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Cytotoxicity of mitochondria-targeted resveratrol derivatives: Interactions with respiratory chain complexes and ATP synthase

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ABSTRACT

We recently reported that mitochondria-targeted derivatives of resveratrol are cytotoxic in vitro, selectively inducing mostly necrotic death of fast-growing and tumoral cells when supplied in the low μM range (N. Sassi et al., Curr. Pharm. Des. 2014). Cytotoxicity is due to H_2O_2 produced upon accumulation of the compounds into mitochondria. We investigate here the mechanisms underlying ROS generation and mitochondrial depolarization caused by these agents. We find that they interact with the respiratory chain, especially complexes I and III, causing superoxide production. "Capping" free hydroxyls with acetyl or methyl groups increases their effectiveness as respiratory chain inhibitors, promoters of ROS generation and cytotoxic agents. Exposure to the compounds also induces an increase in the occurrence of short transient [Ca^{2+}] "spikes" in the cells. This increase is unrelated to ROS production, and it is not the cause of cell death. These molecules furthermore inhibit the F_0F_1 ATPase. When added to oligomycin-treated cells, the acetylated/methylated ones cause a recovery of the cellular oxygen consumption rates depressed by oligomycin. Since a protonophoric futile cycle which might account for the uncoupling effect is impossible, we speculate that the compounds may cause the transformation of the ATP synthase and/or respiratory chain complex(es) into a conduit for uncoupled proton translocation. Only in the presence of excess oligomycin the most effective derivatives appear to induce the mitochondrial permeability transition (MPT) within the cells. This may be considered to provide circumstantial support for the idea that the ATP synthase is the molecular substrate for the MPT pore.

1. Introduction

Cancer cells have an altered redox homeostasis [1–5] and hyperpolarized mitochondria [6–9]. This latter characteristic is a consequence of the shift from mitochondrial respiration to aerobic glycolytic ATP synthesis (the so-called Warburg effect), which leads to reduced oxidative phosphorylation activity, less efficient dissipation of the mitochondrial electrochemical proton gradient, and thus to an increased transmembrane potential [6,8]. ROS-generating agents targeted to mitochondria thanks to conjugation with lipophilic cations (which accumulate in the mitochondrial matrix according to the Nerst's law) have thus been developed, and are emerging as a promising class of mitocans, i.e., anticancer agents acting on mitochondria and destabilizing them [10,11]. Mitochondrial hyperpolarization leads to preferential accumulation of the pro-oxidant agents in cancer cells. Furthermore, the additional oxidative stress they cause may push cancerous cells over the brink of death more easily than normal cells [2–5]. A recent paper has indeed shown that healthy primary B cells can be sensitized to certain pro-apoptotic drugs by induction of a mild mitochondrial oxidative stress with a mitochondriotropic quercetin derivative. Conversely, cancerous

Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; BSA, bovine serum albumin; BTPI, 4-triphenylphosphoniumbutyl; CoQ, coenzyme Q; CsA, cyclosporin A; DCPI, 2,6-dichlorophenolindophenol; $\Delta\psi_m$, mitochondrial membrane potential; DMEM, Dulbecco's Modified Eagle Medium; $\Delta\tilde{\mu}_H$, proton electrochemical gradient; ECAR, extracellular acidification rate; FACS, fluorescence-activated cell scanner; FBS, fetal bovine serum; FCCP, carbonyl cyanide p-trifluoromethoxy-phenylhydrazone; HBSS, Hank's Balanced Salt Solution; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein-diacetate; IMM, inner mitochondrial membrane; LDH, lactate dehydrogenase; MPT, mitochondrial permeability transition; OCR, oxygen consumption rate; OL, oligomycin; PEG-SOD, polyethyleneglycol-superoxide dismutase; PEG-CAT, polyethyleneglycol-catalase; PEP, phosphoenolpyruvate; PK, pyruvate kinase; R-4′BTPI, 4′-(4-triphenylphosphoniumbutyl) resveratrol iodide; R-3BTPI, 3-(4-triphenylphosphoniumbutyl) resveratrol iodide; RDA-4′BTPI, 3,5-diacetyl-4′-(4-triphenylphosphoniumbutyl) resveratrol iodide; RDA-3BTPI, 4′,5-diacetyl-3-(4-triphenylphosphoniumbutyl) resveratrol iodide; RDM-4′BTPI, 3,5-dimethyl-4′-(4-triphenylphosphoniumbutyl) resveratrol iodide; RDM-3BTPI, 4′,5-dimethyl-3-(4-triphenylphosphoniumbutyl) resveratrol iodide; RLM, rat liver mitochondria; ROI, regions of interest; ROS, reactive oxygen species; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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B-CLL cells lose their peculiar sensitivity to the same drugs if treated with permeant ROS scavengers PEG-SOD and PEG-CAT [5].

We previously reported the synthesis and in vitro activity of a group of resveratrol derivatives targeted to mitochondria via a 4-triphenylphosphoniumbutyl (BTPI) group O-linked at either position -3 or -4′ [12,13]. These derivatives, in the low-μM range, induced necrotic cell death, selectively killing cancerous and fast-growing cells in vitro [13]. Resveratrol itself has previously been shown to be cytotoxic, but only at much higher doses (tens of μM) [14]. Mitochondrial targeting thus appears to enhance the cytotoxicity exhibited by resveratrol itself. Cytotoxicity was found to be due to H_2O_2 produced upon accumulation of the derivatives into mitochondria; ROS-independent mitochondrial depolarization was also induced. We investigate here the mechanisms underlying these effects.

Since effectiveness increased if the two free hydroxyls of the derivatives were acetylated or methylated [13], autooxidation of the polyphenolic nucleus, which depends on the presence of free hydroxyls and is favored by their ionization [15], could be excluded. Oxidation of the resveratrol "kernel" could be ruled out as the source of superoxide also because cyclic voltammetry experiments showed that resveratrol and its mitochondriotropic derivatives are not readily oxidizable at physiologically relevant voltages (Mattarei, A. et al., unpublished results). A protonophoric cycle, such as that envisioned for mitochondriotropic quercetin derivatives [16], can be dismissed as the cause of depolarization because of the lack of proton-carrying groups in the most effective, methylated, molecules. Thus we thought that ROS were likely to be generated downstream of the interaction of our molecules with redox-active proteins. The most likely candidates were components of the respiratory chain. Indeed some evidence for a modulation of respiratory chain complexes by resveratrol has already been presented [17,18].

Ca^{2+} is a major second messenger modulating many cellular physiological functions. Calcium and ROS are linked by a reciprocal interaction: Ca^{2+} overload may cause ROS generation [19], and in turn ROS may have an impact on Ca^{2+} homeostasis. Acute as well as chronic oxidative stress can result in inhibition/downregulation/modulation of the plasma membrane Ca^{2+} ATPase, reticular Ca^{2+} ATPase, Na^+/Ca^{2+} exchanger, and other components of the Ca^{2+} homeostasis machinery [20]. Depending on ROS levels, these effects may be mediated by the action of redox-sensitive kinases, by direct modification of the "pumps" and transporters, or both [21].

Resveratrol is furthermore known to bind to the F_1 portion of the mitochondrial ATP synthase inhibiting the enzyme [22–24]. We therefore focused on Ca^{2+} levels and on ATPase functionality as well as on respiration and ROS production.

2. Materials and methods

Experiments were repeated at least three times. Averages ± s.d. are reported.

2.1. Materials

Resveratrol was purchased from Waseta Int. Trading Co. (Shanghai, P.R. China). R-4′BTPI, R-3BTPI, RDA-4′BTPI, RDA-3BTPI, RDM-4′BTPI and RDM-3BTPI were synthesized as described in [12,13]. 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.

2.2. Cells and mitochondria

Mouse colon cancer CT-26 cells (doubling time 24 h) were grown in high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), 10 mM HEPES buffer (pH 7.4), 1 mM sodium pyruvate, 100 U/mL penicillin G, 0.1 mg/mL streptomycin, 2 mM glutamine (GIBCO) and 1% nonessential amino acids (100× solution; GIBCO), in a humidified atmosphere of 5% CO_2 at 37 °C.

Rat liver mitochondria (RLM) were isolated by conventional differential centrifugation procedures [25]. The standard isolation medium was: 250 mM sucrose, 5 mM HEPES, 1 mM EGTA (pH 7.4). Protein content was quantified with a BCA Protein Assay Kit (Thermo Scientific) using an albumin calibration curve.

2.3. Fluorescence microscopy

An Olympus Biosystems apparatus comprising an Olympus IX71 microscope and MT20 light source was used; images were acquired automatically at 1-min intervals for 70 min, and processed with CellR® software. CT-26 cells were seeded on a XF24-well microplate at a density of 5×10^4 cells/well in DMEM + FBS 10% (200 μL) and incubated overnight in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were then washed with HBSS (in mM units: NaCl 136.9, KCl 5.36, $CaCl_2$ 1.26, $MgSO_4$ 0.81, KH_2PO_4 0.44, Na_2HPO_4 0.34, glucose 5.55, pH 7.4 with NaOH) and loaded with dye. All experiments were performed in DMEM without Phenol red and FBS, at room temperature.

2.3.1. Intracellular Ca^{2+} levels

The non-ratiometric dye Fluo-4 was used to follow [Ca^{2+}] changes in cultured cells under the same experimental conditions used in oxygen consumption assays. Cells were loaded with 20 nM Fluo-4 methyl ester, in the presence of 0.04% Pluronic acid, 100 μM Probenecid and 2 μM CsA, at 37 °C for 30 min. The medium was then replaced with 670 μL of pre-warmed (37 °C) DMEM supplemented with 0.04% Pluronic acid, 100 μM Probenecid and 2 μM CsA; cells were then incubated for 30 min at 37 °C to allow complete hydrolysis of Fluo-4 ester bonds. Excitation wavelength was 480–500 nm and fluorescence was collected at λ > 510 nm. Additions were performed by withdrawing 0.5 mL of incubation medium, adding to this aliquot the desired compound as a solution in 70 μL of DMEM and adding back the solution into the chamber at a peripheral point.

2.3.2. $O_2^{-\bullet}$ generation

$O_2^{-\bullet}$ generation in cells was followed using MitoSOX Red™ (Life Technologies). CT-26 cells were loaded with 1 μM MitoSOX Red™, in the presence of 2 μM CsA, at 37 °C for 30 min. The medium was then replaced with 670 μL of pre-warmed (37 °C) DMEM containing 2 μM CsA. Excitation was at 500–520 nm, and fluorescence was collected at λ > 570 nm. Additions were performed as described above.

2.4. Oxygen consumption assay by CT-26 cells

Respiration by adherent cells was measured using a Seahorse XF24 (Seahorse Bioscience) which measures the oxygen consumption rate (OCR) in the medium immediately surrounding adherent cells cultured in an XF24-well microplate. Adherent CT-26 cells were seeded at a density of 5×10^4 cells/well in DMEM + FBS 10% (200 μL) and incubated overnight in a humidified atmosphere with 5% CO_2 at 37 °C The medium was then replaced with 670 μL of pre-warmed (37 °C) high-glucose DMEM supplemented with 1 mM sodium pyruvate, 4 mM L-glutamine, and without FBS. Microplates were incubated at 37 °C for 30 min to allow temperature and pH to stabilize before the measurements. OCR was measured at preset time intervals while the instrument automatically carried out the pre-programmed additions of the various compounds (final concentrations unless otherwise specified: oligomycin A 1 μg/mL, FCCP 0.2 μM, antimycin A 1 μM), added as a solution in 70 μL of DMEM. All measurements were carried out in quadruplicate (4 wells per condition). For presentation purposes the data were normalized to the initial OCR baseline measurement for each set of wells and are presented as % changes with respect to that level. ECAR (extracellular acidification rate) was also automatically measured during the same experiments.

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