Mitochondria-targeted Resveratrol Derivatives Act as Cytotoxic Pro-oxidants

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Abstract: Resveratrol derivatives bearing an O-linked mitochondria-targeting 4-triphenylphosphoniumbutyl group at either position 3 or position 4 are prooxidant and cytotoxic for cultured cells, selectively killing fast-growing cells when supplied in the low μM range. Resveratrol is essentially without effect under these experimental conditions, while the cytotoxicity of the mitochondriotropic derivatives increases if they are methylated on the remaining hydroxyls. Experiments with Bax−/Bak− cells and a pan-caspase inhibitor show that cell death is mostly of the necrotic type. Cytotoxicity is due to ROS produced upon accumulation of the compounds into mitochondria, and specifically to H2O2, since externally added membrane-permeant catalase largely prevents cell death while superoxide dismutase potentiates toxicity. The mitochondriotropic compounds cause ROS-independent depolarization of in situ mitochondria. Effectiveness is increased if resveratrol hydroxyls are acetylated or methylated; this excludes the involvement of autooxidation of the polyphenolic nucleus and a protonophoric cycle as the causes of ROS generation and of depolarization, respectively. Resveratrol-triphenylphosphonium conjugates may thus represent a new class of chemotherapeutic agents, redox-active “mitocans”, whose mechanisms of action and in vivo activity are worthy of further investigation.

Keywords: Resveratrol, mitochondria, mitocans, cancer, reactive oxygen species.

INTRODUCTION

Modulating mitochondrial redox processes is an attractive challenge. These organelles are deeply involved in key cellular functions, such as ATP production, Ca2+ homeostasis and cell death [2, 3], and are the subcellular compartment in which most Reactive Oxygen Species (ROS) are produced [4]. Enhanced ROS production is the common theme in the pathophysiology of many diseases, including cancer, I/R damage, diabetes, aging, and neurodegeneration [5]. ROS are believed to be a factor in carcinogenesis [6, 7]. The metastatic potential of cell lines is correlated to the level of ROS production by mitochondria [8]. Mitochondrial ROS are involved in the activation of Hypoxia Inducible Factor (HIF) [9], which influences angiogenesis and other aspects of tumor growth [10]. On the other hand, oxidative stress can activate pro-apoptotic signalling involving kinases (i.e., ASK1, JNK, p38, MAPK), transcription factors (i.e., p53, -Jun) and members of the Bel-2 family [5, 11, 12]. Oxidation of cardiolipin is needed for the release of cytochrome c [13]. Furthermore, ROS are known to induce the Mitochondrial Permeability Transition (MPT) [14]; MPT is now believed to have a fundamental role in necrotic death, such as occurs, e.g., upon reoxygenation following ischemia [15, 16]. Hinder ing ROS production may thus limit damage due to ischemia, neurodegeneration or aging, and oppose cancer metastasis, while targeted ROS enhancement may provide a way to eliminate cancer cells.

This has led to the birth of a new sector of pharmacology, which targets mitochondria to prevent or to induce, as the case may be, cell death [17, 18]. The most versatile strategy to target compounds to mitochondria involves coupling to a membrane-permeable lipophilic cation such as triphenylphosphonium which will drive accumulation in compartments held at negative relative voltage, such as the mitochondrial matrix, according to Nernst’s law [19].

These agents may exhibit selectivity towards tumor cells because of the higher mitochondrial potential (ΔΨm) of the latter compared to their non-tumoral counterpart [20, 21]. Cancer cells produce their ATP mostly by glycolysis (the Warburg effect), exhibiting reduced oxidative phosphorylation activity; the mitochondrial electrochemical proton gradient is thus dissipated less efficiently, and the transmembrane potential is increased [22]. Moreover, mitochondrial membrane potential seems to be correlated to tumor aggressiveness. Cells with higher ΔΨm have higher levels of Vascular Endothelial Growth Factor (VEGF) and matrix metalloproteinase 7 (MMP7), and increased invasive behavior compared to cancer cells with lower ΔΨm [23].

Polyphenols are plant-derived redox-active compounds with potentially useful biological properties, including the modulation of mitochondrial biogenesis and functionality via AMPK [5]. They can interact with mitochondrial components and modulate redox processes. To enhance their therapeutic potential, we are developing derivatives targeted to mitochondria by conjugation with triphenylphosphonium [24, 25]. Quercetin and resveratrol were chosen as model compounds. This study utilizes derivatives of resveratrol.

Several publications have reported that resveratrol at high dosages (e.g. 100 μM) can be cytotoxic for cancer cells (e.g.: [26]; rev.: [27]), “Intrinsic” apoptosis is generally reported to occur (e.g. [28]). Mitochondrial depolarization (e.g. [26, 28, 29]) and ROS production (e.g. [26, 29]) are observed. Cytotoxicity appears to be a dose-dependent effect; at lower concentrations, resveratrol may instead have a protective action on mitochondria (revs.: [5, 30]). These results and considerations suggest that targeting resveratrol to mitochondria and increasing its concentration in the organelles may enhance its cytotoxic activity and may possibly lead to oncological applications. In fact, mitochondria-targeted liposomes containing resveratrol have provided encouraging results [31].

We previously showed that a mitochondriotropic derivative of quercetin, 7-O-(4-triphenylphosphoniumbutyl)quercetin iodide (Q-7BTP), in the low-μM range, displays selective cytotoxicity vs. cancerous and fast-growing cells in vitro. Cytotoxicity is due to hydrogen peroxide (H2O2) produced upon its accumulation into mitochondria. Effectiveness is increased if resveratrol hydroxyls are acetylated or methylated; this excludes the involvement of autooxidation of the polyphenolic nucleus and a protonophoric cycle as the causes of ROS generation and of depolarization, respectively. Resveratrol-triphenylphosphonium conjugates may thus represent a new class of chemotherapeutic agents, redox-active “mitocans”, whose mechanisms of action and in vivo activity are worthy of further investigation.

Keywords: Resveratrol, mitochondria, mitocans, cancer, reactive oxygen species.
mitochondria [32]. The mechanism of ROS generation appears to be a chain “autooxidation” mechanism, which requires that at least part of the hydroxyl groups in the molecule should be free. Preliminary work showed that also two mitochondriotropic derivatives of resveratrol - 4′-O-(4-triphenylphosphoniumbutyloxy) resveratrol iodide (R-4′BTPI) and its diacetylated analog 3,5-diacetyl-4′-O-(4-triphenylphosphoniumbutyloxy) resveratrol iodide (RDA-4′BTPI) - are selectively cytotoxic at low μM concentrations [25]. We report here a study of structure-activity on mitochondriotropic resveratrol derivatives, as well as on the basic mechanisms underlying their cytotoxic behavior.

MATERIALS AND METHODS

Experiments were performed at least in triplicate, and averages ± s.d are reported.

Materials. Resveratrol was purchased from Waseta Int. Trading Co (Shangai, P.R.China). Other chemicals were purchased from Sigma-Aldrich (Milan) unless otherwise specified. All chemicals for buffer preparation were of laboratory grade, obtained from Merck, J.T. Baker or Sigma.

O-(4-chlorobutyloxy) resveratrol was the starting reagent for the synthesis of all mitochondriotropic derivatives described in this work. The alklylation reaction was performed as described in [25], and yielded a mixture of 24-hour cells and resuspended in HBSS at a density of 3 × 10^6 cells/mL and loaded with 1 μM MitoSOX™ Red in the presence of Cyclosporin A (CsA; 5 μM) (37°C, 20 min). After loading, cells were diluted 1:5 in HBSS (plus CsA) and divided into identical aliquots. At time zero the various compounds were added and the value measured immediately after addition of the compounds (time = 0).

Mitochondrial potential assays. Tetramethylrhodamine methyl ester (MRM; Invitrogen/Molecular Probes) staining was used to monitor mitochondrial transmembrane potential of Jurkat lymphocytes by FACS. Cells were loaded with 20 nM MRM for 20 min, in the presence of CsA. Procedures were then the same as described above for superoxide production assays.

Annexin/PI labelling assays. For the evaluation of cell death, Jurkat cells were labelled with Annexin V-FLUOS (Roche) and Propidium Iodide (PI), and analyzed by FACS. Briefly, cells were washed, resuspended in HBSS at a density of 3 × 10^6 cells/mL, and subsequently treated with compounds for 3 hours at 37°C, 5% CO₂. A 200 μl aliquot of each sample was then incubated with PI (final concentration 1 μg/mL) and Annexin V-FLUOS (1 μL/sample) in Annexin V-FLUOS (1 mL/sample) in the dark at 37°C, 15 min. Samples were finally analyzed by FACS (5000 cells for each measurement). Results are presented either as percentage of cells labelled by either Annexin V-FLUOS, PI, or both (Fig 2), or alternatively as percentage of cell not labelled by either reagent (Fig 4). In this latter case the percentage of unlabelled cells in the control sample of each experiment is set at 100%.

Stability. To assess the stability of the derivatives under the conditions used in FACS experiments, Jurkat cells were washed and resuspended in HBSS at a density of 3 × 10^6 cells/mL. 1 mL aliquots were transferred to a 24-well plate, and incubated with the various mitochondriotropic resveratrol derivatives (5 μM, 0.1% final DMSO) for 0.5, 1, 2 and 3 hours. At the end of the experiment, cells and medium were collected together for each experimental condition; 10 μL Acetic acid (6 M), 10 μL Ascorbic acid (100 mM) and 1 mL acetone were added. The mixture was sonicated 2 min, and then centrifuged 8 min, 10000 g; supernatant was collected and stored at -20°C. Samples (2 μl) were analyzed by HPLC (Agilent 1290 Infinity LC System) using a reversed phase column (Zorbax Extend C18, 3.5 μm, 50 × 2.1 mm i.d.) and a UV diode array detector. Solvents A and B were water containing 0.1% trifluoroacetic acid (TFA) and acetonitrile, respectively. The gradient for B was as follows: 30% for 1 min, from 30% to 65% in 3 min, then from 65% to 100% in 1 min; the flow rate was 0.8 mL/min. The eluate was preferentially monitored at 300 and 320 nm.

Fluorescence microscopy. The weak intrinsic fluorescence emission (exciting at 300-340 nm) of the derivatives was exploited to follow their localization in cultured cells. Slow-growing MEF cells were seeded (75000 cells/well) onto 24-mm coverslips in 6-well plates and grown for about two days in DMEM + 10%FBS. Coverslips were then mounted onto holders, washed twice with HBSS, and covered with 1 ml of DMEM without FBS and Phenol Red and supplemented with 2 μM CsA. An Olympus Biosystems
apparatus comprising an Olympus IX71 microscope and MT20 light source was used; images were acquired automatically at 1-min intervals and processed with CellR software. Excitation was at 340 ± 15 nm and fluorescence was collected at λ > 400 nm. Additions were performed by withdrawing 0.5 ml of incubation medium, and adding back the solution into the chamber at a peripheral point. All images are presented using the same display parameters, and thus fluorescence intensities can be compared.

RESULTS

The redox properties of a polyphenol may be altered by the introduction of a substituent, and they may depend on the position occupied [33]. We thus synthesized and tested both mitochondriotropic resveratrol isomers, bearing the 4-triphenylphosphoniumbutyl (BTPI) group at either position -3 or -4’. To study the role of free hydroxyls, which were proven to be essential for the effectiveness of the quercetin mitochondriotropic derivative Q-7BTPI [32], we also synthesized their analogs with the remaining hydroxyls blocked by acetyl or methyl groups, respectively (Scheme 1). While the acetylated compounds can undergo hydrolysis with regeneration of the free hydroxyls (see below), the methyl ether bond is stable under all our experimental conditions.

The six compounds were tested for their cytotoxic/cytostatic action on three cultured cell lines: CT-26 (a murine colon cancer cell line), and fast- or slow-growing MEFs (non-tumoral cell lines). The tetrazolium salt reduction (MTT) assay was used to quantify cell growth and viability. Figure 1 illustrates the results. Confirming the observations by [25], the mitochondriotropic resveratrol derivatives with free or acetylated hydroxyls turned out to be cytotoxic for tumoral (CT-26; Fig. 1A) and fast-growing MEF cells (Fig. 1B), with a much milder effect on slow-growing MEF cells (Fig. 1C). The effect was dose-dependent.

Methylated mitochondriotropic derivatives proved to be more toxic than the others, giving similar experimental readouts at a 5-fold lower concentration (1 μM vs. 5 μM, respectively). All compounds displayed selectivity, killing cancerous CT-26 and fast-growing MEFs more efficiently than slow-growing MEFs. The isomeric structure of the compounds did not make a major difference. Furthermore, the effects of the acetylated derivatives were similar to those of R-3BTPI and R-4’BTPI. This latter observation may be considered to reflect the experimental conditions of these assays, which include a 72 h incubation period and the necessary use of FBS-supplemented culture medium. Serum albumin is known to have an intrinsic esterase activity [34], and the incubation of RDA-BTPIs with cells in the presence of FBS led to complete hydrolysis to R-BTPIs within 2 h (not shown), so that the effects observed are essentially those of R-3BTPI and R-4’BTPI. These experiments therefore did not actually allow a comparison of the effects of non-acetylated and acetylated derivatives. Resveratrol and a methyl-triphenylphosphonium (TPMP) salt at the same concentrations did not have any impact on the cells.

Fig. (1). Effects of resveratrol derivatives on the readout of MTT assays. Cells were allowed to grow for 3 days in the presence of the specified compounds (see Materials and Methods for details). A) CT-26 mouse colon tumor cells; B) Fast-growing Mouse Embryonic Fibroblasts (MEF); C) Slow-growing MEF. RESV = Resveratrol.
We studied cell death also in FACS experiments, staining with Annexin V-FLUOS and PI after a 3h incubation with the compounds. We used Jurkat lymphocytes because these cells grow in suspension, and thus possible side effects of traumatic detachment were avoided. Also using this experimental approach all six compounds proved to be cytotoxic, the methylated ones being the most effective (Fig. 2): in these experiments the RDM-BTPIs were used at 1 µM, while R-BTPIs and RDA-BTPIs were used at 5 µM. These concentrations are high enough to have clear cytotoxic effects in MTT and Annexin/PI labelling assays, but do not cause major changes in forward- and side-scatter parameters during the course of the FACS experiment.

We verified whether oxidative stress and autooxidation might be the cause of the cytotoxicity of mitochondriotropic resveratrol derivatives, as is the case for Q-7BTPI [32]. Mitochondrial superoxide production was monitored in FACS experiments with Jurkat cells, using the mitochondria-targeted fluorescent indicator MitoSOX™ Red. The selectivity of MitoSOX™ Red for superoxide was confirmed using PEG-SOD (40 U/ml), a membrane-permeant form of SuperOxide Dismutase, which markedly suppressed the fluorescence increase (not shown). Cyclosporine A (CsA) was routinely provided with MitoSOX™ Red to block MDR pumps, thus increasing dye load. Its replacement by CsH, which inhibits MDR pumps but not the MPT, had no influence on the MitoSOX™ Red response (not shown). To exclude possible contributions of processes associated with cell death to ROS production, only vital cells were selected for analysis on the basis of forward- and side-scatter parameters. The results are presented in (Fig. 3). All compounds induced superoxide generation, RDA-4’BTPI and methylated derivatives being the most effective. Acetyl groups were only partially hydrolyzed under the conditions of FACS experiments in the presence of cells (Supplementary Fig. 1; about 10 or 40% of RDA-3BTPI or RDA-4’BTPI was completely hydrolyzed to R-3BTPI or R-4’BTPI, respectively, over 2h). Methylated derivatives were completely stable in the same experimental conditions. Resveratrol, a TPMP salt or a combination of them did not affect the MitoSOX™ Red response (Supplementary Fig. 2).

We verified whether ROS generation was the cause of cell death. In FACS experiments with Annexin/PI staining of Jurkat cells, PEG-SOD did not have any protective effect, but actually increased staining, while PEG-CAT was able to rescue cells from death (Fig. 2). We can thus conclude that hydrogen peroxide is the toxic specie.

ROS can induce both necrotic or apoptotic cell death; quantitative aspects and concurrent cellular signalling are very relevant for the outcome. MTT assays with double knock-out cells lacking both key pro-apoptotic proteins Bax and Bak, and FACS experiments in the presence of the pan-caspase inhibitor z-VAD-fmk showed that the derivatives bearing the mitochondria-targeting group at position 3 and RDM-4’BTPI mainly caused necrotic cell death, since the lack of Bax and Bak or the presence of z-VAD-fmk had little effect on the readout. By the same criteria, R-4’BTPI and RDA-4’BTPI additionally induced apoptosis in a fraction of the cells (Fig. 4).

Resveratrol at high (e.g. 100 µM) concentrations has been reported to induce mitochondrial depolarization [26, 28, 29]. Mitochondrial potential was therefore monitored in FACS experiments with Jurkat cells labelled with TMRM. TMRM fluorescence decrease was in fact observed. RDM-BTPIs and RDA-BTPIs were more effective (Fig. 5B, C) than their counterparts with free hydroxyls (Fig. 5A).

Mitochondriotropic quercetin derivatives cause a loss of TMRM fluorescence by competing for the binding sites of the dye on membranes, independently of effects on the transmembrane potential [32]. This complication can be eliminated by performing the measurements in the presence of tetramethyl-7-O-(4-triphenylphosphoniumbutyl) quercetin iodide (QTMM-7BTPI), which is inert and at 20 µM saturates the binding sites involved [32]. When we included QTMM-7BTPI in the assays with the resveratrol derivatives, loss of TMRM fluorescence in excess of that determined by QTMM-7BTPI was still evident (particularly in the case of the acetylated and methylated compounds), confirming that in this case loss
Fig. (4). Cell death is mainly necrotic. A) Cytotoxic effect (MTT assay readout) of mitochondriotropic resveratrol derivatives on cells expressing or not the pro-apoptotic proteins Bax and Bak. Wild type or Bax -/-/Bak-/- (DKO) MEF cells were treated with the indicated derivatives for 72 h (see Materials and methods for details). B) Effects of the pan-caspase inhibitor z-VAD-fmk 100 /g1M on Annexin/PI labelling of Jurkat cells treated for 3h with the indicated compounds. Staurosporine is used as a positive control for apoptosis. For coherence with panel A, the percentage of cells not labelled by either Annexin or PI is shown, with the percentage of unlabelled cells in the control sample of each experiment set as 100%.

of transmembrane potential is in fact taking place (Supplementary Fig. 3). Depolarization was also observed exploiting the intrinsic fluorescence of our derivatives (especially RDM-BTPIs) upon excitation at 340 nm. As can be seen in (Fig. 6), addition of 1 /g1M RDM-4'BTPI to cultured cells caused a progressive increase of fluorescence in intracellular structures (compare panel B to panel A), which corresponds to a voltage-dependent accumulation of the compound into mitochondria, since addition of a transmembrane potential-dissipating protonophore (carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)) causes a rapid redistribution of fluorescence due to efflux of the derivative (not shown). Even if no uncoupler was added, a spontaneous loss of fluorescence from the mitochondria developed after several minutes (compare panels B and C), with little visible effect by a subsequent addition of FCCP (compare panels C and D).

The demonstration that resveratrol derivatives induce mitochondrial depolarization has implications for the quantitative evaluation of superoxide production, since MitoSOX™ Red is a mitochondriotropic probe; fluorescence increase upon superoxide generation may be underestimated when mitochondria are uncoupled, since MitoSOX™ Red is expected to be released from the matrix.

To evaluate if the depolarization might be induced by H₂O₂, we compared the results in the presence or in the absence of a high concentration of PEG-CAT, and found that the enzyme did not affect the TMRM fluorescence decrease (Fig. 5). CsA, which decreases the probability of MPT pore opening, was also without a clear effect on TMRM fluorescence redistribution.

Fig. (5). Mitochondriotropic resveratrol derivatives cause a decrease of fluorescence of TMRM-labelled cells, which is not due to hydrogen peroxide. TMRM fluorescence was measured in FACS experiments using Jurkat cells treated with: A) 5 /g1M non-acetylated, or B) 5 /g1M acetylated, or C) 1 /g1M methylated mitochondriotropic derivatives. Treatments were performed in the absence or presence of PEG-CAT (500 U/ml).

DISCUSSION

The mitochondriotropic resveratrol derivatives we have studied are cytotoxic in vitro when used in the low /g1M range, showing a vast increase in effectiveness over unmodified resveratrol, presumably reflecting their electrophoretic accumulation. They are
“nucleus” is not the source of superoxide. The most promising current candidates, compounds which cause the death of cancer cells via oxidative stress at the mitochondrial level, are a recognized mitogenic factor: rapidly proliferating cells have higher levels than quiescent ones [10,45] and cancerous cells in particular are characterized by an altered redox homeostasis and by an accentuated oxidative stress (e.g.: [46-50]). On the other hand a vast consensus exists that ROS can induce apoptosis [51] and, at higher levels, necrosis [52,53]. Activation of phosphorylation cascades is the key feature of apoptotic induction by ROS [12]. Necrosis is now understood to comprise a set of interacting signaling cascades and biochemical phenomena forming a continuum with other forms of cell death like apoptosis or autophagic death and comprising a variety of subtypes such as, e.g., “necroptosis”, “parthanatos”, “autoschizis”, etc. [54]. ROS, along with Ca²⁺ dysregulation, are a key factor in inducing the mitochondrial permeability transition and/or lysosomal rupture, characteristic features of necrotic death. Thus, despite their enhanced redox defences, an additional oxidative stress may push cancerous cells over the brink of death more easily than normal cells (e.g. [47-49,55]). In this context, it is not surprising that cell death, mostly of a necrotic character under our conditions, may also include an apoptotic component in some cases (Fig. 4).

Besides oxidative stress, our derivatives also cause loss of TMRE fluorescence from (the mitochondria of) treated cells (Fig. 5). Even though depolarization was found to be CsA-insensitive, an involvement of the MPT cannot be ruled out. Permeabilization of the inner membrane may be the common outcome of distinct processes, regulation of this phenomenon is complex, and CsA is far from being a fail-safe inhibitor [58]. For example, its effects have been found to depend on Pi [59]. Cyclophilin D-dependent and –

α-TOS and mitoVES induce the generation of ROS via interaction with succinate dehydrogenase, suggesting that our compounds may also act as inducers of an “electron leak” from the respiratory chain. Resveratrol has been reported to inhibit respiratory chain complexes I and III [41]. Our mitochondrial tropic derivatives may be expected to interact with these complexes as well, and the interaction might well lead to excess ROS generation, since complexes I and III represent the two major sites of superoxide production in mitochondria (e.g. [4]).

The conclusion that mitochondrial-level oxidative stress determines cell death provides a tentative explanation – in addition to that involving the mitochondrial transmembrane potential, mentioned in the introduction - for the selectivity of death induction in certain cell types. ROS, in particular H₂O₂, act as intracellular messengers, with effects that depend on the cellular context and on the quantitative and temporal aspects of their production (e.g. [42-44]). They are a recognized mitogenic factor: rapidly proliferating cells have higher levels than quiescent ones [10,45] and cancerous cells in particular are characterized by an altered redox homeostasis and by an accentuated oxidative stress (e.g.: [46-50]). On the other hand a vast consensus exists that ROS can induce apoptosis [51] and, at higher levels, necrosis [52,53]. Activation of phosphorylation cascades is the key feature of apoptotic induction by ROS [12]. Necrosis is now understood to comprise a set of interacting signaling cascades and biochemical phenomena forming a continuum with other forms of cell death like apoptosis or autophagic death and comprising a variety of subtypes such as, e.g., “necroptosis”, “parthanatos”, “autoschizis”, etc. [54]. ROS, along with Ca²⁺ dysregulation, are a key factor in inducing the mitochondrial permeability transition and/or lysosomal rupture, characteristic features of necrotic death. Thus, despite their enhanced redox defences, an additional oxidative stress may push cancerous cells over the brink of death more easily than normal cells (e.g. [47-49,55]). In this context, it is not surprising that cell death, mostly of a necrotic character under our conditions, may also include an apoptotic component in some cases (Fig. 4).

Besides oxidative stress, our derivatives also cause loss of TMRE fluorescence from (the mitochondria of) treated cells (Fig. 5). Since it is unaffected by PEG-SOD (not shown) or PEG-CAT (Fig. 5) this uncoupling is not downstream of ROS. It cannot be attributed to protonophoric cycling of the compounds between the cytoplasm and the mitochondrial matrix [56], since the acetylated and methylated derivatives, which have no protons to carry, are much more effective than their analogs R-3BTPI and R-4’BTPI. Resveratrol’s phenolic hydroxyls, furthermore, would be only slightly deprotonated at physiological pH values (pKa: 8.8; [57]). Even though depolarization was found to be CsA-insensitive, an involvement of the MPT cannot be ruled out. Permeabilization of the inner membrane may be the common outcome of distinct processes, regulation of this phenomenon is complex, and CsA is far from being a fail-safe inhibitor [58]. For example, its effects have been found to depend on Pi [59]. Cyclophilin D-dependent and –

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Fig. (6). RDM-4’BTPI induces mitochondrial depolarization. Representative fluorescence microscope images of cultured slow-growing MEF cells A) just before addition of 1 μM RDM-4’BTPI; B) 15 min and C) 25 min after addition of RDM-4’BTPI; D) 2 min after addition of 2 μM FCCP. Fluorescence is rendered on the greyscale for clarity.

furthermore selective, acting preferentially cancerous and fast-growing cells. Death induction is observed both in long-term (three days) MTT viability assays (Fig. 1) and in short-term Annexin/VI labelling experiments (Fig. 2). Our data indicate that cytotoxicity and the upstream effects (ROS generation, depolarization) are specific of the molecules formed by the covalent linkage of the resveratrol moiety and of the mitochondria-targeting triphenylphosphonium cation, since a 1:1 mixture of the two has little effect at the concentrations used (Fig. 1). Concentrations required for resveratrol-induced toxicity (50-100 μM; [26-29]) can hardly be reached or maintained in vitro, due to the poor bioavailability of this natural compound [35,36]. Mitochondria targeting allows high local concentrations of the derivatives to be reached, and thus may represent a strategy to exploit the toxic/chemotherapeutic properties of resveratrol. In vivo behavior of these new candidate chemotherapeutic agents remains to be explored.

It is furthermore clear that the cytotoxic agent is H₂O₂ produced by dismutation of superoxide, since the presence of PEG-CAT rescues the cells (Fig. 2). This prooxidant and cytotoxic effect is observed in vitro with the mitochondriotropic derivatives used in the low μM range. Concentrations in this range favor prooxidant behavior also for mitochondria-targeted quinones such as MitoQ or SkQ1 which act as antioxidants at lower levels [37,38]. While the possibility that mitochondriotropic polyphenols may act as protective antioxidants when used at very low concentrations remains to be explored, these compounds join the ranks of redox-active “mitochondriotropic derivatives are not readily oxidizable at physio-

Cyclic voltammetry experiments showed that resveratrol and its methylated derivatives, which have no protons to carry, are much more effective than their analogs R-3BTPI and R-4’BTPI. Resveratrol’s phenolic hydroxyls, furthermore, would be only slightly deprotonated at physiological pH values (pKa: 8.8; [57]). Even though depolarization was found to be CsA-insensitive, an involvement of the MPT cannot be ruled out. Permeabilization of the inner membrane may be the common outcome of distinct processes, regulation of this phenomenon is complex, and CsA is far from being a fail-safe inhibitor [58]. For example, its effects have been found to depend on Pi [59]. Cyclophilin D-dependent and –
independent mechanisms of pore activation may exist [60], and expression levels of Cyp D may vary depending on cell type, reflecting on the sensitivity of the MPT to CsA [61].

A fuller understanding of cytotoxicity thus requires an understanding of the mechanisms underlying ROS production and depolarization. An investigation of these aspects is under way. In vivo studies on mice tumor models will determine if these resveratrol mitochondriotropic derivatives may indeed qualify as candidates for a new class of chemotherapeutic agents.

CONFLICT OF INTEREST
The authors confirm that this article content has no conflicts of interest.

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