Intracellular metabolism and bioactivity of quercetin and its *in vivo* metabolites

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Understanding the cellular effects of flavonoid metabolites is important for predicting which dietary flavonoids might be most beneficial *in vivo*. Here we investigate the bioactivity in dermal fibroblasts of the major reported *in vivo* metabolites of quercetin, i.e. 3′-*O*-methyl quercetin, 4′-*O*-methyl quercetin and quercetin 7-*O*-β-D-glucuronide, relative to that of quercetin, in terms of their further metabolism and their resulting cytotoxic and/or cytoprotective effects in the absence and presence of oxidative stress. Uptake experiments indicate that exposure to quercetin led to the generation of two novel cellular metabolites, one characterized as a 2′-glutathionyl quercetin conjugate and another product with similar spectral characteristics but 1 mass unit lower, putatively a quinone/quinone methide. A similar product was identified in cells exposed to 3′-*O*-methyl quercetin, but not in the lysates of those exposed to its 4′-*O*-methyl counterpart, suggesting that its formation is related to oxidative metabolism. There was no uptake or metabolism of quercetin 7-*O*-β-D-glucuronide by fibroblasts. Formation of oxidative metabolites may explain the observed concentration-dependent toxicity of quercetin and 3′-*O*-methyl quercetin, whereas the formation of a 2′-glutathionyl quercetin conjugate is interpreted as a detoxification step. Both *O*-methylated metabolites conferred less protection than quercetin against peroxide-induced damage, and quercetin glucuronide was ineffective. The ability to modulate cellular toxicity paralleled the ability of the compounds to decrease the level of peroxide-induced caspase-3 activation. Our data suggest that the actions of quercetin and its metabolites *in vivo* are mediated by intracellular metabolites.

Key words: fibroblast, flavonoid, glucuronide, MS, *O*-methylated, oxidative stress.

INTRODUCTION

Few investigations have studied the influence of metabolism on the bioactivity of flavonoids [1–4]. Thus most of the effects reported based on *in vitro* experiments with natural flavonoid forms cannot be extrapolated to *in vivo* situations. One of the most intensively investigated flavonoids, quercetin, has often been used as the aglycone in cell culture systems, which has led to conflicting reports of its activity. Reported biological activities of quercetin include essentially cytotoxic effects, such as induction of apoptosis [5–8], cell cycle arrest [9] and anti-proliferative effects [10] on the one hand, to inhibition of apoptosis [11], anti-inflammatory effects [12] and protection against oxidative stress [13,14] on the other. However, intestinal metabolism [15,16], colonic studies [17] and *in vivo* bioavailability reports [18] suggest that quercetin is metabolized to the extent that little or no free quercetin aglycone is present in the circulation. Rather, it will be in the form of quercetin glucuronides and sulphates, *O*-methylated quercetin and *O*-methylated quercetin glucuronides [19] formed in the small intestine and liver, as well as hydroxyphenylacetate and other degradation products formed in the large intestine by action of the colonic microflora [17]. It is therefore important for the relevance of flavonoid–cell interactions to involve the *in vivo* metabolites of the flavonoid under investigation.

Another important consideration when attempting to determine the biological mechanism of flavonoid action *in vivo* is whether a specific cell type is able to metabolize further the compounds intracellularly, and the physiological and cellular effects of the resulting metabolites. Little attention has been given to potential intracellular metabolism of flavonoids, other than that well reported in cells of the gastrointestinal tract, such as enterocytes [15] and Caco-2 cells [20], or the hepatic system [21]. Recent observations from our laboratory have suggested that the citrus flavanone hesperetin is susceptible to intracellular glucuronidation and subsequent export from human dermal fibroblasts in culture (A. R. Proteggente, S. Basu-Modak. G. Kuhnle, M. J. Gordon, K. Youdim, R. Tyrrell and C. Rice-Evans, unpublished work). Previously we have reported the uptake of epicatechin and one of its *in vivo* metabolites, 3′-*O*-methyl epicatechin, into both dermal fibroblasts [1,2] and cortical neurons [1], where no further metabolism was detected, but the levels of uptake were an order of magnitude lower than those of hesperetin. In contrast, epicatechin 5-*O*-β-D- and 7-*O*-β-D-glucuronides were unable to enter cells. Interestingly, the glucuronides also failed to protect against cytotoxicity induced by oxidative stress, whereas both epicatechin and its *O*-methylated form were protective, suggesting that intracellular access is a requirement for the protective mechanism, or that the structural modification caused by glucuronidation on the A-ring inhibits binding to the appropriate receptor [1].

Here we investigate the bioactivity of quercetin and its major reported *in vivo* metabolites, 3′-*O*-methyl quercetin, 4′-*O*-methyl quercetin and quercetin 7-*O*-β-D-glucuronide, on dermal fibroblasts, in terms of their further metabolism by the cells and their resulting cytotoxic and/or cytoprotective effects in the absence and presence of oxidative stress. Possible cellular transformation of these compounds would be expected to play a significant role in the overall positive or negative effects that are observed in cell systems and *in vivo* [22].

Abbreviations used: DCDHF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; HBM, Hepes-buffered medium; LC, liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl; RT, retention time.

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MATERIALS AND METHODS

Synthesis of quercetin glucuronide

The method employed was developed for the glucuronidation of epicatechin as described previously [1]. The supernatant of a 50 % (w/v) liver homogenate containing dithiothreitol (0.5 mM) and a β-glucuronidase inhibitor was isolated, and UDP-glucuronic acid (2.5 mM) and quercetin (1.5 mM) were added and incubated in a shaking water bath at 37 °C for 6 h under nitrogen. During incubation, two further aliquots of UDP-glucuronic acid were added. Isolation and purification were performed as described previously [1], and characterization of quercetin glucuronides was by HPLC/β-glucuronidase treatment and by electrospray MS using an LCQ Deca XP quadrupole ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, U.S.A.). In addition, the position of glucuronidation was investigated by ^{1}H NMR. Spectra were recorded using a Bruker DRX 500 spectrometer with an operating frequency of 500.13 MHz (Bruker Biospin, Rheinstetten, Germany).

Cell culture and assessment of fibroblast uptake/association and metabolism

Normal human dermal fibroblasts derived from foreskin were used between passages 8 and 14 [2] and were routinely cultured in fibroblast growth medium (PromoCell®, Heidelburg, Germany) supplemented with insulin (5 μg/ml), basic fibroblast factor (1 ng/ml), amphotericin (50 ng/ml) and gentamicin (50 μg/ml).

The cell-associated levels of quercetin, quercetin 7-*O*-β-D-glucuronide and 3′-*O*-methyl and 4′-*O*-methyl quercetin were assessed by exposure of dermal fibroblasts (2×10^6 cells/plate) following a 2, 6, 12 or 18 h incubation in fibroblast growth medium as described previously [1,2]. Following exposure, cells were washed four times with ice-cold PBS and rapidly lysed on ice using aqueous methanol (50 %, v/v) containing HCl (0.1 %). Lysed cells were scraped and left on ice to solubilize for 45 min, and then centrifuged at 2000 ***g*** for 5 min at 4 °C to remove unbroken cell debris and nuclei. The supernatants were recovered and analysed by HPLC with photodiode array detection and by liquid chromatography (LC)–MS [1]. The protein concentration in the supernatants was determined by the Bio-Rad Bradford protein assay® (Bio-Rad).

Synthesis of quercetin–glutathione conjugate

2′-Glutathionyl quercetin was prepared by a method similar to that reported for catecholamine conjugates [23], with some modifications. GSH (0.4 g) and 20 mg of mushroom tyrosinase (2000 units/ml) were incubated in ammonium acetate buffer (0.1 M), pH 5.8, at 25 °C (total volume of 50 ml) before the dropwise addition of quercetin (0.1 g) under constant stirring for 2 h. The sample mixture was filtered through a 0.22 μm Centriprep® particle separator (Amicon), and de-protonated by addition of an equal volume of ice-cold methanol. The de-protonated preparation was rotary-evaporated to remove the methanol and then freeze dried. The dried residue was dissolved in 10 ml of aqueous methanol (50 %, v/v) and 2 ml fractions were purified by preparative reverse-phase HPLC, using a Zorbax ODS column (21.2 mm × 250 mm; HPLC Technology, Macclesfield, Cheshire, U.K.), eluted isocratically at a flow rate of 4 ml/min with 6 % (v/v) methanol in 10 mM ammonium acetate buffer, pH 4.7. Fractions containing the glutathione conjugates of quercetin were lyophilized and dissolved in methanol (50 %, v/v) to obtain a 10 mM stock solution.

Characterization of the glutathionyl conjugate of quercetin was performed by LC–MS and ^{1}H NMR. LC–MS was performed as described above, and ^{1}H NMR spectra were recorded using a Bruker DRX 500 spectrometer with an operating frequency of 500.13 MHz (Bruker Biospin, Rheinstetten, Germany). The samples were dissolved in deuterated DMSO (Cambridge Isotopes, Cambridge, U.K.). The toxicity of the quercetin–glutathione conjugate (0.1–10 μM) was assessed by exposure of fibroblasts for 18 h and application of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl] assay.

Assessment of cell damage

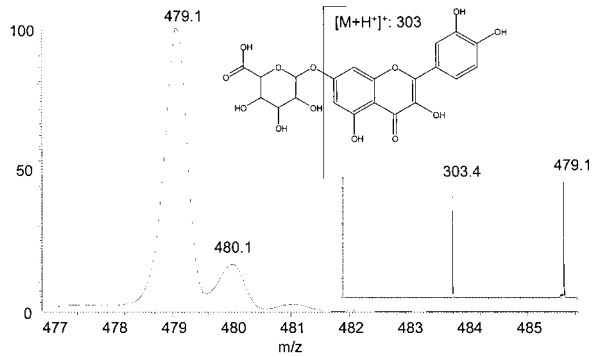
Cells were exposed to H_2O_2 (50 μM) in Hepes-buffered medium (HBM; pH 7.4; 5 mM Hepes, 154 mM NaCl, 4.6 mM KCl, 2.3 mM $CaCl_2$, 33 mM glucose, 5 mM $NaHCO_3$, 1.1 mM $MgCl_2$, 1.2 mM Na_2HPO_4) for 2 h, after which time the peroxide was removed and the HBM was replaced with fresh medium. To evaluate the potential protective effects of quercetin and its glucuronidated and *O*-methylated metabolites, fibroblast cultures were pretreated for 18 h at concentrations of 0.3–30 μM. Following pretreatment, both cultures were washed twice with PBS (to prevent direct extracellular interactions between the compounds and H_2O_2) prior to the addition of H_2O_2 as detailed above. Vehicle controls containing equivalent volumes of methanol were carried out. Cellular damage elicited by H_2O_2 treatment was evaluated by measuring MTT reduction and Trypan Blue exclusion, as described previously [2]. Following exposure to H_2O_2 and replacement of the medium (as detailed above), MTT reduction in neurons and fibroblasts was determined after a further 21 h incubation. Additional experiments to assess the direct toxic effects of quercetin and its metabolites (0.3–30 μM; 18 h) were also carried out.

Caspase-3 activity in fibroblasts (2×10^6) was analysed as described previously [1,2]. After pretreatment with quercetin, quercetin glucuronide, quercetin 3-glucoside, 3′-*O*-methyl quercetin or 4′-*O*-methyl quercetin at 1, 3 and 10 μM for 18 h, cultures were washed twice with ice-cold HBM and exposed to H_2O_2 (50 μM) in HBM as detailed above. Caspase-3-like protease activity in fibroblast lysates was assessed 12 h after peroxide treatment, and was based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp *p*-nitroanilide by caspase-3, resulting in the release of the *p*-nitroaniline moiety, which absorbs at 405 nm.

2′,7′-Dichlorodihydrofluorescein diacetate (DCDHF-DA) assay for oxidative stress

Fibroblasts were cultured for 7 days on black 24-well plates. Cells were pretreated with quercetin or one of its metabolites (10 μM) as described above and then washed twice before addition of HBM containing DCDHF-DA (100 μM). Plates were incubated for 30 min in 5 % CO_2/95 % air at 37 °C. After the incubation, the cells were washed three times with HBM and the fluorescence was measured for up to 6 h using a SPECTRAmax® Gemini microplate spectrofluorimeter (Molecular Devices). The temperature was maintained at 37 °C and the emission was recorded at 530 nm after excitation at 500 nm. Each well was scanned in the instrument's well scan mode, accumulating data from 21 independent points per well, which were then transformed into an average signal expressed in relative fluorescent units. All data were calculated and normalized with respect to the increase in fluorescence of a control sample.

A



B

Proton	Quercetin [δ in ppm]	Quercetin-7-O-β-D-glucuronide [δ in ppm]
HO-3'	9.391	9.557
HO-4'	9.625	9.693
HO-3	9.337	9.345
HO-5	12.509	12.547
HO-7	10.813	—

Figure 1 Mass spectrum and ^{1}H NMR of the synthesized glucuronide of quercetin

(**A**) Mass spectrum of synthesized quercetin glucuronide. The large spectrum shows the quasi-molecular ion ($[M+H^+]^+$) of quercetin glucuronide (molecular mass 478 Da). The inset shows the fragmentation of quercetin *O*-glucuronide with a fragmentation energy of 25 %. The main fragment formed is the aglycone, quercetin. (**B**) ^{1}H NMR data indicating the position of conjugation on the quercetin structure.

Statistical analysis

Data were expressed as means ± S.D. Statistical comparisons were made using an unpaired two-tailed Student's *t* test with a confidence level of 95 %. The significance level was set at $P < 0.05$.

RESULTS

Quercetin glucuronide was synthesized and purified by preparative HPLC, and characterization was confirmed by mass spectrometric analysis (Figure 1A). The $[M+H^+]^+$ ion of a quercetin glucuronide was detected at *m/z* 479, which generated an intense product ion at 303 that could be assigned to the quercetin aglycone (*m/z* 303). ^{1}H NMR analysis of the purified conjugate was undertaken to determine the position of glucuronidation, which was assigned to the 7-position of the A-ring (Figure 1B).

Human dermal fibroblasts were treated with quercetin, 3′-*O*-methyl quercetin, 4′-*O*-methyl quercetin or quercetin 7-*O*-β-D-glucuronide in a dose-dependent manner for 18 h to investigate their effects on cell integrity/damage. None of the compounds displayed any toxicity up to 10 μM. In addition, 4′-*O*-methyl quercetin and quercetin 7-*O*-β-D-glucuronide did not induce toxicity at 30 μM. In contrast, direct exposure to quercetin or to its 3′-*O*-methylated metabolite at a concentration of 30 μM resulted in a decrease in the ability of the cells to reduce MTT (a marker of cell damage), to the extent of 73.2 ± 3.6 % (untreated control cells = 100 %) ($P < 0.05$) for quercetin and 84.6 ± 2.8 % ($P < 0.05$) for 3′-*O*-methyl quercetin.

Table 1 Characterization of the glutathionyl conjugate of quercetin by ^{1}H NMR

^{1}H NMR spectra were recorded with an operating frequency of 500.13 MHz. The samples were dissolved in deuterated DMSO. Q-GSH, quercetin–glutathione conjugate.

	Quercetin			Q-GSH	
	δ (p.p.m.)	$J_{H2'H6'}$ (Hz)	$J_{H6'H5'}$ (Hz)	δ (p.p.m.)	$J_{H6'H5'}$ (Hz)
H2′	7.715	2.056			
H5′	6.919		8.475	6.843	8.091
H6′	7.575	2.059	8.469	6.896	8.190

Fibroblast uptake and metabolism of quercetin, its two *O*-methylated metabolites and quercetin 7-*O*-β-D-glucuronide (all present at 10 μM concentration) were monitored following 2, 6, 12 and 18 h exposures (Figure 2). Reverse-phase HPLC with photodiode array detection revealed quercetin [retention time (RT) 42.5 min] and two new major peaks at RT 33.7 min and 39.6 min. These two new compounds, which were observed at all time points post-exposure, had similar spectral characteristics to quercetin and were detected at earlier RTs (Figure 2, left panels; 12 h). The presence of quercetin (RT 42.5 min) in lysates was confirmed by mass spectrometric analysis (*m/z* of $[M+H^+]^+$ ion = 303), and was greatest in fibroblasts at around 6 h exposure, after which levels fell. The two peaks at RT 33.7 min and 39.6 min were observed to increase in amount between 2 and 18 h. The HPLC RT, spectral characteristics and MS fragmentation spectrum (Figure 3A) of the peak at 33.7 min (*m/z* of $[M+H^+]^+ = 608$) allowed characterization of the peak as a predicted quercetin–glutathione adduct. A standard of quercetin glutathione adduct was synthesized chemically and was proven to be a 2′-glutathionyl quercetin conjugate by LC–MS and ^{1}H NMR investigation (Table 1). The properties of the intracellularly formed adduct correlated precisely with the RT and MS fragment ion spectrum of this synthesized standard, allowing structural assignment of the adduct as 2′-glutathionyl quercetin. Examination of the toxicity of this 2′-glutathionyl quercetin adduct added to fibroblasts was undertaken over the concentration range 0.3–30 μM for 18 h exposures by applying the MTT assay. This conjugate was found to have no significant toxicity itself on exposure to the fibroblast system (30 μM gave 98.6 ± 1.2 % MTT reduction relative to untreated cells). However, it should be noted that no uptake was detected for the exogenously added 2′-glutathionyl quercetin adduct. A third, smaller peak was also observed at RT 30.9 min in cells exposed to quercetin. This peak, which appeared only at later time points, is most probably the result of oxidative metabolism of quercetin, as a similar product has been observed on exposure of peroxynitrite to quercetin (J. P. E. Spencer, R. J. Williams and C. Rice-Evans, unpublished work).

LC–MS analysis of the peak at 39.6 min (M1) revealed a compound with an *m/z* for $[M+H^+]^+$ of 302 (one mass unit lower than that of quercetin; $[M+H^+]^+ = 303$) with a different fragment ion spectrum from that of quercetin itself (Figure 3B). Due to the nature of the cell experiments, only low amounts of this product could be prepared for structural characterization, and ^{1}H NMR analysis was not possible. However, the formation of M1 only occurred during fibroblast exposure, and it was not found in control incubations performed in cell-free medium (2–18 h). As quercetin is highly autoxidizable and cell lysis took place in acid, with the subsequent HPLC run in an acidified mobile phase, it is likely that M1 is an intracellularly formed quinone species that has been trapped by protonation.

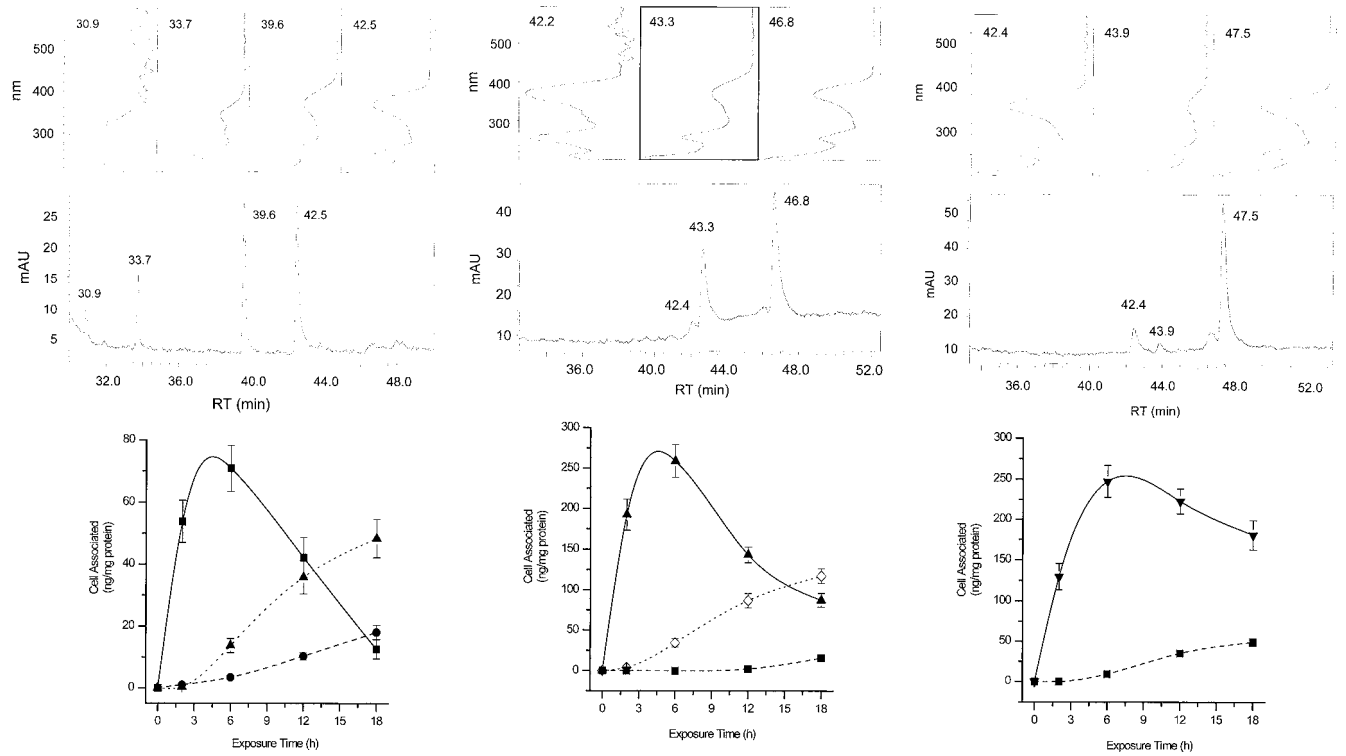


Figure 2 Association of quercetin and its metabolites with fibroblasts

Cells were exposed to the various compounds (10 μM) for 2, 6, 12 and 18 h, after which the cells were lysed, de-protonated and analysed by HPLC with photodiode array detection. Left panels: typical HPLC trace of a cell lysate from fibroblasts exposed to quercetin for 12 h. Quercetin (RT 42.5 min) and three new peaks at 30.9, 33.7 and 39.6 min were detected at all time points. Middle panels: typical HPLC trace of a cell lysate from fibroblasts exposed to 3′-*O*-methyl quercetin (RT 46.8 min) for 12 h. Right panels: typical HPLC trace of a cell lysate from fibroblasts exposed to 4′-*O*-methyl quercetin (RT 47.5 min) for 12 h. mAU, m-absorbance units.

Both *O*-methylated forms of quercetin were observed to be taken up by fibroblasts at all exposure time points (Figure 2, middle and right panels), although the patterns of association differed in a time-dependent manner. Exposure of cells to 3′-*O*-methyl quercetin also resulted in detection of a major peak at 43.3 min (M2), which had similar spectral characteristics to 3′-*O*-methyl quercetin (Figure 2, middle panels) and a *m/z* for the $[M + H^+]^+$ ion of 316 (Figure 3C), again suggesting that it is likely to be a 'proton-stabilized' quinone species, as described above for the oxidized quercetin. This product increased in amount with time, in parallel with the degradation of 3′-*O*-methyl quercetin (Figure 2, middle panels), in a similar manner to that observed with the $[M + H^+]^+$ ion at *m/z* 302 peak in quercetin-exposed cells. Also, as with quercetin, 3′-*O*-methyl quercetin was degraded between 6 and 18 h, with the increased production of this compound consistent with a proton-stabilized quinone species. Interestingly, a similar product was not observed in fibroblasts exposed to 4′-*O*-methyl quercetin, and levels of 4′-*O*-methyl quercetin were sustained with time up to 18 h relative to those of quercetin or the 3′-*O*-methyl metabolite. Since 4′-*O*-methyl quercetin is structurally incapable of autoxidation due to the methyl substitution on the 4′-hydroxy group of the B-ring, this observation supports the assignment of the above-mentioned peaks M1 and M2 as oxidation products. In addition, there were small but measurable levels of quercetin detected in the 12 h and 18 h lysates from cells exposed to both *O*-methylated quercetin metabolites (Figures 3B and 3C), although this was most significant in cells exposed to 4′-*O*-methyl quercetin. The detection of free quercetin in these lysates suggests the intracellular cleavage of the B-ring methyl moiety. In contrast with these metabolites, there was no detectable uptake of quercetin 7-*O*-β-D-glucuronide into fibroblasts up to 18 h of exposure.

As well as cell-associated levels of these products, there were also significant amounts measurable in the medium following exposure. While the concentration of quercetin in the medium fell from 9.5 ± 0.4 to 1.5 ± 0.2 μM over 18 h of exposure to fibroblasts, the levels of both 2′-glutathionyl quercetin and the unidentified product (concentration calculated using quercetin as standard) increased in a time-dependent manner, reaching concentrations of 0.9 ± 0.1 and 1.9 ± 0.1 μM respectively after 18 h. The levels of both *O*-methylated metabolites in the medium fell less rapidly between 0 and 18 h compared with that of quercetin, with that of 3′-*O*-methyl quercetin declining significantly ($P < 0.05$) from 9.7 ± 0.5 to 6.9 ± 0.5 μM and that of 4′-*O*-methyl quercetin falling from 9.8 ± 0.6 to 8.5 ± 0.6 μM (not significant). Levels of product M2 also increased in a time-dependent manner, reaching 2.7 ± 0.2 μM at 18 h. No free quercetin was detected in the medium after exposure of cells to either *O*-methylated metabolite. Whereas no glucuronide was detected in the cells, levels of quercetin 7-*O*-β-D-glucuronide in the medium declined steadily from 9.9 ± 0.7 to 3.5 ± 0.2 μM at 18 h.

In order to assess the consequences of the observed metabolism for the fibroblasts, the potential protective abilities of quercetin and its *in vivo* metabolites against fibroblast damage induced by peroxide were investigated. Exposure to peroxide (50 μM; 2 h) resulted in a 56.7 ± 2.1% decrease in the ability of the fibroblasts to reduce MTT, a measure of cell damage. Pretreatment

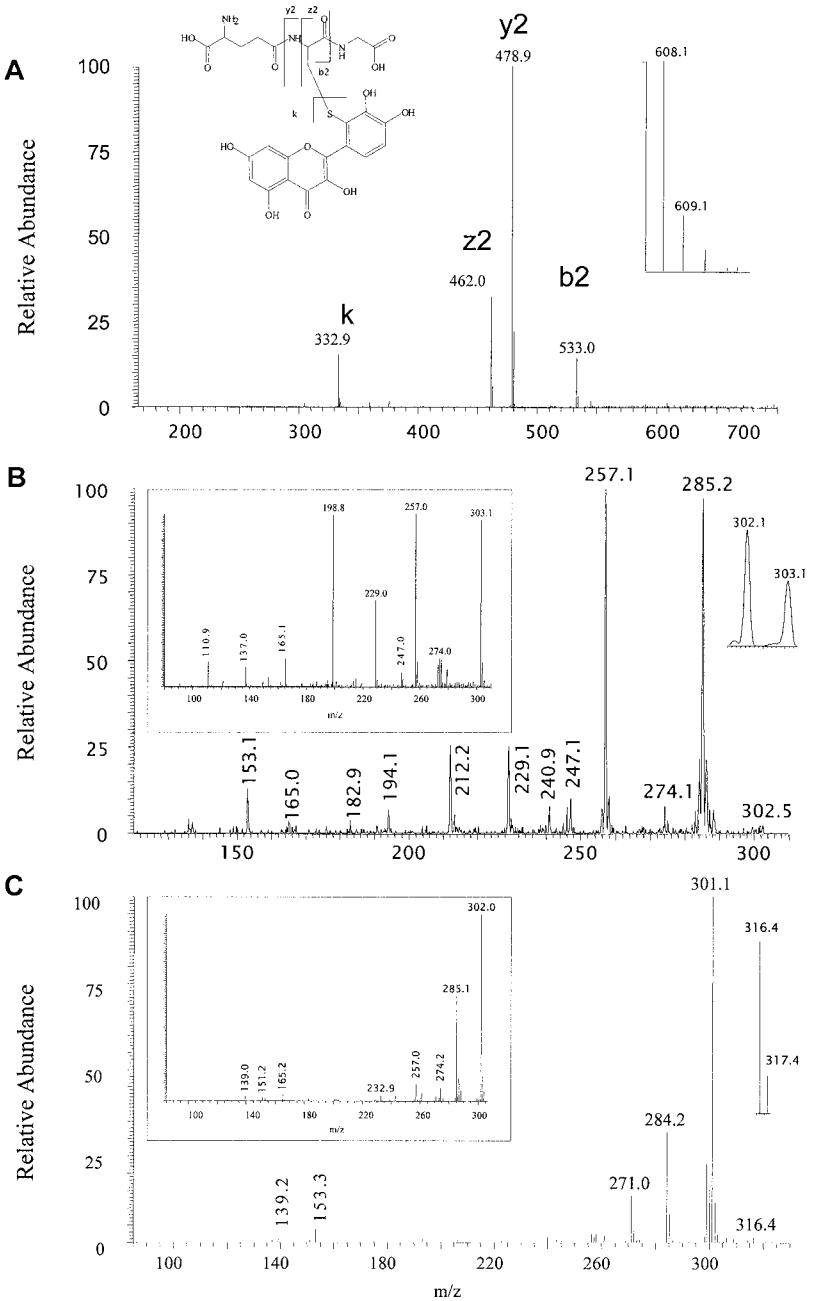


Figure 3 Mass spectra of intracellularly formed metabolites

(**A**) MS/MS spectrum of the peak at 33.7 min relating to a 2′-glutathionyl quercetin conjugate. The fragment ion spectrum of the quercetin–glutathione conjugate was recorded at a collision energy of 30 %. The structure of the conjugate is shown after further confirmation by ^{1}H NMR. The inset spectrum shows the quasi-molecular ion ($[M + H^+]^+$) of the conjugate. (**B**) MS/MS spectrum of the unknown compound M1 with an *m/z* of 302. The small inset shows the signal for the $[M + H^+]^+$ of the compound, and the large inset shows the product ion spectrum of quercetin. (**C**) MS/MS spectrum of the unknown compound M2 with an *m/z* of 316. The small inset shows the signal of the $[M + H^+]^+$ ion, and the large inset shows the product ion spectrum of 3′-*O*-methyl quercetin.

of fibroblasts with quercetin resulted in dose-dependent protection against peroxide-induced damage, which peaked at 10 μM quercetin (Figure 4A). Pretreatment with higher concentrations of quercetin (30 μM) resulted in enhanced cell damage compared with peroxide-treated cells. The *O*-methylated metabolites of quercetin with substituted catechol groups, i.e. 3′-*O*-methyl and 4′-*O*-methyl quercetin, also conferred protection against the oxidative stress-induced damage, although this was less extensive than that observed with quercetin. Furthermore, whereas for 4′-*O*-methyl quercetin the protection was concentration-dependent up to 30 μM, the 3′-*O*-methyl metabolite was less protective at concentrations above 10 μM. The synthesized quercetin 7-*O*-β-D-glucuronide conferred no protection against the peroxide insult (Figure 4A). Furthermore, the glucuronide had no significant effects in protecting against peroxide-induced (50 μM; 2 h) caspase-3 activation in fibroblasts (Figure 4B). In contrast,

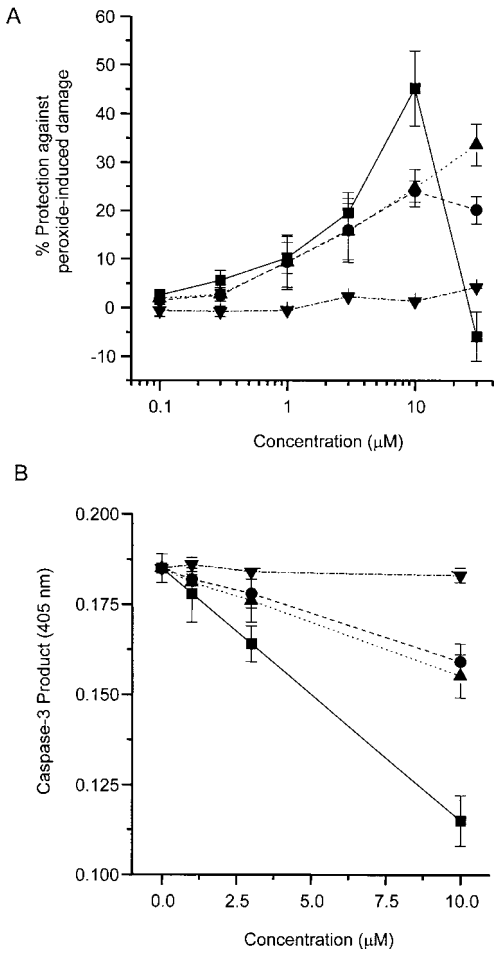


Figure 4 Protection against peroxide-induced cell damage by quercetin and its metabolites

(**A**) Dermal fibroblasts were pretreated with the compounds (0–30 μM) for 18 h before the addition of H_2O_2 (50 μM; 2 h), and cellular damage was assessed by the MTT assay 24 h post-stress. The zero point indicates the addition of H_2O_2 only and represents the mean loss of MTT reduction (56.7 $\pm$ 2.1 %). Data are means $\pm$ S.D. of three separate experiments, each performed in quadruplicate. (**B**) Increased activity of caspase-3-like proteases induced by H_2O_2 (50 μM) and inhibition of this effect by pretreatment with quercetin, 3′-*O*-methyl quercetin, 4′-*O*-methyl quercetin, quercetin glucuronide and quercetin 3-glucoside (18 h). Caspase-like protease activity is represented as an increase in absorbance at 405 nm due to the increased liberation of the caspase-3 cleavage product *p*-nitroaniline. Fibroblasts were analysed for caspase-3 activity 12 h after exposure to H_2O_2 (50 μM; 2 h). Cells were lysed and the activity of caspase-3-like proteases was measured spectrophotometrically using the caspase-3 substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide. ■, Quercetin; ●, 3′-*O*-methyl quercetin; ▲, 4′-*O*-methyl quercetin; ▼, quercetin 7-*O*-β-D-glucuronide.

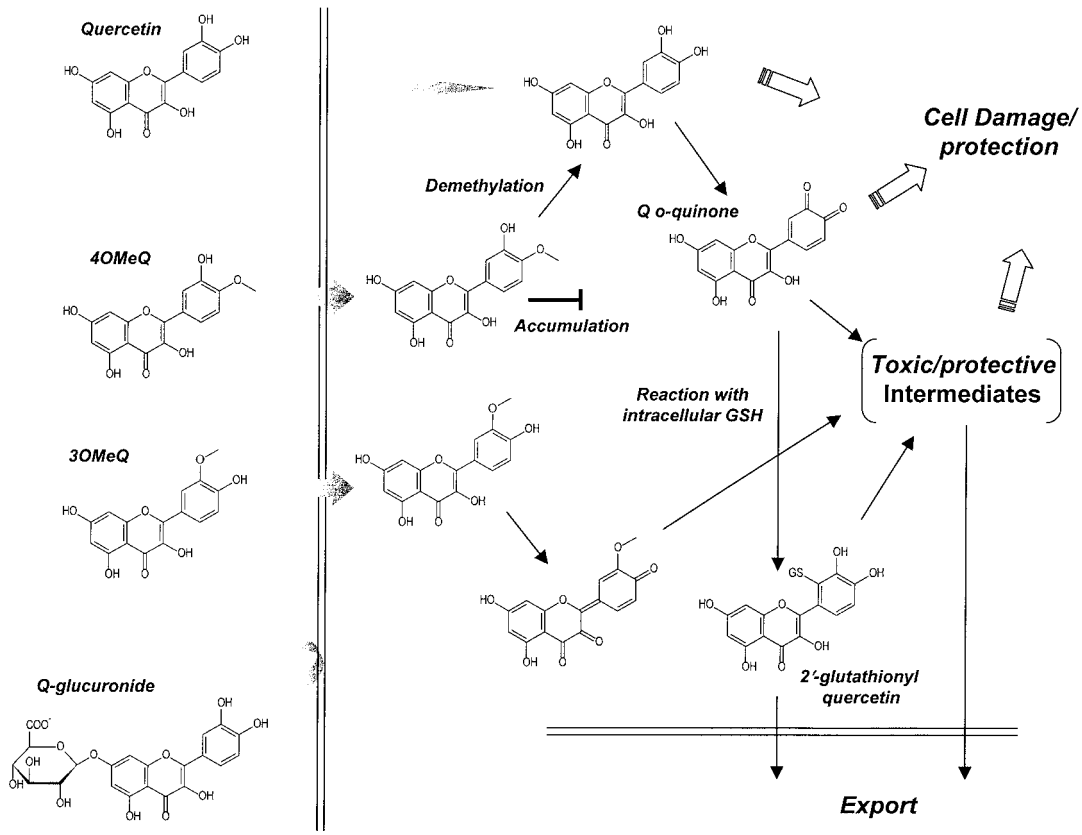
this caspase-3 activation was attenuated by quercetin and its 3′-*O*-methyl and 4′-*O*-methyl metabolites in a concentration-dependent manner (Figure 4B). Control experiments indicated that none of the compounds had a direct effect on the activation of caspase-3 in these cells up to a concentration of 10 μM.

Exposure of fibroblasts to quercetin or its metabolites (10 μM; 6 h) did not result in a decrease in intracellular oxidative stress, either alone or following peroxide treatment, as assessed using DCDHF-DA. Addition of peroxide (50 μM; 2 h) to cells provoked an increase in overall intracellular fluorescence over 6 h (63.1 $\pm$ 7.3 relative light units) in the absence of any loss of cell integrity, as determined by parallel Trypan Blue exclusion assays (4.2 % Trypan Blue-positive cells). There was no significant difference in the increase in intracellular fluorescence between untreated fibroblasts and those pre-exposed to quercetin (10 μM; 56.3 $\pm$ 8.6 relative light units) or its metabolites (61.9–62.5 relative light units) following peroxide addition. Furthermore, control experiments showed that pretreatment with the compounds alone up to a concentration of 10 μM had no effect on the basal level of intracellular oxidative stress or on cell integrity.

DISCUSSION

In considering the possible bioactive mechanisms of action of flavonoids and their *in vivo* metabolites in cell systems, it is important to consider their uptake and possible further metabolism by the cells. Here we have examined both the intracellular metabolism and bioactivity of quercetin and its *in vivo* metabolites in human dermal fibroblasts. The studies described indicate that the ability of flavonoids to protect fibroblasts from oxidative stress is ultimately dependent on their intracellular metabolism. In particular, the cells handle the metabolism of the flavonoid catechol moiety quercetin and its *in vivo* metabolite forms, 3′-*O*-methyl quercetin, 4′-*O*-methyl quercetin and quercetin 7-*O*-β-D-glucuronide, through different metabolic mechanisms. Uptake of quercetin leads to the time-dependent appearance of three products: quercetin itself, a 2′-glutathionyl quercetin adduct and an unidentified product (M1) consistent with an assignment as quercetin quinone/quinone methide. Previous investigations with chemical systems have shown that quercetin [24–28] and other catechol-containing flavonoids, such as catechin [21], taxifolin, luteolin [28], fisetin and 3,3′,4′-trihydroxyflavone [29], are capable of reaction with GSH to generate mono- and di-glutathionyl adducts. The conjugation of glutathione with quercetin has been shown to be pH-dependent in cell-free systems, with conditions of low pH favouring the formation of quinone methide intermediates and A-ring conjugates, and less acidic conditions leading to the generation of B-ring adducts [25], as observed in our fibroblast cell system. The formation of two A-ring monoglutathione conjugates of quercetin (8-glutathionyl quercetin and 6-glutathionyl quercetin) has recently been observed in a mouse melanoma cancer cell model (B16F-10) exposed to quercetin [24]. However, in those experiments the formation of adducts with glutathione was to be expected, due to the significant levels of tyrosinase in the cells, which will catalyse quercetin quinone/quinone methide formation prior to their reaction with GSH [24]. The formation of a 2′-glutathionyl quercetin conjugate in our cell system may suggest that the oxidative metabolism of quercetin in fibroblasts occurs in such a physiological intracellular environment, via enzymic processes or the non-enzymic oxidation of quercetin.

For the 4′-*O*-methyl metabolite, a greater level of time-dependent uptake was observed, accompanied by minor decomposition to quercetin. This metabolite cannot take the same metabolic route as quercetin, since there are no conjugating hydroxy groups allowing oxidation across the molecule (Scheme 1). In contrast, 3′-*O*-methyl quercetin has a free 4′-OH group on the B-ring and is able to autoxidize or be subject to the action of peroxidases to form oxidation products such as 3′-*O*-methyl quercetin 5-quinone methide. Thus uptake of this methylated form leads to the progressive formation of an unidentified product consistent with assignment as a 3′-*O*-methyl quercetin quinone methide. However, in contrast with quercetin aglycone, no glutathione adduct was detected (although its formation might be postulated via reaction of the thiol with the quinone form). No metabolism of quercetin 7-*O*-β-D-glucuronide was observed, presumably due to its inability to enter the cells.



Scheme 1 Summary of the cellular metabolism of quercetin and its *O*-methylated metabolites

O-methylated forms of quercetin associate with fibroblasts to a greater extent than does quercetin, whereas the glucuronide (Q-glucuronide) does not enter cells. Quercetin and 3′-*O*-methyl quercetin (3OMeQ) undergo oxidative metabolism within the cell to yield as yet unidentified intermediates, and a small amount of 4′-*O*-methyl quercetin (4OMeQ) is demethylated. Quercetin *o*-quinone (Q *o*-quinone) reacts with GSH to form a 2′-glutathionyl conjugate. Both the GSH conjugate and oxidative products are exported from cells.

As mentioned above, the cellular products, M1 and M2, seem likely to be quinone and quinone methide species respectively that are trapped by protonation in the acidic lysis, extraction and analysis environment. Both M1 and M2 were observed at time points up to 18 h, indicating they are relatively long-lived species. Interestingly, *o*-quinones have been shown to have differing lifetimes. For example, *o*-quinone formed from the catechol oestrogen, 2-hydroxyoestrone, has a half-life of 47 s, whereas the 4-hydroxyoestrone *o*-quinone is considerably longer-lived ($t_{1/2} = 12$ min) [30]. Such types of products may be linked to the toxicity of quercetin and 3′-*O*-methyl quercetin at high concentrations, and indeed many studies have shown that flavonoid catechol moieties can autoxidize *in vitro*, as well as acting as substrates for peroxidases, and other metalloenzymes, yielding quinone or quinone methide pro-oxidant and alkylating agents [27,31,32]. Quinones are Michael acceptors, and can exert pro-oxidant activities after metabolic activation to semiquinone and quinoidal products, with ensuing cellular damage from the alkylation of cellular proteins or DNA [31]. Quercetin is known to be mutagenic [33], presumably in part through the formation of quinone or quinone methide-type metabolites [25,27,29], whereas the physiological form, 4′-*O*-methyl quercetin, would not undergo such reactions and elicit these effects. Because of the inherent nucleophilicity of the thiol group, protein and non-protein thiols represent a major target for quinones, and the detoxification of quinones by GSH is generally considered to be cytoprotective [34–37]. In fact, glutathionyl conjugates from a variety of polyphenol quinones have been observed to display a wide array of biological activities [34]. Indeed, the redox activity of polyphenols is frequently enhanced following conjugation with GSH [34,35] and thus does not necessarily result in detoxification. Only when GSH conjugation is coupled with the subsequent export of the adduct from cells, so-called phase III metabolism [38], will detoxification be the predominant pathway. In the studies described here, export of 2′-glutathionyl quercetin is observed; in addition, when it was exposed extratracellularly, it did not induce cytotoxicity. However, we cannot rule out possible deleterious reactions by intracellular 2′-glutathionyl quercetin, which is detected in cells at all time points.

Although the cytotoxicity of polyphenolic–GSH conjugates is effectively decreased by their active export from the cells in which they are formed, as we observed in our study with 2′-glutathionyl quercetin, once in the extracellular environment and/or circulation they may be transported to tissues that are capable of accumulating these metabolites [34]. For example, glutathionyl conjugates of 3,4-methylenedioxymethamphetamine (MDMA; ecstasy) express strong nephrotoxic effects due to redox cycling reactions at the apical membrane of renal proximal tubular cells [39]. In addition, cytotoxicity may occur as a result of the intracellular formation of these conjugates, possibly due to a lowering of cellular thiol levels [40] or to the binding of quinone intermediates to cysteine residues at the active site of specific enzymes [41]. Similar chemistry is involved in the reaction of other catechols, such as the catecholamines dopamine and

L-dopa. Their conjugation with cysteine or GSH [23,41] can lead to decreases in GSH levels and to the generation of mitochondrial toxins with relevance to Parkinson's disease [42]. The formation of a quercetin–glutathione conjugate in our cell system may provide an insight into the toxic effects of quercetin, and to a lesser extent of its 3′-*O*-methyl metabolite, that were observed at higher concentrations (30 μM; Figure 2). However, it is possible that flavonoids, such as quercetin, may also act beneficially by competing with catecholamines and acting to limit the formation of potent cytotoxic catecholamine–thiol adducts in dopaminergic cells, in a similar way as dihydrolipoic acid has been observed to do [23]. Because of the structural similarity of the flavonoid B-ring and their ability to donate electrons efficiently to form quinones, it is conceivable that specific flavonoids may be of use to prevent neurotoxic compounds such as 5-*S*-cysteinyl dopamine from being formed *in vivo* [43].

There is a limited amount of information on the effects of the metabolites of quercetin on cells [4], although a wealth of data exists in favour of both the protective [11–13] and the cytotoxic [5,6,8,9] nature of the parent flavonoid quercetin. In our studies, concentrations of quercetin up to 10 μM conferred protection against fibroblast damage induced by oxidative stress. This protective effect was replaced by cytotoxicity following pretreatment with 30 μM quercetin, which was presumably due to the deleterious effects observed during a quercetin-only exposure at this concentration. Previous investigations have indicated the potential of quercetin aglycone to induce fibroblast injury [44], which could be associated with disturbances in cell division due to effects on proteins, DNA and membranes. Furthermore, quercetin has been observed to cause DNA damage [45], cell cycle arrest [9,10] and increases in caspase-3 activity [8,46], and lead to the induction of apoptosis in various cell lines, particularly cancer cells [5,7,10]. It has been postulated that the cytotoxicity of quercetin may be mediated by its cellular metabolic activation to a semiquinone and/or quinone, which are known to facilitate the formation of superoxide and the depletion of GSH [31]. On the other hand, quercetin has been observed to confer protection against oxidative stress-induced fibroblast injury [47,48], similar to that observed at concentrations below 10 μM in our present study. The effects of quercetin on cells seem to be dependent both on cell type and in particular on the concentration of quercetin. The concentration of quercetin aglycone *in vivo* is very low due to its extensive metabolism in the small intestine, liver and colon to *O*-methylated and glucuronidated metabolites [15] and phenolic acid derivatives [17].

Both 3′-*O*-methyl and 4′-*O*-methyl quercetin were less efficacious than quercetin against the peroxide challenge, with the 4′-*O*-methyl metabolite effective in a dose-dependent manner up to 30 μM, whereas the 3′-*O*-methyl quercetin caused toxicity above 10 μM. Interestingly, the protective effects expressed by quercetin and its *O*-methylated metabolites were different from those observed with epicatechin and 3′-*O*-methyl epicatechin, which both prevented peroxide-induced toxicity and caspase-3 activation to exactly the same extent in the same cellular model [1,2]. One explanation may be that oxidative metabolism, glutathione conjugation and even demethylation reactions were apparent in the fibroblasts exposed to flavonols, as described here, whereas no such reactions were observed in associated experiments with the flavanols [1]. Demethylation of 4′-*O*-methyl quercetin to yield free quercetin may occur due to the action of cytochrome P450s [49], and could act to aid further metabolism and clearance of the *O*-methylated compound from the cell. The lack of protective or cytotoxic activity of quercetin 7-*O*-β-D-glucuronide is in agreement with similar studies with epicatechin 5-*O*-β-D- and 7-*O*-β-D-glucuronides [1], and is likely to be due to the increased polarity of flavonoid glucuronides hindering their uptake or association with fibroblasts. These observations may point to an inability of quercetin glucuronides to act *in vivo*. However, cleavage to yield free quercetin may occur under local conditions of inflammation where β-glucuronidases are released by neutrophils and macrophages into the extracellular environment. For example, cleavage of luteolin monoglucuronide has been observed in a system where β-glucuronidase is released from neutrophils stimulated with ionomycin [50].

Our data suggest that quercetin and its two *O*-methylated metabolites may provide protection against oxidatively induced cellular damage *in vivo*. Although the *O*-methylated metabolites were less efficacious in our fibroblast model, it should be noted that *in vivo* they would be present at greater concentrations than quercetin aglycone. The precise mechanism of the protective action observed here remains unclear; however, effects may be mediated by the cellular metabolism of these compounds (Scheme 1). The protective properties of the flavonols seemed to be due to the original compounds to which the cells were exposed; however, at higher exposure concentrations, at which quercetin and 3′-*O*-methyl quercetin showed some toxicity towards the cells, this could be mediated by compounds such as M1, M2 and 2′-glutathionyl quercetin that are formed intracellularly. The inability of the glucuronide to exert protection is almost certainly linked to its inability to associate with the cells. Further investigations are necessary to elucidate the precise mechanisms of action of these compounds.

This research was supported by the Biotechnology and Biological Sciences Research Council (grant no. 18/D14751) and by a European Union Fifth Framework RTD Programme Grant (grant no. QLK4-1999-01590).

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Received 20 December 2002/28 January 2003; accepted 11 February 2003
Published as BJ Immediate Publication 11 February 2003, DOI 10.1042/BJ20021972