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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 mitochondriotropic antioxidant decylquinone–TPP ($MitoQ_{10}$) was administered to rats before explanting the hearts and subjecting them to ischemia and reperfusion (I/R).[28] Treatment resulted in a significant reduction of the necrotic area. A similar protective effect was afforded by high doses of intravenous resveratrol in models of cerebral[29] and cardiac[30] ischemia.

Polyphenols can also induce a potentiation, rather than a reduction, 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 maintaining a redox cycle and/or of redox-active enzymes, such as tyrosinases ("polyphenol oxidases") and peroxidases, on the ion-chelating properties of the polyphenols themselves, and on pH. Additional factors could include the concentration of the polyphenol[33] and even the subcellular compartment involved.[34] Depending on such factors and on the particular polyphenol involved, polyphenol–TPP conjugates might act as cytotoxic, apoptosis or necrosis-inducing pro-oxidants.

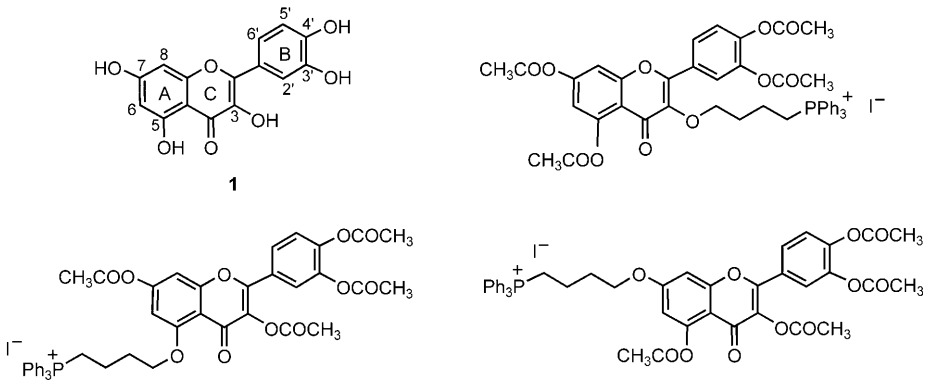
The pro-oxidant mode of action is as potentially useful as the antioxidant one, because it can be exploited to induce the death of unwanted, that is, cancerous, cells. Cancer cells live under oxidative stress,[35] which, in principle, makes them more vulnerable to ROS-mediated damage. Cell death and/or MPT induction by "redox-cycling" compounds, such as menadione or adriamycin, are well known, and antitumour, mitochondrion-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] Mitochondriotropic polyphenols might provide significant oncological benefits also if they turn out to act as antioxidants. Recent studies have shown that mitochondrial ROS production is an important 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 polyphenols, their varied reactivity and the relevance of the pathophysiological processes for which they offer promise, suggest that mitochondriotropic polyphenol derivatives could find clinically relevant applications that are not necessarily limited to circumstances that involve redox processes.

Here we report the proof-of-principle synthesis, characterisation and initial biological assessment of triphenylphosphonium-comprising derivatives of quercetin, a widely used model polyphenol.

Results

Quercetin (**1**) and the potentially useful target mitocondriotropic 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 following considerations: 1) the hydroxyls of quercetin provide convenient sites to connect—through an ether bond—with a linker that bears the TPP group; 2) because the catecholic hydroxyls determine to a large extent the redox properties of quercetin, they should be maintained as such in the target derivative. 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 removed 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 protection 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 nonacetylated 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

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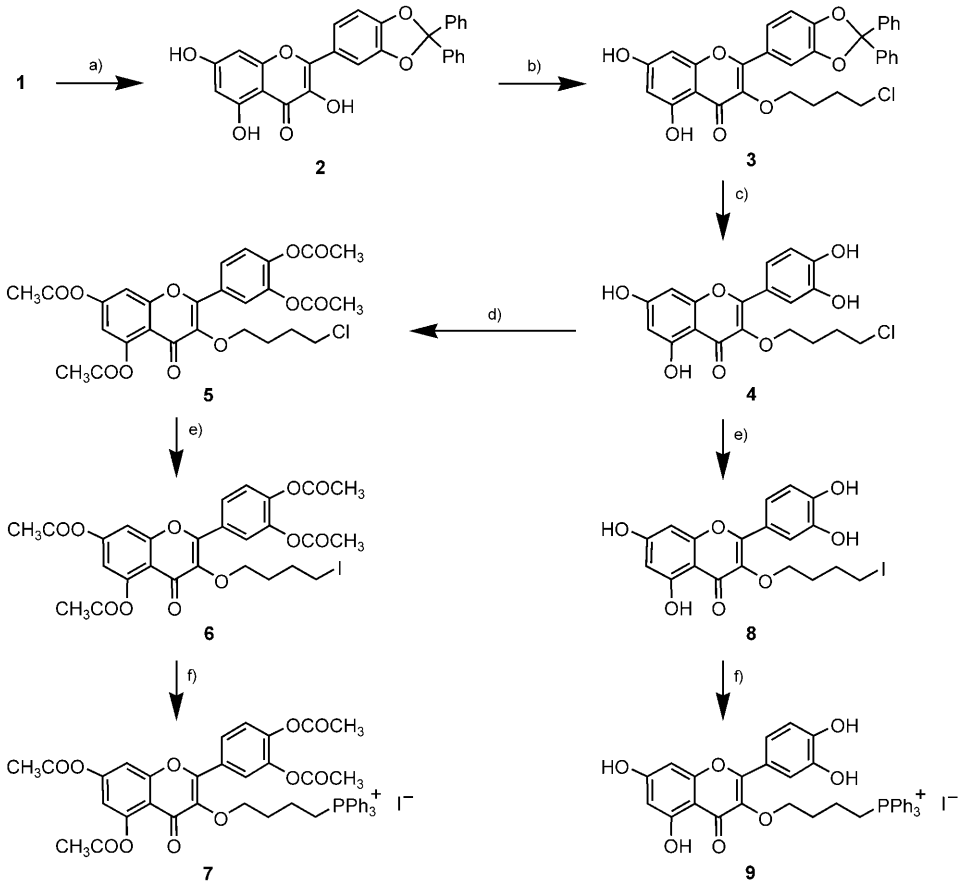

Scheme 2. Synthesis of mitochondriotropic derivatives **7** and **9**. a) Ph_2CCl_2 (3 equiv), 180 °C, 10 min; b) 1-bromo-4-chlorobutane (1.2 equiv), K_2CO_3 (1.3 equiv), DMF, Ar, room temperature, 20 h; c) AcOH/H_2O 8:2, reflux, 2 h; d) $CH_3C(=O)Cl$, pyridine, room temperature, 24 h; e) NaI, acetone,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

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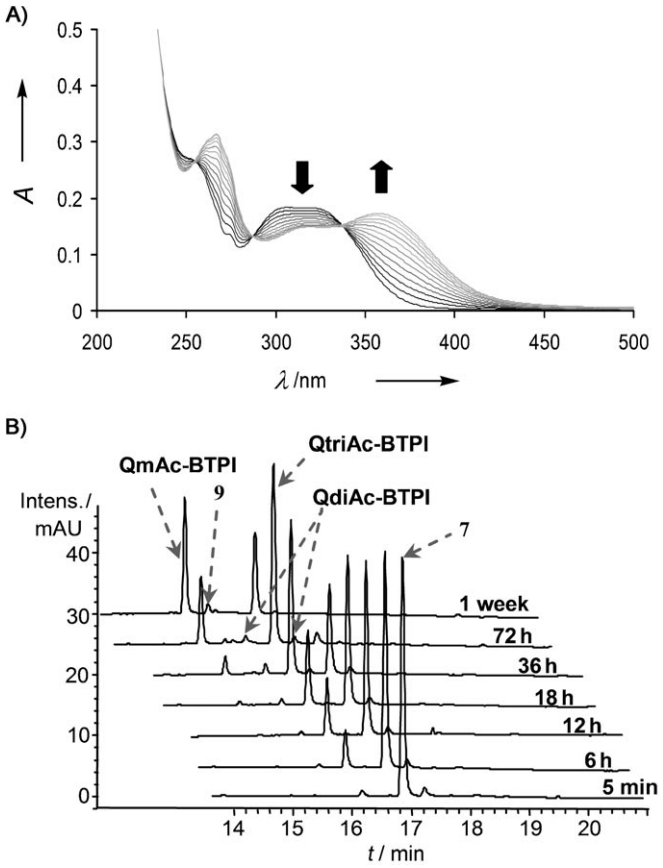

Figure 1. Hydrolysis of **7** in HBSS/CH_3CN (9:1). A) UV/Vis spectra were recorded every 6 h for 72 h. B) HPLC traces were recorded at 300 nm at different reaction times. Abbreviations: QmAc-BTPI: acetyl-3-(4-*O*-triphenylphosphoniumbutyl) quercetin iodide; QdiAc-BTPI: diacetyl-3-(4-*O*-triphenylphosphoniumbutyl) quercetin iodide; QtriAc-BTPI: triacetyl-3-(4-*O*-triphenylphosphoniumbutyl) quercetin iodide.

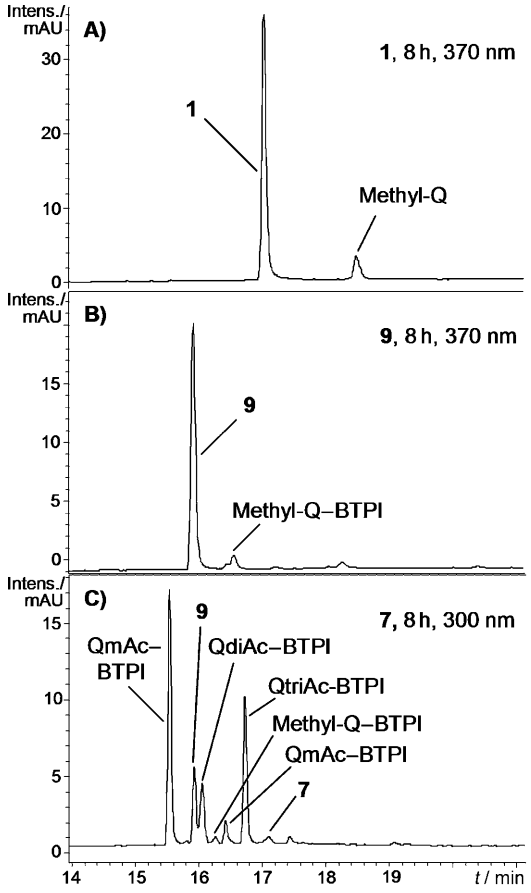

Figure 2. HPLC chromatograms that were recorded at 300 or 370 nm, as indicated, for the extracts obtained after 8 h of incubation of: A) **1**, B) **9** and C) **7** with HCT116 cells. See the Experimental Section for details. Abbreviations as in Figure 1; methyl-Q: methylquercetin; methyl-Q-BTPI: methyl-3-(4-*O*-triphenylphosphoniumbutyl)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

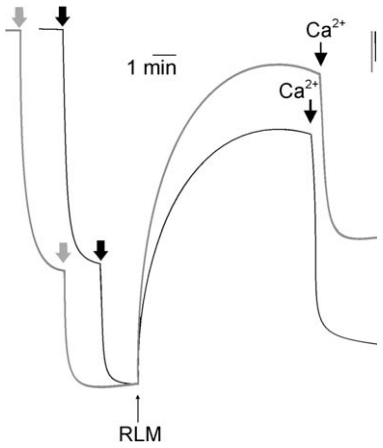

Figure 3. Accumulation of tetraphenylphosphonium (Ph_4P^+; black trace) and **7** (grey trace) by rat liver mitochondria (RLM). Thick arrows indicate the addition of 0.16 μM $Ph_4P^+I^-$ or **7** to the medium (200 mM sucrose, 10 mM HEPES/K^+, 5 mM succinate/K^+, 1 mM NaH_2PO_4, (1.25×10^{-3}) mM rotenone, pH 7.4). Addition of RLM (1 mg protein mL^{-1}) and of $CaCl_2$ (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.

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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).

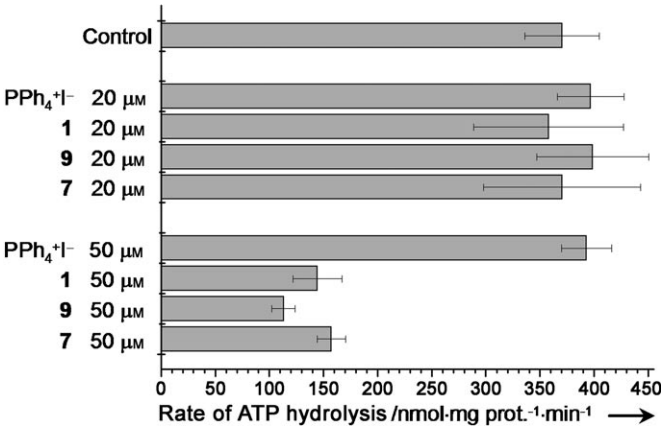

Figure 5. Effects of **7**, **9**, quercetin and tetraphenylphosphonium on the rate of ATP hydrolysis by permeabilised rat liver mitochondria. See the Experimental Section for details. Determinations were carried out in triplicate and averages ±s.d. are reported.

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 $Ph_4P^+I^-$ 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).

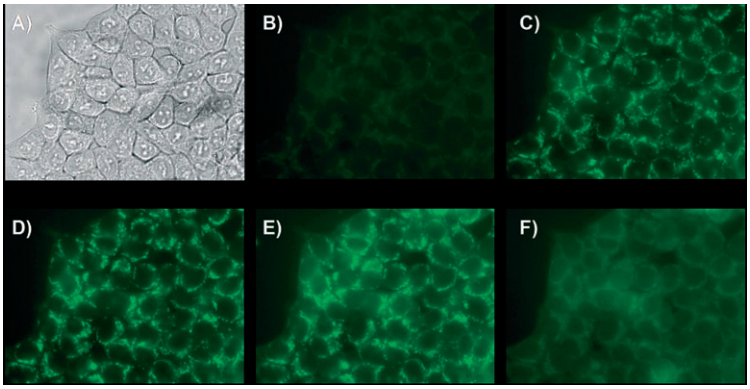

Figure 4. $\Delta\psi_m$-dependent accumulation of compound **7** in the mitochondria of cultured HCT116 cells. A) Phase contrast image taken shortly before the start of the sequential automatic acquisition of 20 fluorescence images that were taken 1 min apart. About 20 s after the first image was recorded, **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 $\Delta\psi_m$-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.

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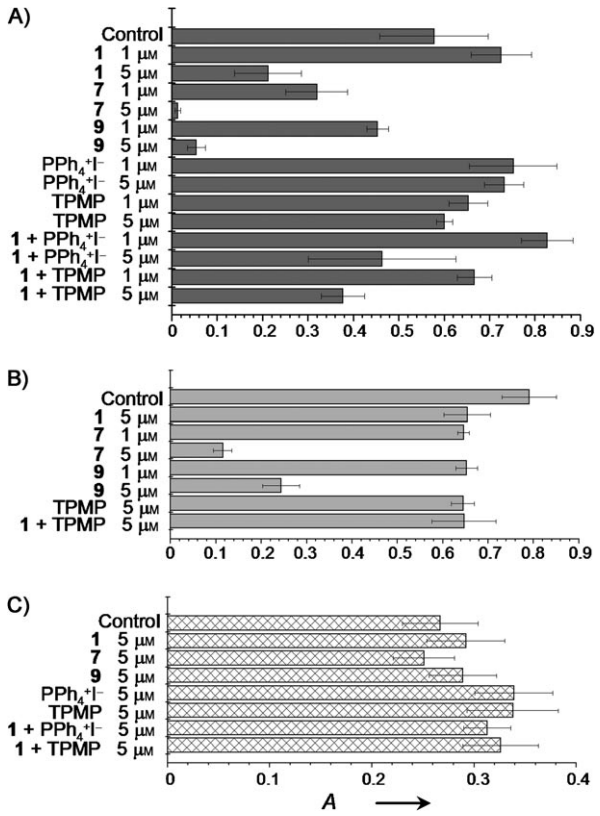

Figure 6. Effect of the mitochondriotropic quercetin derivatives and control compounds on the readout of tetrazolium reduction cell proliferation assays. Cells were allowed to grow for three days in the presence of the specified compounds (see the Experimental Section for details). The panels show the 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

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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): δ = 6.22 (d, J = 2.0 Hz, 1H; Ar), 6.49 (d, J = 2.0 Hz, 1H; Ar), 7.20 (d, J = 8.0 Hz, 1H; H5′), 7.39–7.60 (m, 10H; Ar), 7.78–7.86 (m, 2H; H2′, H6′), 9.68 (br s, 1H; OH), 10.87 (br s, 1H; 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, $CDCl_3$): δ = 1.84 (m, 4H; CH_2), 3.46 (t, 2H; CH_2), 3.94 (t, 2H; CH_2), 6.32 (d, J = 2.0 Hz, 1H; Ar), 6.42 (d, J = 2.0 Hz, 1H; Ar), 6.98 (d, J = 8.25 Hz, 1H; H5′), 7.34–7.44 (m, 5H; Ar), 7.55–7.69 (m, 7H; Ar), 12.60 ppm (s, 5-OH); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): δ = 27.0 (CH_2), 28.9 (CH_2), 45.2 (CH_2Cl), 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_2O (4:1; 50 mL) and the solution was heated at reflux for 2 h. Then H_2O (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): δ = 1.82 (m, 4H; CH_2), 3.66 (t, 2H; CH_2), 3.94 (t, 2H; CH_2), 6.19 (d, J = 1.75 Hz, 1H; Ar), 6.40 (d, J = 1.75 Hz, 1H; Ar), 6.89 (d, J = 8.25 Hz, 1H; H5′), 7.44 (dd, J = 8.25, 2.0 Hz, 1H; H6′), 7.51 (d, J = 2.0 Hz, 1H; H2′,), 12.72 ppm (s, 5-OH); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): δ = 27.0 (CH_2), 28.9 (CH_2), 45.3 (CH_2Cl), 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_2Cl_2 (50 mL) was added, and the organic layer was washed with 0.5 N HCl (3×50 mL) and H_2O (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_2Cl_2/petroleum ether/EtOAc (6:3:1) as eluent to afford the acetylated product **5** in 78% yield. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 1.85 (m, 4H; CH_2), 2.33 (m, 9H; OAc), 2.45 (s, 3H; OAc), 3.56 (t, 2H; CH_2), 3.99 (t, 2H; CH_2), 6.82 (d, J = 2.25 Hz, 1H; Ar), 7.30 (d, J = 2.25 Hz, 1H; Ar), 7.35 (d, J = 8.5 Hz, 1H; H5′), 7.92 (d, J = 2.0 Hz, 1H; H2′), 7.96 ppm (dd, J = 8.5, 2.0 Hz, 1H; H6′); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): δ = 20.5 (CH_3), 20.6 (CH_3), 21.1 (CH_3), 26.9 (CH_2), 28.8 (CH_2), 45.3 (CH_2Cl), 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_2O (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, $CDCl_3$): δ = 1.74–2.00 (m, 4H; CH_2), 2.35 (m, 9H; OAc), 2.47 (m, 3H; OAc), 3.22 (m, 2H; CH_2), 3.99 (m, 2H; CH_2), 6.83 (m, 1H; Ar), 7.32 (m, 2H; Ar), 7.95 ppm (m, 2H; H2′, H6′); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): δ = 8.6 (CH_2I), 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_2Cl_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): δ = 1.62–1.96 (m, 4H; CH_2), 2.17 (s, 3H; OAc), 3.32 (m, 9H; OAc), 3.68 (t, 2H; CH_2), 4.00 (t, 2H; CH_2), 7.10 (d, J = 2.25 Hz, 1H; Ar), 7.34 (d, J = 9.25 Hz, 1H; H5′), 7.62 (d, J = 2.25 Hz, 1H; Ar), 7.72–8.04 ppm (m, 17H; H2′, H6′); ^{13}C NMR (62.9 MHz, [D$_6$]DMSO): δ = 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,

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$[M]^+$; 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. ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 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): δ = 8.7 (CH_2I), 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. ^{1}H NMR (250 MHz, [D$_6$]DMSO): δ = 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): δ = 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 ± 0.009)×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 ± 0.011, from which a concentration of (3.23 ± 0.06)×10^{-5} M was interpolated by using the calibration curve. By correcting for the dilution factor, a solubility of (4.96 ± 0.21)×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_2PO_4 (0.44 mM), Na_2HPO_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

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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_2PO_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 transmembrane 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.

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