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Selective targeting of synthetic antioxidants to mitochondria: towards a mitochondrial medicine for neurodegenerative diseases?

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Abstract

Mitochondria are the major source of superoxide, and are responsible for activating apoptosis and oxidative damage during acute neuronal cell death and neurodegenerative disorders like Alzheimer and Parkinson diseases. While the molecular mechanisms by which mitochondrial oxidative stress triggers apoptosis are still investigated, attempts to achieve neuroprotection using antioxidant molecules have already been successful in several models of neuronal cell death. To increase the availability of antioxidant drugs at the mitochondrial level within cells, Michael P. Murphy recently proposed to covalently couple antioxidant molecules to a membrane-permeable lipophilic cation serving as carrier. Since mitochondria maintain at rest a potential of −180 mV, the diffusible cationic moiety drives the accumulation of the complex inside the matrix towards a diffusion equilibrium: for a monovalent cationic carrier, a thousand-fold accumulation of the complex is theoretically achievable; for a divalent cation, a million-fold accumulation is expected. Such mitochondria-targeted versions of natural antioxidants have successfully been synthesized and were found to counteract the pro-apoptotic effects of exogenous oxidative insults, while having no effects in models mimicking physiological apoptosis. Based on these observations, we carried out the synthesis of targeted variants of the artificial free radical scavengers 4-hydroxy-2,2,6,6-tetramethylpiperidin-*N*-oxide (TEMPOL) and Salen–Mn(III) complex of *o*-vanillin (EUK-134). Our preliminary results indicate that these targeted compounds, while delaying apoptosis after an exogenous oxidative insult, are not more active than their untargeted variants. This questions the general efficiency of the targeting procedure used and/or suggests that the main pro-apoptotic effector targets of exogenous oxidative insults are not located within mitochondria.

Keywords: Apoptosis; Antioxidant; Drug targeting; Mitochondrion

1. Introduction

Mitochondria are respiring organelles that continuously oxidize substrates to build up and maintain a proton gradient that polarizes their inner membrane at a value of around −180 mV. This value is without equivalent in other sub-cellular compartments, and represents about the double of the potential recorded across the plasma membrane of excitable cells, and is approximately six times higher than the plasma membrane potential of common nonexcitable cells. Mitochondria adapt their respiration rate to exactly compensate the diverse dissipative events that constantly tend to collapse the proton gradient: ATP synthesis, cation uptake (e.g., Ca^{2+} and K^{+}), acid uptake (e.g., pyruvate and phosphate), etc. Therefore, by clamping the electrical potential of their inner membrane, mitochondria constitute a well for membrane-permeant cations that will tend to concentrate in the mitochondrial matrix and to equilibrate according to the equation: $[\text{cation}]_{\text{mitochondria}}/[\text{cation}]_{\text{cytosol}} = 10^{3n}$, where n represents the net charge of the cation. This means that, in living cells, membrane-permeable cations rapidly get sequestrated by mitochondria with much less than 5% of the intracellular cation contents remaining outside mitochondria. This process is operating, for instance, during the accumulation of fluorescent cations like Rhodamine 123 or Mitotracker® stains, or of the older membrane potential probe tetraphenylphosphonium (TPP^{+}) (Liberman et al., 1969). Based on these observations, the idea pioneered by

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Murphy was to develop mitochondria-targeted variants of drugs by simple covalent coupling to TPP^+ (Murphy, 1997). The prototype drugs chosen by Murphy were antioxidants. Indeed, the maintenance of a membrane potential by means of respiration, e.g., electron transfer, has a cost for mitochondria: electrons leaking out of the respiratory chain produce superoxide. Thus, and in spite of a specific detoxification machinery (e.g., the mitochondrial manganese superoxide dismutase), oxidative damage accumulates more rapidly in mitochondria than in the rest of the cell (Beckman and Ames, 1998; Shigenaga et al., 1994). For several reasons (e.g., mitochondrial calcium overload and respiratory chain deficiency), this problem is exacerbated during neurodegenerative disorders like Parkinson or Alzheimer diseases, and is responsible for the triggering of cell death/apoptosis (Matthews et al., 1998; Baker et al., 1998). Thus, the idea of Murphy was to develop mitochondria-targeted antioxidants that could specifically concentrate inside the mitochondrial matrix to relieve such pathological oxidative burden and prevent or delay cell death. Murphy and his group prepared TPP^+-coupled versions of the antioxidant moiety of vitamin E and ubiquinone (MitoQ) (Kelso et al., 2001). As predicted, accumulation of the drugs in the mitochondria of living cells took place according to the mitochondrial membrane potential and increased the antioxidant power of the mitochondrial matrix compartment. In addition, MitoQ efficiently antagonized apoptosis induced by exogenous H_2O_2 in cultured cells, while untargeted ubiquinone was without effect at a similar dose. Finally, and most importantly, MitoQ was reported to have no effect on apoptosis induced by staurosporine and tumor necrosis factor-α (TNF-α), suggesting that this type of drug could specifically antagonize "accidental" apoptosis due to oxidative stress without interfering with "physiological" apoptosis, thus, without risk of promoting cancer. Based on these observations, we carried out the synthesis of two new mitochondria-targeted antioxidants based on synthetic drugs. We coupled to TPP^+ (i) the nitroxide radical 4-hydroxy-2,2,6,6-tetramethylpiperidin-*N*-oxide (TEMPOL), which reacts with and scavenges superoxide (Krishna et al., 1998), and (ii) the catalyst Salen–Mn(III) complex of *o*-vanillin (EUK-134), which exhibits combined catalase and superoxide dismutase activities (Baker et al., 1998). The resulting compounds were tested for their antiapoptotic activity in cultured HeLa cells treated with staurosporine (model of physiological apoptosis–protein kinase inhibition) or sodium selenite (model of accidental apoptosis–oxidative stress).

2. Materials and methods

2.1. Organic syntheses

All reactions were monitored by thin-layer chromatography carried out on 0.2-mm Merck silica gel plates (60F-254 nm) that were visualized by UV light using molybdic acid as the developing agent. Flash chromatography was carried out using silica gel 60 (230–400 mesh) from ROCC (Belgium). ^1H, ^{13}C and ^{31}P nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 400 NB spectrometer, and chemical shifts were expressed in parts per million (δ, ppm) relative to tetramethylsilane (TMS); multiplicity was indicated as singlet (s), doublet (d), triplet (t), multiplet (m) and broad singlet (brs). All starting chemicals were purchased from commercial companies (Aldrich, Acros). Solvents were dried as described elsewhere prior to use. All the obtained compounds were high-pressure liquid chromatography (HPLC) analysed using a Waters Alliance working with Millenium software: separation module W2690 and detector W996 set at 254 nm, Nova-Pak C18 column (40 μm, 3.9∗150 mm). The average purity after purification was superior to 95%. Centesimal analysis was performed by Dr. A. Stones (University College London). High-resolution mass spectra were recorded with a VARIAN MAT-44 and a FINNIGAN-MAT TSQ-70 equipped with a Xenon ION TECH 8 KV source (electronic impact 70 eV), at the Mass Spectrometry Laboratory (Université catholique de Louvain, Belgium). Mass spectrometry analysis was obtained with an electrospray LCTOF Micromass.

Nitroxide radicals were reduced to the corresponding hydroxylamines prior to recording the NMR spectra: 10 mg of the compound was introduced in a 0.5-ml phenylhydrazine 0.03 M solution in $CDCl_3$.

Salen–Mn(III) complexes EUK-134 and JD 29 were prepared according to the procedure published by Jacobsen et al. (1991).

2.1.1. 4-(4-Bromobutyloxy)-2,2,6,6-tetramethylpiperidin-N-oxide

A mixture of TEMPOL (0.50 g, 1 eq.) and sodium hydride (0.35 g, 6 eq.) in DMF (12 ml *N*,*N*-dimethylformamide) was stirred during 1 h at 0 °C under an argon atmosphere, at which point 1,4-dibromobutane (3.13 g, 5 eq.) was added. The reaction mixture was stirred overnight at 50 °C, then allowed to cool down to room temperature, and the solvent was evaporated. The obtained oil was purified by flash column chromatography using a gradient dichloromethane/methanol (100:0 to 95:5) to yield the desired compound as an orange oil (0.69 g, 77%). ^1H NMR ($CDCl_3$) δ (ppm): 1.25 (s, 3H, CH_3), 1.33 (s, 3H, CH_3), 1.70 (m, 1H, CH_2–CH–O), 1.83 (brs, 1H, CH_2–CH_2–Br), 1.94 (m, 1H, CH_2–CH–O), 2.07 (brs, 1H, CH_2–CH_2–CH_2–Br), 3.46 (m, 4H, CH_2–Br and CH_2–O), 3.63 (m, 1H, CH–O). ^{13}C NMR ($CDCl_3$) δ (ppm): 1.5 (CH_3), 29.1 (CH_2–CH_2–Br), 30.2 (CH_2–CH_2–CH_2–Br), 34.2 (CH_2–Br), 44.07 ($C(CH_3)_2$), 45.0 (CH_2–CH–O), 67.6 (CH_2–O), 70.7 (CH–O). High-resolution mass spectroscopy (EI): calculated for $C_{13}H_{25}BrNO_2$: 306.106865; measured: 306.107070; $\Delta = -0.7$ ppm.

2.1.2. [4-(2,2,6,6-Tetramethyl-piperidin-4-yloxy)-butyl]triphenylphosphonium bromide-N-oxide (TEMPOL–TPP)

To a solution of the previous bromide (0.50 g, 1 eq.) in ethanol (14 ml) was added triphenylphosphine (0.85 g, 2 eq.) under an argon atmosphere, then the reaction mixture was refluxed during 72 h. The solvent was removed under reduced pressure, and the brown solid was dissolved in chloroform and precipitated in diethyl ether. The desired compound was obtained as a beige crystalline powder after drying under vacuum (0.64 g, 70%). ^{1}H NMR ($CDCl_3$) δ (ppm): 0.85 (m, 2H, CH_2–CH–O), 1.25 (s, 3H, CH_3), 1.36 (s, 3H, CH_3), 1.74 (m, 2H, CH_2–CH–O), 1.94 (m, 2H, CH_2–CH_2–P), 2.11 (m, 2H, CH_2–CH_2–CH_2–P), 3.63 (m, 2H, CH_2–P), 3.70 (m, 2H, CH_2–O), 3.86 (m, 1H, CH–O), 7.22–7.87 (m, 15H, PPh_3). ^{13}C NMR ($CDCl_3$) δ (ppm): 22.2 (d, J=58.2 Hz, CH_2–P), 22.7 (*C*H_2–CH_2–P), 29.86 (*C*H_2–CH_2–CH_2–P), 30.3 (CH_3), 30.5 (CH_3), 43.3 (*C*H_2–CH–O), 58.1 (*C*$(CH_3)_2$), 67.1 (CH_2–O), 69.8 (CH–O, 119.0 (d, J=85.7 Hz, *C*P), 130.9 (d, J=12.2 Hz, CH_{Ar} ortho/meta), 134.9 (d, J=9.8 Hz, CH_{Ar} ortho/meta), 135.3 (CH_{Ar} para). ^{31}P NMR ($CDCl_3$) δ (ppm): 24.9 (*P*Ph_3). Mass (IE, 70 eV): 489.5 (100, M^+). Mass (FAB+): 489 (100, M^+). High-resolution mass spectroscopy (EI): calculated for $C_{31}H_{40}BrNO_2P$: 489.279668; measured: 489.280843; Δ = – 2.4 ppm. Elemental analysis C, H, N: calculated for $C_{31}H_{40}BrNO_2P$, H_2O: C: 63.34%, H: 7.22%, N: 2.39%; found: C: 63.53%, H: 7%, N: 2.20% (Fig. 1A).

2.1.3. Salen ligand of o-vanillin

To a solution of ethylenediamine (0.15 g, 0.5 eq.) in ethanol (13 ml) was added *o*-vanillin (0.76 g, 1 eq.) and the mixture was refluxed 60 min under an argon atmosphere. The rapidly appeared yellow precipitate was filtered off and washed with cold ethanol to give the desired bisimine ligand (0.84 g, 97%). ^{1}H NMR ($CDCl_3$) δ (ppm): 3.88 (s, 3H, OCH_3), 3.96 (s, 2H, CH_2–N), 6.75–6.92 (m, 3H, Ar), 8.33 (s, 1H, CH=N). ^{13}C NMR ($CDCl_3$) δ (ppm): 56.4 (OCH_3), 59.9 (CH_2–N), 114.4 (C_{Ar}), 118.4 (C_{Ar}), 118.8 (CH–C_{Ar}), 123.5 (C_{Ar}), 148.6 (HO–C_{Ar}), 151.8 (CH_3O–C_{Ar}), 167.0 (CH=N).

2.1.4. Salen–Mn(III) complex of o-vanillin (EUK-134)

A round bottom flask was charged with the previous bisimine (0.2 g, 1 eq.) in ethanol (5 ml), then manganese acetate tetrahydrate (0.3 g, 2 eq.) was added. A dark precipitate appeared immediately and the mixture was refluxed in air for 60 min, at which point lithium chloride (0.07 g, 3 eq.) was introduced in the reaction mixture and the reflux was maintained for 30 min. After solvent removal, water was added and the desired compound crystallized slowly overnight to give a dark solid (0.2 g, 77%). High-resolution mass spectroscopy (EI): calculated for $C_{18}H_{18}ClMnN_2O_4$: 416.033556; measured: 416.032287; Δ = 3.1 ppm. Elemental analysis C, H, N: calculated for $C_{18}H_{18}ClMnN_2O_4$, $4H_2O$: C: 44.20%, H: 5.37%, Cl: 7.25, N: 5.73%; found: C: 44.56%, H: 5.05%, Cl: 7.49, N: 5.61% (Fig. 1B).

2.1.5. 4-(4-Bromobutoxy)-2-hydroxybenzaldehyde

A solution of 2,4-dihydroxybenzaldehyde (1 g, 1 eq.) and potassium hydroxide (0.41 g, 1 eq.) in ethanol (20 ml) was gently refluxed under argon in order to dissolve the base,

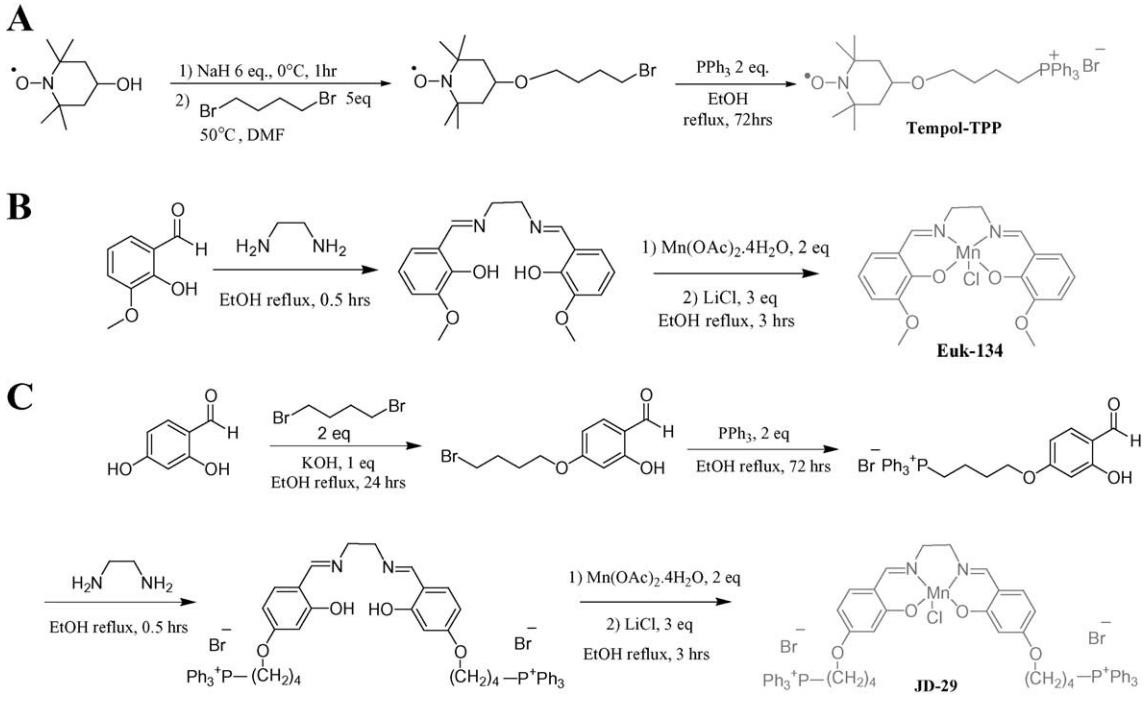

Fig. 1. Synthesis pathways of TEMPOL–TPP (A), EUK-134 (B) and JD-29 (C) used in this study.

then 1,4-dibromobutane (6.2 g, 4 eq.) was added and the reaction mixture was refluxed 24 h. After cooling down, water (15 ml) was added and the organic phase was extracted three times with dichloromethane, dried over magnesium sulfate and concentrated under reduced pressure. The obtained purple oil was purified by flash chromatography using cyclohexane/ethyl acetate (90:10) as eluent. The desired bromide was obtained as a clear yellow oil (0.5 g, 25%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.97 (m, 2H, CH_2–CH_2–CH_2–Br), 2.05 (m, 2H, CH_2–CH_2–Br), 3.48 (t, J=6.8 Hz, 2H, CH_2–Br), 4.05 (t, J=6.0 Hz, 2H, CH_2–O), 6.40 (d, J=2.4 Hz, 1H, H_{Ar}-3), 6.40 (dd, J_{3-5}=2.4 Hz, J_{6-5}=8.8 Hz, 1H, H_{Ar}-5), 7.42 (d, J=8.8 Hz, 1H, H_{Ar}-6), 9.71 (s, 1H, ald.), 11.47 (s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 27.9 (CH_2–CH_2–Br), 29.6 (CH_2–CH_2–CH_2–Br), 33.7 (CH_2–Br), 67.8 (CH_2–O), 101.5 (C_{Ar}-3), 108.9 (C_{Ar}-5), 115.5 (C_{Ar}-1), 135.7 (C_{Ar}-6), 164.7 (C_{Ar}-2), 166.4 (C_{Ar}-4), 194.7 (C_{ald}). Mass (IE, 70 eV): 272.8 (20, M^+). High-resolution mass spectroscopy (EI): calculated for $C_{11}H_{13}BrO_3$: 272.004806; measured: 272.005182; Δ = − 1.4 ppm.

2.1.6. 2-Hydroxy-4-[4-(1,1,1-triphenylphosphonio)butoxy]-benzaldehyde bromide

The previous bromide (0.5 g, 1 eq.) was introduced in a round bottom flask along with triphenylphosphine (0.96 g, 2 eq.) and ethanol (20 ml). The reaction mixture was refluxed under argon for 72 h, then allowed to cool down before removing the solvent under vacuum. The residual oil was purified by flash chromatography using dichloromethane/methanol (95:5 to 90:10) as eluent. The desired phosphonium bromide was obtained as a clear oil (0.89 g, 90%), which gave whitish crystals in diethyl ether. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.81 (m, 2H, CH_2–CH_2–P), 2.20 (m, 2H, CH_2–CH_2–CH_2–P), 3.88 (m, 2H, CH_2–P), 4.09 (t, J=6.0 Hz, 2H, CH_2–O), 6.28 (d, J=2.4 Hz, 1H, H_{Ar}-3), 6.45 (dd, J_{3-5}=2.4 Hz, J_{6-5}=8.8 Hz, 1H, H_{Ar}-5), 7.39 (d, J=8.8 Hz, 1H, H_{Ar}-6), 7.60–7.85 (m, 15H, PPh_3), 9.66 (s, 1H, ald.), 11.29 (s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ (ppm): 19.6 (d, J=3.7 Hz, CH_2–CH_2–P), 22.5 (d, J=50.5 Hz, CH_2–P), 29.4 (d, J=16.8 Hz, CH_2–CH_2–CH_2–P), 67.6 (CH_2–O), 102.0 (C_{Ar}-3), 108.4 (C_{Ar}-5), 115.7 (C_{Ar}-1), 118.5 (d, J=85.3 Hz, C_{Ar}–P), 130.9 (d, J=12.7 Hz, C_{Ar}–P ortho), 134.0 (d, J=9.7 Hz, C_{Ar}–P meta), 135.5 (d, J=2.9 Hz, C_{Ar}–P para), 135.8 (C_{Ar}-6), 164.5 (C_{Ar}-4), 166.3 (C_{Ar}-2), 194.7 (C_{ald}). ^{31}P NMR ($CDCl_3$) δ (ppm): 24.6 ($P$$Ph_3$). Mass (IE, 70 eV): 455.0 (100, M^+). High-resolution mass spectroscopy (EI): calculated for $C_{29}H_{28}O_3P$: 455.177608; measured: 455.177188; Δ = 0.9 ppm. Elemental analysis C, H, N: calculated for $C_{29}H_{28}BrO_3P$, H_2O: C: 65.03%, H: 5.28%, Br: 14.92; found: C: 64.35%, H: 5.30%, Br: 15.09%.

2.1.7. Salen ligand of the phosphinylated benzaldehyde

The solid phosphonium bromide (0.3 g, 1 eq.) was solubilized with ethanol (5 ml) and ethylenediamine (0.5 eq.) was added. The reaction mixture was refluxed under argon for 90 min, then allowed to cool down. The yellow solid was filtered off and identified as the desired bisimine (0.5 g, 97%). ^{1}H NMR ($CDCl_3$) δ (ppm): 1.79 (m, 2H, CH_2–CH_2–P), 2.14 (m, 2H, CH_2–CH_2–CH_2–P), 3.80 (m, 4H, CH_2–P and CH_2–N), 3.95 (t, J=6.0 Hz, 2H, CH_2–O), 6.20 (dd, J_{3-5}=2.4 Hz, J_{6-5}=8.8 Hz, 1H, H_{Ar}-5), 6.21 (d, J=2.4 Hz, 1H, H_{Ar}-3), 7.02 (d, J=8.8 Hz, 1H, H_{Ar}-6), 7.56–7.86 (m, 15H, PPh_3), 8.21 (s, 1H, CH=N). ^{13}C NMR ($CDCl_3$) δ (ppm): 19.6 (d, J=3.8 Hz, CH_2–CH_2–P), 22.4 (d, J=50.5 Hz, CH_2–P), 29.4 (d, J=16.5 Hz, CH_2–CH_2–CH_2–P), 58.67 (CH_2–N), 66.9 (CH_2–O), 102.5 (C_{Ar}-3), 106.6 (C_{Ar}-5), 112.7 (C_{Ar}-1), 118.5 (d, J=85.3 Hz, C_{Ar}–P), 130.9 (d, J=12.7 Hz, C_{Ar}–P ortho), 134.0 (d, J=9.7 Hz, C_{Ar}–P meta), 134.1 (C_{Ar}-6), 135.8 (d, J=2.9 Hz, C_{Ar}–P para), 162.7 (C_{Ar}-2), 164.6 (C_{Ar}-4), 165.9 (CH=N). ^{31}P NMR ($CDCl_3$) δ (ppm): 24.6 ($P$$Ph_3$). Mass (FAB+):

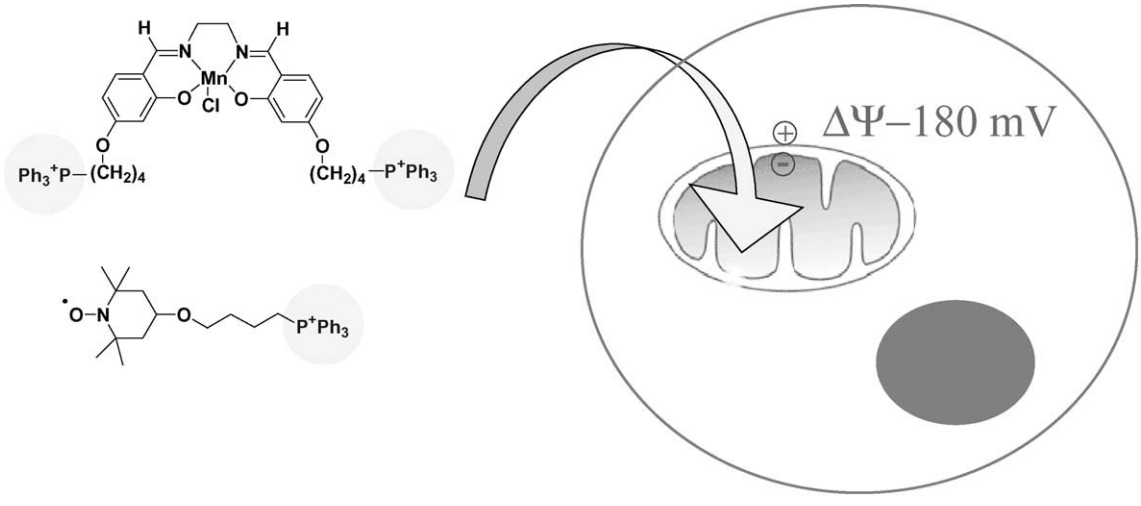

Fig. 2. Membrane potential-dependant accumulation of TEMPOL–TPP and JD-29 in the mitochondrial compartment.

467.2 (40, M^{2+}). High-resolution mass spectroscopy (EI): calculated for $C_{60}H_{59}N_2O_4P_2$: 933.395011; measured: 933.395126; $\Delta = -0.1$ ppm.

2.1.8. Salen–Mn(III) complex of the phosphinylated benzaldehyde (JD-29)

Manganese tetraacetate (0.1 g, 2 eq.) was introduced to a solution of the bisimine (0.2 g, 1 eq.) in ethanol (10 ml) and the reaction mixture was refluxed in air for 60 min, at which point lithium chloride (0.03 g, 3 eq.) was added and the reflux was maintained for an additional 30-min period. The solvent was evaporated, the residual dark solid solubilized in water, and it was allowed to crystallize slowly to give shiny dark crystals (0.14 g, 59%). Mass (FAB+): 1022.1 (55, M^+). High-resolution mass spectroscopy (EI): calculated for $C_{60}H_{58}N_2O_4P_2ClMn$: 1022.294085; measured: 1022.294574; $\Delta = -0.5$ ppm. Elemental analysis C, H, N: calculated for $C_{60}H_{58}Br_2ClMnN_2O_4P_2$, $5H_2O$: C: 56.57%, H: 5.40%, N: 2.20; found: C: 56.72%, H: 5.48%, N: 2.25% (Fig. 1C).

2.2. Apoptosis assays

HeLa cells (human cervix carcinoma) or the derived 2H18 HeLa cell clone (Goldstein et al., 2000) were grown in high-glucose Dulbecco's modified eagle medium (DMEM, Gibco, Life Technologies) supplemented with 10% fetal calf serum, 100 μg/ml streptomicin and 100 U/ml penicillin in six-well plates at 37 °C in an atmosphere of 5% CO_2 95% air. To induce apoptosis, cells were treated either with 40 nM staurosporine for 24 h or with 20 μM sodium selenite for 12 h. When relevant, the antioxidants

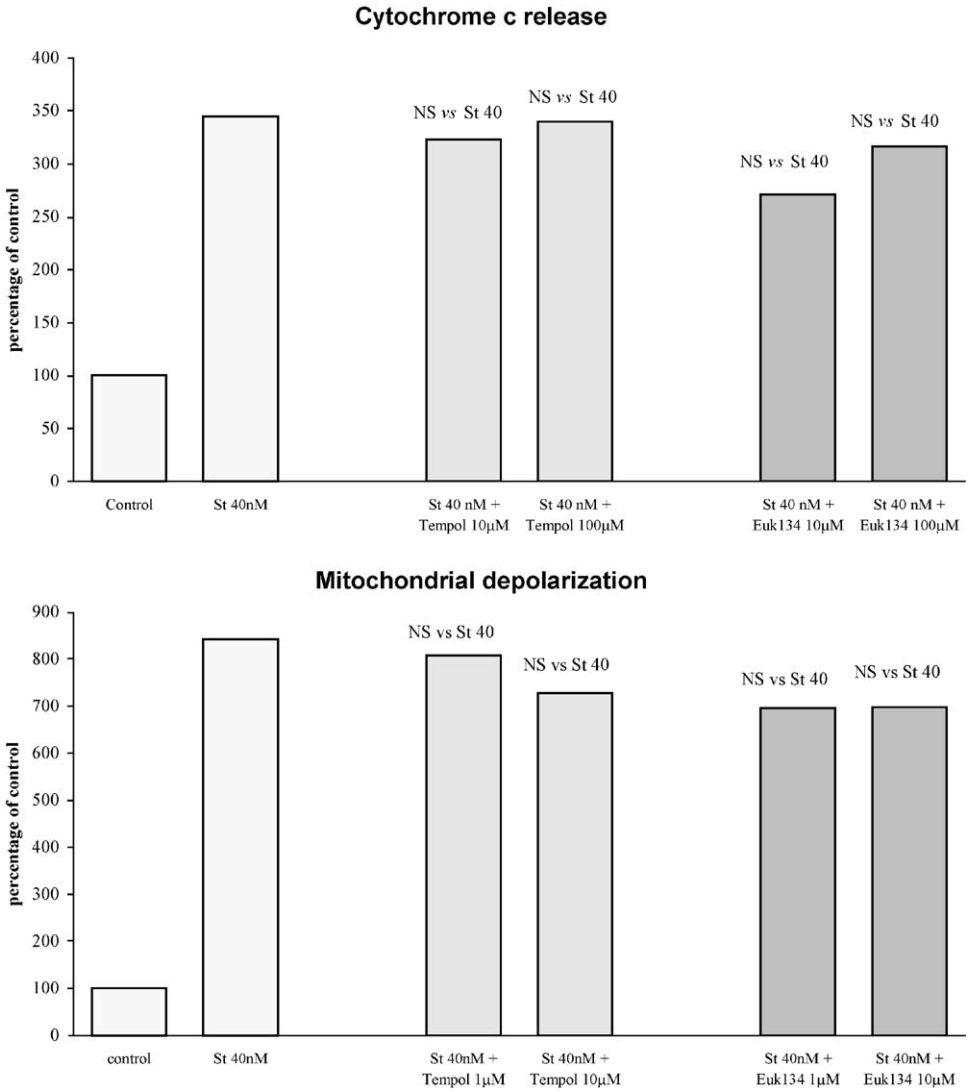

Fig. 3. Flow cytometry reveals that TEMPOL and EUK-134 do not interfere with the release of cytochrome *c* or with the depolarization of the inner mitochondrial membrane that take place during apoptosis induced by the protein kinase inhibitor staurosporine (St). NS: not statistically significant.

were added at the same time as the inducer. Apoptosis was then quantified by flow cytometry after resuspension of the cells. Two parameters were measured: cytochrome *c* release and the mitochondrial membrane potential using tetramethylrhodamine methylester (TMRM) (Goldstein et al., 2000). Statistical analysis of the results was carried out using the chi-square test.

3. Results

We achieved the synthesis of two mitochondria-targeted compounds endowed with a TPP^+ moiety according to the proposal of Murphy (Smith et al., 1999). The first one, TEMPOL–TPP bears a single net positive charge and is expected to concentrate thousand-fold in the mitochondrial matrix; the second, JD-29 has two TPP^+ moieties, thus, bears two positive charges that theoretically should promote a million-fold accumulation of the drug in the mitochondrial compartment (Fig. 2). We first tested the drugs for a possible antiapoptotic activity in cells treated with staurosporine. Measuring either cytochrome *c* release or the loss of mitochondrial membrane potential as markers of apoptosis, we found that the parent compounds TEMPOL and EUK-134 had no effect on these parameters (Fig. 3), and that TEMPOL–TPP and JD-29 tested in the same conditions were also inefficient (data not shown). We then tested the drugs in selenite-induced apoptosis. In this case, TEMPOL and EUK-134 significantly limited apoptosis, and their corresponding targeted variants TEMPOL–TPP and JD-29

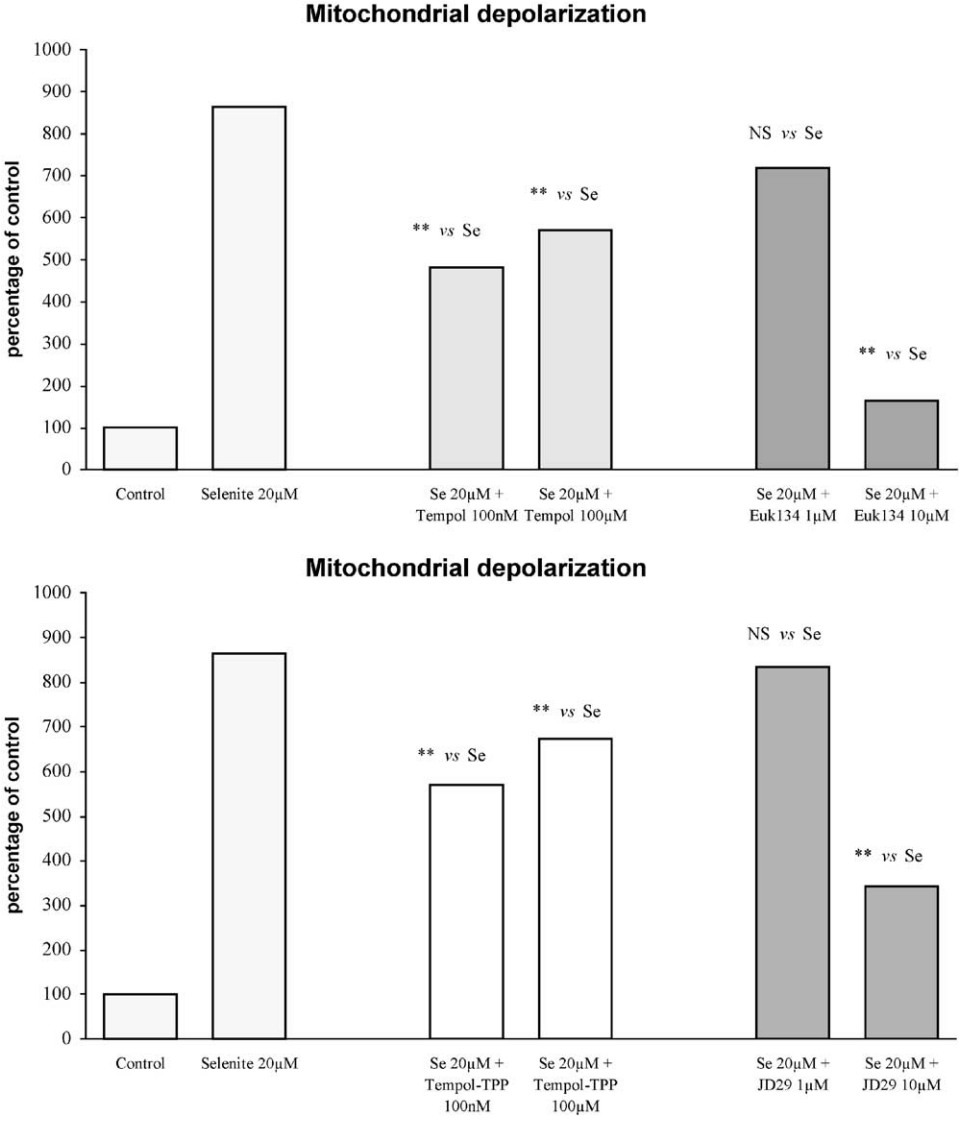

Fig. 4. Flow cytometry reveals that TEMPOL, TEMPOL–TPP, EUK-134 and JD-29 all inhibit the depolarization of the inner mitochondrial membrane that take place during apoptosis induced by the oxidative stress-inducing agent selenite (Se). ** Significant at $P<0.001$. The mitochondria-targeted drugs TEMPOL–TPP and JD-29 do not appear, however, to be more potent than their corresponding untargeted parent drugs.

had a comparable inhibiting activity (Fig. 4). However, it is clear that TEMPOL–TPP and JD-29 did not exhibit any increase in their antiapoptotic activity against sodium selenite as a result of their covalent coupling to TPP^+.

4. Discussion

Since staurosporine treatment somehow mimics the protein kinase inhibition induced by growth factor withdrawal, the first series of results suggests that free radicals are not involved in staurosporine-induced apoptosis, this can be taken to indicate that antioxidants (targeted or not) do not inhibit physiological apoptosis. On the other hand, sodium selenite treatment increases the cellular concentration of superoxide and promotes ''accidental'' apoptosis by mitochondrial oxidative damage (Shen et al., 2001). Our results indicate that there is no significant difference between the targeted and untargeted compounds against apoptosis induced by selenite. Several suggestions can be made to account for this observation: (i) the coupling to TPP^+ was inefficient at driving the mitochondrial accumulation of the drugs, and/or (ii) the coupling to TPP^+ impaired the free radical scavenging activity of the compounds, and/or (iii) the oxidative stress generated by selenite and its primary effector targets are located outside mitochondria, and are thus out of reach of drugs accumulated in the mitochondrial matrix compartment. While these diverse hypotheses are currently being investigated, it must be noted that the possibility that the covalent coupling to TPP^+ was inefficient at driving the mitochondrial accumulation of the drugs has a precedent. Indeed, it was observed that, in spite of a very good membrane solubility, fullerenes coupled to TPP^+ failed to get accumulated by mitochondria (Coulter et al., 2000). Thus, TPP^+ is probably not universal in conferring possible mitochondrial targeting. In addition, it is likely that sodium selenite treatment is not correctly mimicking endogenous mitochondrial oxidative stresses like those taking place during neurodegenerative disorders, and thus that the targeted drugs need to be evaluated in a more relevant context in which the oxidative damage originates from mitochondria.

In any case, there is no doubt that the general targeting principles put forward by Murphy will promote the development of new carriers and targeting procedures aimed at conferring a mitochondrial tropism to drugs active on accidental apoptosis, and preventing or delaying oxidative neuronal cell death.

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