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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.

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 → –I → – 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 ^1H 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_3CN (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 cultured 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

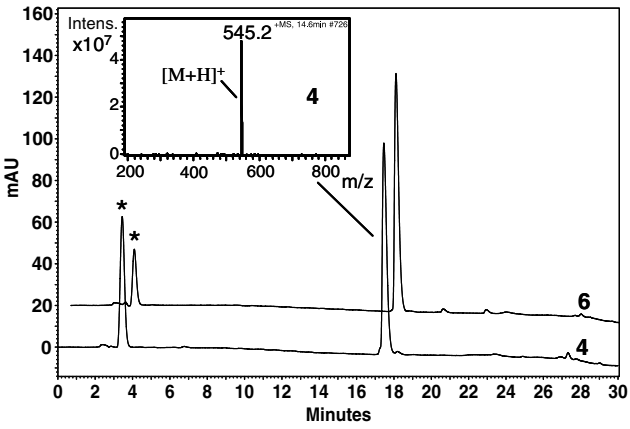
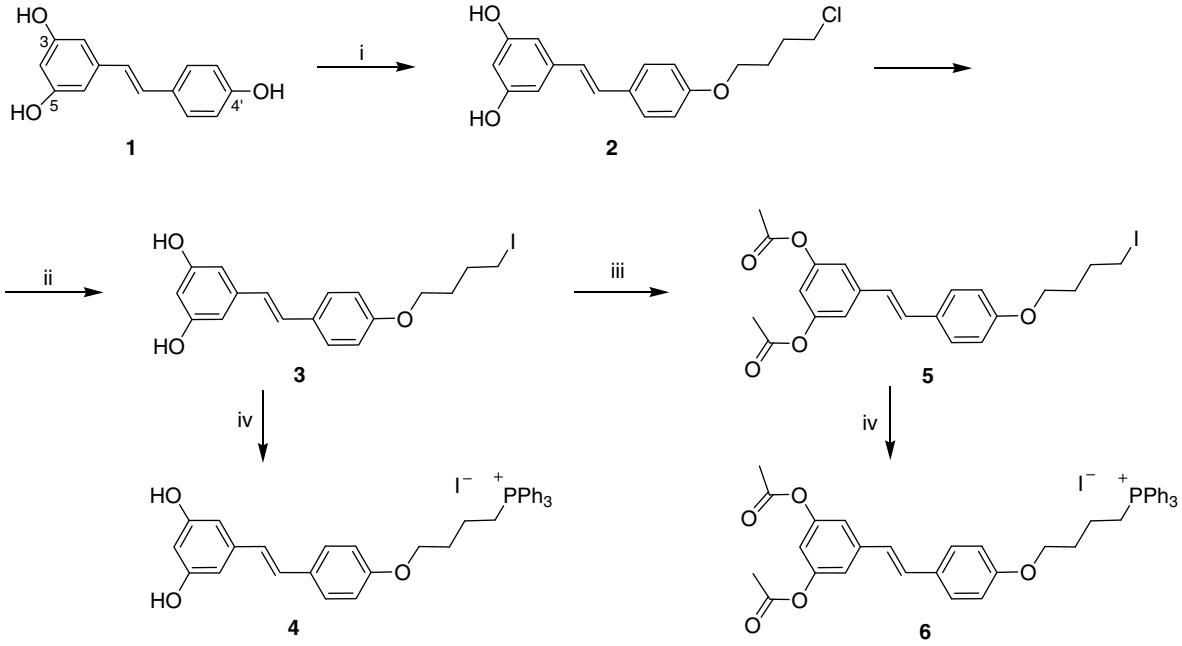


Figure 1. HPLC chromatograms recorded at 320 nm of the extracts obtained after incubation of **4** (lower trace) and **6** (upper trace) with HCT116 cells for 6 h. Inset: positive ESI-MS spectrum of **4**. For clarity, the upper trace was shifted slightly to the right along the time axis: the retention 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.



Scheme 1. Synthesis of mitochondriotropic derivatives **4** and **6**. Reagents and conditions: (i) 1-Bromo-4-chlorobutane (1.5 equiv), K_2CO_3 (1.1 equiv), DMF, Ar, rt, 20 h, yield 33%; (ii) NaI, acetone, reflux, 20 h, yield 89%; (iii) $CH_3C(=O)Cl$ (20 equiv), pyr, CH_2Cl_2, yield 73%; (iv) PPh_3 (5 equiv), toluene, 100 °C, 6 h, yield 78%.