria, lack or have a decreased cytotoxic activity; 7-QP represents an obvious exception. Accumulation of a molecule in a specific cell compartment may result in loss or potentiation of its action, depending on the location of its target(s). The potential advantages of accumulating polyphenols in mitochondria to strengthen their action have been already discussed at some length, and this hypothesis has been supported by these initial studies on their cytotoxic action. Thanks to the alkyl chain, pentyl derivatives can easily interact with cell membranes; since flavonoids (but not resveratrol) are well-known kinase inhibitors, and the 3-OH has been recognized as crucial for this inhibition (see introduction), this can realistically explain the results obtained with quercetin and resveratrol pentyl-derivatives.

Paying due attention to the substitution position, anchoring flavonoids in membranes might be a successful strategy for effective inhibition of vital membrane-associated kinases. Other (iso)flavone derivatives, in particular genistein, are as good or better kinase inhibitors than quercetin, and might be used to advantage.

Interesting perspectives are opened by these initial observations. In the short term, to confirm that 7-alkylquercetin is more cytotoxic than 3-alkylquercetin because its 3-OH is free to participate in enzyme inhibition we shall synthesise 7-pentyl-luteolin (luteolin: 5,7,3',4'-teytraxydoxyflavone; commercially available) and compare its effectiveness as a cell death inducer to that of the other compounds. Optimization of the membrane-anchoring side chain, and extension to other kinase-inhibiting polyphenols (and other inhibitors) are also obvious developments. Increasing solubility without unduly affecting membrane association and achieving specific delivery to the desired site of action are, as very often in pharmacology, two major problems that will have to be faced if our observations are to find application.

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14. Determination of mitochondriotropic quercetin and resveratrol derivatives in whole blood

Abstract

The approach used to develop an analytical protocol and validate it in the case of quercetin and resveratrol (Chapt. 3) was applied also to the analysis of their mitochondriotropic derivatives.

Introduction

We have already synthesized resveratrol and quercetin derivatives bearing a lipophilic permeant triphenylphosphonium cation (TPP). The charged moiety leads to the accumulation of the compounds in regions held at negative potential (i.e. the cytoplasm and especially mitochondria), according to Nernst's law. (Chapts. 8, 9, 10). Two different isomers have been produced for both quercetin and resveratrol, to take into account the possibility that the conjugation position might influence redox and other properties of the derivative and for use in metabolism studies (Scheme 1).
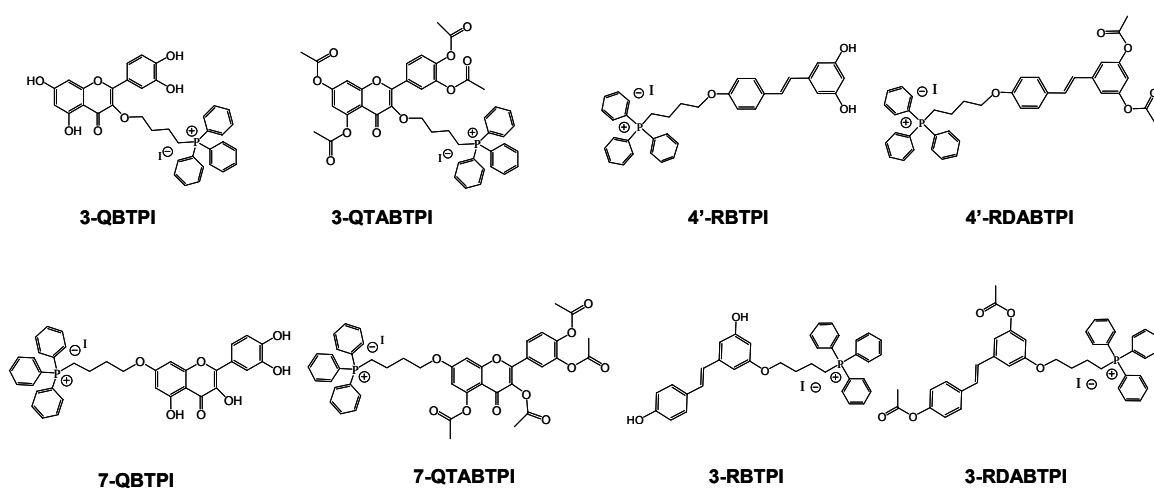
Scheme 1. Mitochondriotropic quercetin and resveratrol derivatives already synthesised.

These compounds accumulate into energized mitochondria as expected and exhibit a cytotoxic effect when tested on cultured cells (Chapts. 8, 9, 13), suggesting their potential use as chemotherapeutic agents.

Absorption and pharmacokinetics studies will be a logical step before testing their anticancer properties on an *in vivo* model. However, a validated protocol for extracting

mitochondriotropic derivatives from biological matrices, such as blood, is currently lacking. In previous work (Chapt. 3) we developed a method for resveratrol and quercetin extraction from blood. The importance of analyzing whole blood emerged comparing the recovery of the analytes from whole blood and from plasma. The incomplete recovery from the latter, with the missing material being associated with the cellular fraction, is explained by the known propensity of polyphenols to bind to cell membranes, platelets, haemoglobin and blood proteins.

In the work presented here we verified the suitability of the same method also for mitochondriotropic derivatives. Analysis of whole blood seemed to be preferable also in this case, since the derivatives have the same molecular kernel of their parent polyphenols (and thus may associate similarly to different blood components), and furthermore they accumulate in the cytoplasm and mitochondria (i.e. in the cellular fraction). We tested only the non-acetylated derivatives, because their peracetylated analogs are expected to undergo hydrolysis of ester bonds in biological systems, with the eventual generation of the non-acetylated species.

As for quercetin and resveratrol, a known amount of internal standard was added to the blood sample. We then determined the recovery of the internal standards and analytes, and the reproducibility of the recovery ratio of analyte to the standard. We furthermore checked whether recovery depended on the concentration of the compounds, confirming that the method can be reliably extended to mitochondriotropic derivatives.

Material and methods

Instrumentation. HPLC-UV analyses were performed by a Thermo Separation Products Inc. system equipped with a P2000 Spectra System pump, a UV6000LP diode array detector (190-500 nm) and a reverse phase column (Gemini C18, 3 μm, 150 x 4.6 mm i.d.; Phenomenex).

Chemicals. Materials were purchased from Sigma/Aldrich/Fluka/Riedel de-Haen, Merk-Novabiochem, and were used as received. 2',5,7-trihydroxyflavone was from Indofine (Hillsborough, NJ, USA).

Preparation of samples. Rats were sacrificed by decapitation (under anaesthesia) and blood was withdrawn from the jugular vein, heparinized and transferred to tubes containing EDTA. Blood samples (1 ml) were spiked with 4'-RBTPI, 3-RBTPI, 3-QBTPI or 7-QBTPI (dilution from 1000× stock solutions in DMSO, 0.1% final DMSO). 200 μl aliquots were taken and processed. Before starting the treatment, internal standard was

added (4,4' dihydroxybiphenyl and 2',5,7-trihydroxyflavone, respectively for resveratrol- and quercetin- derivatives; dilution from 50× stock solutions in Methanol, 25 μM final concentration). Samples were then treated as described for resveratrol and quercetin; cleared samples were analyzed by HPLC-UV.

HPLC-UV analysis. Samples (20 μl) were analyzed using a reversed-phase column (Gemini C18, 3 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: 30% (5 min), 60% in 15 min, 100% in 5 min (3 min). The flow rate was 0.7 mL/min. The eluate was preferentially monitored at 286 nm for 4,4'-dihydroxybiphenyl, 320 nm for resveratrol derivatives, and 370 nm for quercetin derivatives and 2',5,7-trihydroxyflavone.

Calibration curves. 0.2, 0.5, 1, 5 and 25 μM solutions in $H_2O:CH_3CN$ 7:3 of 4'-RBTPI, 3-RBTPI, 3-QBTPI or 7-QBTPI were analyzed by HPLC-UV as described above, without any treatment; peak area (at 320 and 370 nm, respectively for resveratrol- and quercetin-derivatives) was plotted against concentration to establish the correlation between peak area and amount analyzed.

Results and discussion

Recovery of each analyte was determined as described in Chapter 3. The treatment ensured a mean recovery of 92.3$\pm$1.8 %, 75.8$\pm$9.8%, 95.3$\pm$4.1 % and 94.0$\pm$5.4 %, for 4'-RBTPI, 3-RBTPI, 3-QBTPI and 7-QBTPI, respectively (means from 3, 3, 7 and 4 independent treatments, respectively).

Analytes and internal standards were recovered with a reproducible ratio: 1.08$\pm$0.05 for 4'-RBTPI: 4,4'dihydroxybiphenyl (N = 3); 1.02$\pm$0.12 for 3-RBTPI: 4,4'dihydroxybiphenyl (N = 3); 1.07$\pm$0.11 for 3-QBTPI : 2',5,7-trihydroxyflavone (N = 7); 0.81$\pm$0.10 for 7-QBTPI : 2',5,7-trihydroxyflavone (N = 4).

Whole blood was spiked with different concentrations of resveratrol- or quercetin-derivatives, and treated as described above. Plots of the peak area ratio of the derivatives to their standards vs. analyte concentration were linear, with a correlation coefficient of 0.9953, 0.9971, 0.9932 and 0.9970 for 4'-RBTPI, 3-RBTPI, 3-QBTPI and 7-QBTPI, respectively.
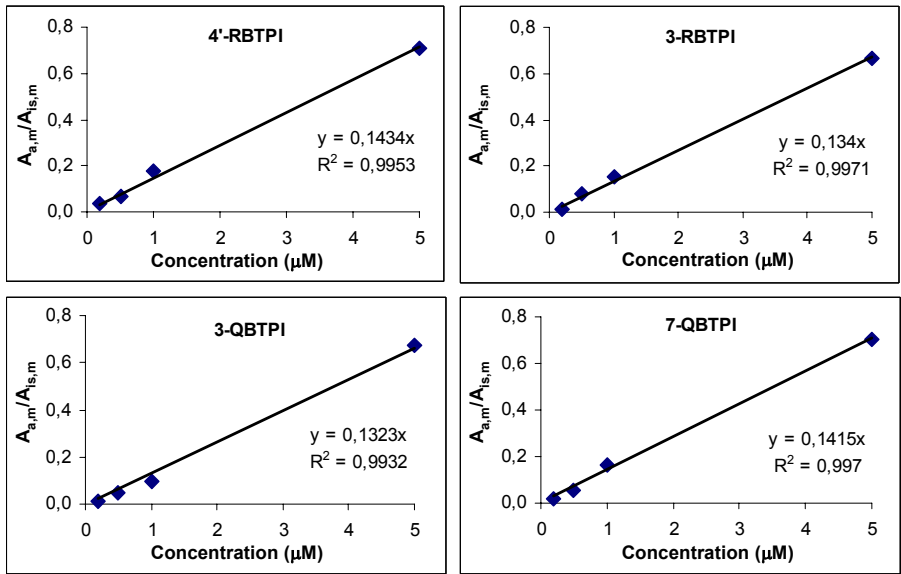
Fig. 1. Linear regression of the peak area ratio of 4'-RBTPI, 3-RBTPI (at 320 nm), 3-QBTPI and 7-QBTPI (at 370 nm) to that of their internal standards (at 286 and 370 nm, respectively), as a function of the concentration of the former. $A_{a,m}/A_{is,m}$: peak area ratio of the analyte to its internal standard.
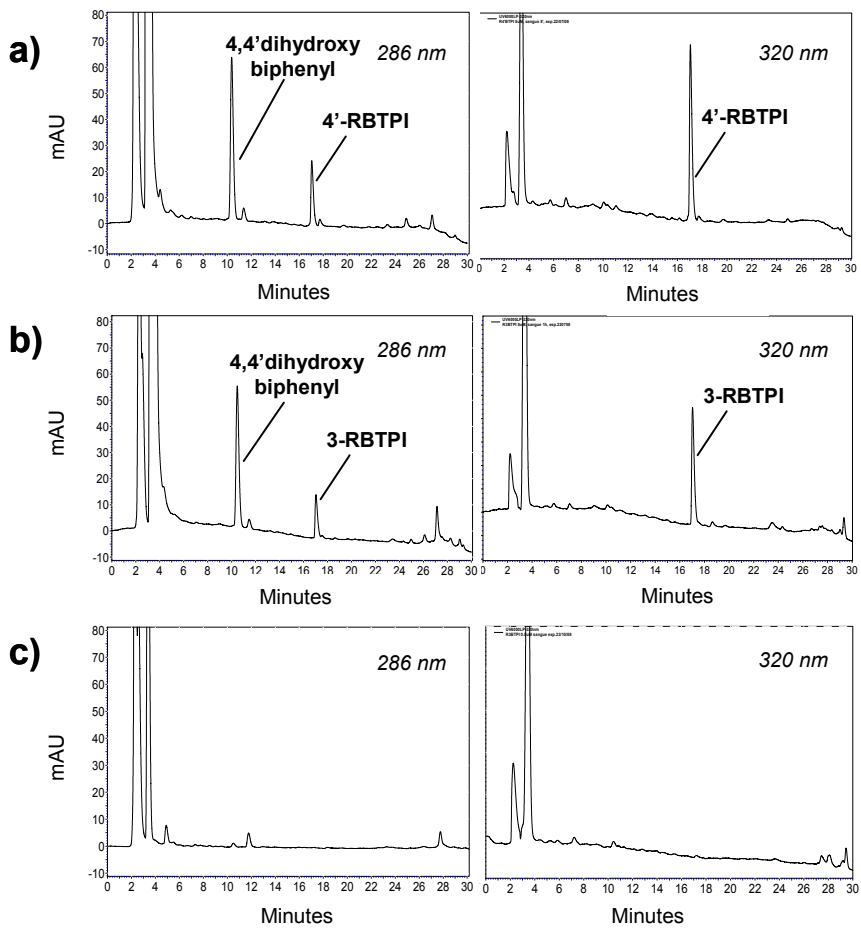

Fig. 2. HPLC chromatograms (recorded both at 286 and 320 nm) of a treated blood samples spiked with 5μM: a) 4'-RBTPI and b) 3-RBTPI; c) sample without any spiking with analytes.

4'-RBTPI, 3-RBTPI, 3-QBTPI, 7-QBTPI and their internal standards were quantified at 320 nm (resveratrol derivatives), 286 nm (4,4' dihydroxybiphenyl) and 370 nm (quercetin derivatives and 2',5,7-trihydroxyflavone). The chomatograms at these wavelengths (fig. 2a,b; fig. 3a,b) show that the analytes are all resolved and free from interfering background peaks of the instrument and the blood matrix (fig. 2c, fig. 3c). Also, the internal standards do not interfere with metabolite analysis (not shown).
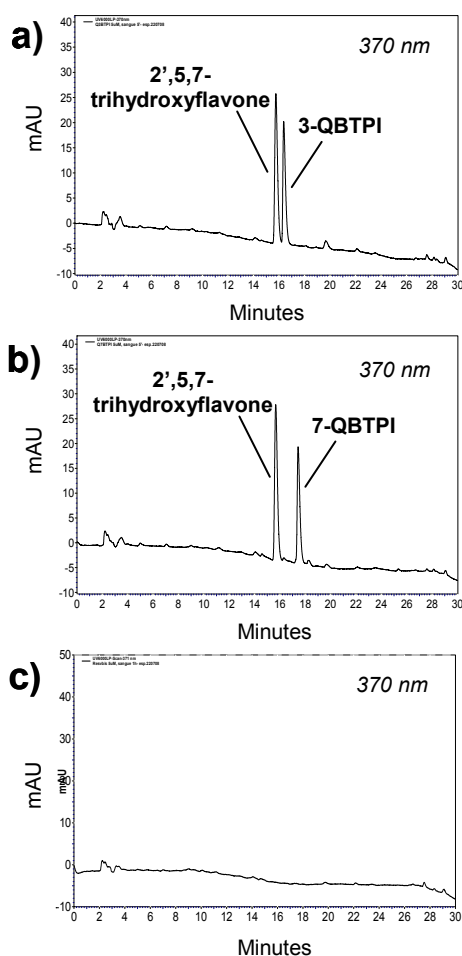

Fig.3. HPLC chromatograms (recorded at 370 nm) of a treated blood samples spiked with 5μM: a) 3-QBTPI and b) 7-QBTPI; c) sample without any spiking with analytes.

Limit of detection (LD) and limit of quantification (LQ) were calculated measuring the average background response at 320 and 370 nm in 10 different HPLC runs. LD and LQ were taken to be 3 and 10 times, respectively, the standard deviation of this background response, according to the definition accepted by both IUPAC and the American Chemical Society. Interpolating the data obtained at 320 and 370 nm respectively with calibration curves in $H_2O:CH_3CN$ for 4'-RBTPI, 3-RBTPI, 3-QBTPI and 7-QBTPI (see above), we obtained: for quercetin derivatives, an LD of 0.07 μM and an LQ of 0.20 μM; for

resveratrol derivatives, an LD of 0.04 μM and an LQ of 0.14 μM. It should be noted that these parameters refer to the actual solution being analysed. Blood concentrations below LQ can still be quantified by the simple expedient of reducing the volume of the extract to be injected.

Conclusions

The method for blood extraction already used for resveratrol and quercetin has proved to be suitable also for mitochondriotropic derivatives. This provides the necessary tool for future assessment of pharmacokinetic profiles of these derivatives.

Abbreviations

AUC: Area Under Curve (in pharmacokinetics)

BTPI: (n-butyl-4-triphenylphosphonium) iodide

COX: Cyclooxygenase

CSP: Cyclosporin A

DMEM: Dulbecco's Modified Eagle Medium

DMF: Dimethylformamide

DMSO: Dimethylsulfoxide

EDC: N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide

EDTA: N,N,N',N'etilendiamminotetraacetic acid

EGCG: EpiGalloCatechinGallate

ER: Endoplasmic reticulum

ESI: ElectroSpray Ionization

EtOAc: Ethyl Acetate

FBS: Fetal Bovine Serum

GSH: Glutatione

GST: Glutathione-S-Transferase

HBSS: Hank's balanced saline solution

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

HPLC: High Performance Liquid Chromatography

IMM: Inner mitochondrial membrane

I/R: Ischemia/Riperfusion

MAPK: Mitogen-Activated Protein Kinase

MDR: Multi-Drug Resistance

MEF: Mouse Embryo Fibroblast

MeOD: Deuterated methanol

MeOH: Methanol

MPT: Mitochondrial Permeability Transition

MPTP: Mitochondrial Permeability Transition Pore

MS: Mass Spectrometry

MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazolium salt)

OAc: Acetyl-

OMM: Outer mitochondrial membrane

Q: Quercetin

QBCl: Chlorobutyl-quercetin

QBI: Iodobutyl-quercetin

QBTPI: (n-butyl-4-triphenylphosphonium)-quercetin-iodide

QTA: Tetraacetyl-quercetin

QTABCl: Chlorobutyl-tetraacetyl-quercetin

QTABI: Iodobutyl-tetraacetyl-quercetin

QTABTPI: (n-butyl-4-triphenylphosphonium)-tetraacety-quercetin-iodide

Pyr: Pyridine

R: Resveratrol

RDA: Diacetyl-resveratrol

RT: Room temperature

TPMP: Methyl-triphenylphosphonium

TPP: Tetraphenylphosphonium

RBCl: Chlorobutyl-resveratrol

RDABCl: Chlorobutyl-diacetyl-resveratrol

PEG: PolyEthyleneGlycol

P_i: Phosphate

PTFE: Politetrafluoroetilene (Teflon®)

RLM: Rat Liver Mitochondria

ROS: Reactive Oxygen Species

TLC: Thin layer chromatography

SOD: Superoxide Dismutase

SULT: Sulfotransferase

UGT: UDP-Glucuronosyltransferase

Partecipation to congresses

Posters

▪ Ester Marotta, Umberto De Marchi, Lucia Biasutto, Cristina Paradisi, Mario Zoratti, "Transepithelial transport of natural and synthetic polyphenols". *Massa 2004 – An international symposium on mass spectrometry*, Bari, 26-30 settembre 2004.

▪ Mario Zoratti, Ester Marotta, Umberto De Marchi, Lucia Biasutto, Cristina Paradisi, "Synthetic polyphenol derivatives to improve bioavailability of neuroactive natural compounds". *Consiglio Nazionale delle Ricerche- Quarto incontro dell'istituto di Neuroscienze*, Roma, 17-18 marzo 2005.

▪ Ester Marotta, Umberto De Marchi, Lucia Biasutto, Mario Zoratti, Cristina Paradisi, "The complex hydrolysis of quercetin esters". *ESOR 10 – 10th European Symposium on Organic Reactivity*, Roma, 25-30 luglio 2005.

▪ Lucia Biasutto, Andrea Mattarei, Ester Marotta, Umberto De Marchi, Alice Bradaschia, Spiridione Garbisa, Cristina Paradisi, Mario Zoratti, "Mitochondriotropic polyphenol derivatives". *COST Action 926 Conference- Benefits and Risks of Bioactive Plant Compounds,* Cracovia, 27-28 marzo 2008. Acta Biochim. Pol. 55 (1, Suppl), 12 (P1.18)

▪ Lucia Biasutto, Andrea Mattarei, Ester Marotta, Umberto De Marchi, Alice Bradaschia, Spiridione Garbisa, Cristina Paradisi, Mario Zoratti, "Mitochondriotropic polyphenol derivatives". *Bari International Symposium on Mitochondrial Physiology and Pathology. IUBMB Symposium S1/2008,* Bari, 22-26 giugno 2008.

▪ Lucia Biasutto, Alice Bradaschia, Andrea Mattarei, Ester Marotta, Spiridione Garbisa, Mario Zoratti, Cristina Paradisi, "The fate of polyphenols and their mitochondriotropic derivatives in blood - accumulation by mitochondria slows metabolic conjugation". *International PSE Symposium on Natural Products in Cancer Therapy,* Napoli, 23-26 settembre 2008.

▪ Andrea Mattarei, Lucia Biasutto, Ester Marotta, Spiridione Garbisa, Mario Zoratti, Giancarlo Sandonà, Cristina Paradisi, "Oxidation potentials and radical-scavenging properties of novel mitochondrion-targeted quercetin derivatives". *International PSE Symposium on Natural Products in Cancer Therapy,* Napoli, 23-26 settembre 2008.

Oral Communications

▪ Mario Zoratti, Lucia Biasutto, Ester Marotta, Andrea Mattarei, Mauro Fallica, Umberto De Marchi, Alice Bradaschia, Spiridione Garbisa, Cristina Paradisi, "Bioavailability-enhancing prodrugs of polyphenols". *COST Action 926 Conference- Benefits and risks of*

bioactive plant compounds, Cracovia, 27-28 marzo 2008. Acta Biochim. Pol. 55 (1, Suppl), 26 (O2.3).

▪ Umberto De Marchi, Lucia Biasutto, Nicola Sassi, Paola Cattelan, Antonio Toninello, Mario Zoratti, "Similarities between the redox activities of polyphenols and polyamines: effects on the mitochondrial permeabilità transition". *International Congress on Biogenic Amines: Biochemical, Physiological and Clinical Aspects*, Albarè di Tenna (TN), 14-18 maggio 2008.

▪ Lucia Biasutto, Andrea Mattarei, Ester Marotta, Umberto De Marchi, Alice Bradaschia, Spiridione Garbisa, Cristina Paradisi, Mario Zoratti, "Mitochondriotropic polyphenol derivatives". *Riunione annuale Gruppo Italiano di Biomembrane e Bioenergetica,* Bari, 20-21 giugno 2008. (Presentazione di Lucia Biasutto).

▪ Andrea Mattarei, Lucia Biasutto, Ester Marotta, Alice Bradaschia, Umberto De Marchi, Nicola Sassi, Spiridione Garbisa, Cristina Paradisi, Mario Zoratti, "Mitochondria-targeted polyphenol derivatives". *International PSE Symposium on Natural Products in Cancer Therapy,* Napoli, 23-26 settembre 2008.

Graduated courses attended

21-25/05/2007: Scuola Nazionale "Metodologie analitiche in spettrometria di massa"; corso organizzato dalla Società Chimica Italiana e dall'Università di Parma.

19/02/2008: Corso pratico di sviluppo metodiche HPLC con le nuove colonne Zorbax RRLC HT.