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RESEARCH PAPER

Olesoxime protects embryonic cortical neurons from camptothecin intoxication by a mechanism distinct from BDNF

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BACKGROUND AND PURPOSE
Olesoxime is a small cholesterol–oxime promoting rat embryonic motor neurons survival in the absence of trophic factors. Because olesoxime can substitute for neurotrophic factors in many situations, and to gain further understanding of its mechanism of action, we wondered if it could prevent neuronal death induced by camptothecin (CPT) and compared its effects with those of brain-derived neurotrophic factor (BDNF).

EXPERIMENTAL APPROACH
E17 rat embryonic cortical neurons were treated with olesoxime, BDNF or vehicle and intoxicated with CPT. Caspase-dependent and caspase-independent death pathways along with pro-survival pathways activation were explored.

KEY RESULTS
As previously reported for BDNF, olesoxime dose-dependently delayed CPT-induced cell death. Both compounds acted downstream of p53 activation preventing cytochrome c release and caspases activation. When caspase activation was blocked, both olesoxime and BDNF provided additional neuroprotective effect, potentially through the prevention of apoptosis-inducing factor release from mitochondria. While BDNF activates both the PI3K/Akt and the ERK pathway, olesoxime induced only a late activation of the ERK pathways, which did not seem to play a major role in its neuroprotection against CPT. Rather, our results favour preserved mitochondrial membrane integrity by olesoxime.

CONCLUSIONS AND IMPLICATIONS
Albeit different, olesoxime and BDNF mechanisms for neuroprotection converge to preserve mitochondrial function. These findings emphasize the importance of targeting the mitochondria in the process of neurodegeneration. Importantly olesoxime, by mimicking neurotrophin pro-survival activities without impacting PI3K/Akt and ERK signalling, may have greater therapeutic potential in many diseases where neurotrophins were considered as a therapeutic solution.

Introduction

Reduced neurotrophic support is implicated in many neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis and peripheral neuropathies (Connor *et al.*, 1996; Zuccato and Cattaneo, 2009). This suggested that therapeutic use of neurotrophic factors could prevent or slow the progression of a number of these diseases. However, the promising results seen in animal models of disease (Sendtner *et al.*, 1992b; Sagot *et al.*, 1995; Tomac *et al.*, 1995; Haase *et al.*, 1997; Bordet *et al.*, 2001; Nagahara *et al.*,

2009; Gonzalez-Aparicio *et al.*, 2010) have not translated well into clinical trials due to the poor pharmacokinetics associated with the intact proteins, in particular, their short *in vivo* half-life, low blood–brain barrier permeability, limited diffusion and undesirable side effects through multiple receptor interactions (Miller *et al.*, 1996; The BDNF Study Group (Phase III), 1999; Apfel, 2001; Lang *et al.*, 2006). This motivated the search for alternative small molecule that could mimic neurotrophic factors (reviewed in Pollack and Harper, 2002; Molina-Holgado *et al.*, 2008; Skaper, 2008).

We previously reported the identification of olesoxime (cholest-4-en-3-one, oxime; TRO19622), a small molecule issued from a drug screening programme searching to identify compounds able to rescue rat embryonic motor neurons in the absence of neurotrophic factors (Bordet *et al.*, 2007). In an *in vivo* model of trophic factor deprivation, olesoxime efficiently prevented motor neuron loss in the facial nucleus after facial nerve axotomy in rat neonates (Bordet *et al.*, 2007). Olesoxime also inhibits neuronal cell death and promotes or preserves neurite outgrowth in multiple preclinical neurodegeneration models (Bordet *et al.*, 2007; 2008; Xiao *et al.*, 2009; Rovini *et al.*, 2010; Sunyach *et al.*, 2012). Olesoxime belongs to a new family of cholesterol-oximes that binds to two outer mitochondrial membrane proteins, the 18 kDa translocator protein (TSPO, formerly named as the peripheral benzodiazepine receptor) and the voltage-dependent anion channel (VDAC or porin) (Bordet *et al.*, 2007). These two proteins have been implicated in many biological processes, including the regulation of mitochondrial metabolism, the response to oxidative stress and the modulation of the mitochondrial permeability transition pore (mPTP; see Gatliff and Campanella, 2012; Shoshan-Barmatz and Ben-Hail, 2012 for recent reviews). This suggests that olesoxime targets mitochondria and modulates mitochondrial-dependent apoptotic pathways. Consistent with this, olesoxime concentrates at the mitochondria (Bordet *et al.*, 2010) and inhibits mPTP-driven apoptosis in neurons in the lateral geniculate nucleus (LGN) following the ablation of the occipital cortex in mouse (Martin *et al.*, 2011). Interestingly, olesoxime shares many of its neuroprotective and neurotrophic effects with those of brain-derived neurotrophic factor (BDNF). Indeed, BDNF exerts neurotrophic effects on primary motor neurons (Oppenheim *et al.*, 1992; Sendtner *et al.*, 1992a; Henderson *et al.*, 1993), cerebellar granule neurons (Kubo *et al.*, 1995; Shimoke *et al.*, 1997; 1999; Yamagishi *et al.*, 2003; Bazan-Peregrino *et al.*, 2007) or LGN neurons (Caleo *et al.*, 2003; Madeddu *et al.*, 2004). BDNF also protects cortical neurons from the DNA damaging agent camptothecin (CPT) through the activation of the ERK1/2 pathway (Hetman *et al.*, 1999).

CPT is a topoisomerase-I inhibitor that causes apoptotic death of post-mitotic neurons (Morris and Geller, 1996) through cyclin-dependent kinases (Park *et al.*, 1997; 1998; Zhang *et al.*, 2006), JNK (Ghahremani *et al.*, 2002) and p53 (Morris *et al.*, 2001; Sedarous *et al.*, 2003) activation-mediating Bax translocation (Xiang *et al.*, 1998; Morris *et al.*, 2001), cytochrome c release (Stefanis *et al.*, 1999) and caspase activation (Keramaris *et al.*, 2000). Interestingly, treatment with caspase inhibitors revealed the involvement of caspase-independent neuronal death resulting from a prolonged loss of mitochondrial function due to cytochrome c release, mitochondrial membrane potential loss, free radical generation and energy depletion (Stefanis *et al.*, 1999; Lang-Rollin *et al.*, 2003). Whether BDNF prevents this caspase-independent death is not known.

Because olesoxime can substitute for neurotrophic factors in many situations, and to gain further understanding of its mechanism of action, we investigated its ability to prevent neuronal death induced by CPT and compared its effects with those of BDNF on various survival or death signalling pathways. We show that both BDNF and olesoxime act downstream of p53 activation and upstream of cytochrome c release preventing both caspase-dependent and caspase-independent death pathways; however, a major difference is that while these effects involve the early activation of ERK by BDNF, this is a late event following olesoxime treatment and is unlikely to play a major role in its neuroprotective activity. Our results rather support preserved mitochondrial membrane integrity and consequently function by olesoxime.

Methods

Drugs

Olesoxime was synthesized by Synkem (Chenôve, France) and dissolved in dimethyl sulfoxide (DMSO) to prepare 10 mM stock solutions that were diluted to their final concentration in the appropriate medium. BDNF was purchased from Tebu-bio (Le Perray-en-Yvelines, France). DMSO, CPT, caspase inhibitor [Boc-aspartyl(OMe)-fluoromethylketone (BAF)], and MEK1 and MEK2 inhibitor (U0126) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Pregnant Sprague-Dawley rats were obtained from Elevage Janvier (Le Genest Saint Isle, France) and maintained in a room with controlled temperature (21–22°C) and a reverse 12 h light/dark cycle with food and water available *ad libitum*. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Primary cortical neuron culture

Primary cortical neurons were prepared from embryonic day 17 (E17) rat embryos with minor changes from a previously described protocol (Friedman *et al.*, 1993). After dissection, brain cortex was dissociated by 0.25% trypsin mild digestion in HBSS medium (Invitrogen, Grand Island, NY, USA) supplemented with 7 mM HEPES and 0.45% glucose (w/v) for 18 min at 37°C. Then tissues were dissociated by mechanical trituration and the cells were resuspended in Neurobasal® medium (Invitrogen) supplemented with 2% B-27 (Invitrogen) and 1% pyruvate (Invitrogen). Primary cortical neurons were seeded into poly-ornithine and laminin coated plates at a density of 6250 cells·cm^{-2} in 96-well plates ('low-density' model) or at a density of 210 000 cells·cm^{-2} in 96- or 6-well plates ('high-density' model). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Treatments

After 6 days in culture (DIV6), cortical neurons were treated with CPT alone (usually 10 μM unless otherwise noted) or in combination with BDNF, olesoxime, 100 μM BAF or 10 μM U0126 in final concentration of 0.5% DMSO and 0.1% BSA (ID Bio, Limoges, France). Control CPT-intoxicated cultures received DMSO only. Drug compounds were prepared as 200× stock solutions in DMSO and diluted first 1:25 in complete Neurobasal medium before addition to the cells without changing the culture medium (one-eighth of the total volume in the well). In separate experiments, we treated cortical neurons with BDNF or olesoxime for 2 h to measure PI3K and ERK activation independent of CPT intoxication.

Assessment of survival

After a 24 h treatment, surviving cortical neurons were labelled by incubating with 2 $\mu g \cdot mL^{-1}$ calcein-AM (Invitrogen) for 30 min at 37°C. Then, automatic fluorescent image acquisition of each individual wells of a 96-well plate was performed using Trophos' Plate RUNNER HD® (formerly named Flash Cytometer, Marseille, France) as previously described (Bordet *et al.*, 2007) and analysed using Tina software (Trophos, Marseille, France). When plated at 'low density', individual surviving cortical neuron in each well were counted. When plated at 'high density', the global fluorescence of surviving cells in each well was measured. Survival was assessed in eight replicate wells per culture plate, expressed as percentage of survival compared with control DMSO-treated cells, and reported as mean ± SEM. A representative experiment of at least three independent experiments, unless mentioned, is shown.

Caspase assay

Caspase activation was assessed after 16 h of CPT intoxication on cortical neurons seeded at 'low density' (6250 $cells \cdot cm^{-2}$) in 96-well plates using Caspase-Glo® proluminescent substrates of caspases (Promega, Madison, WI, USA). Caspases 3/7, caspase 8 and 9 activations were measured according to manufacturer's instructions. Assays were performed in eight replicate wells per culture plate and reported as mean ± SEM. Representative results of three independent experiments are shown.

Biochemistry and immunoblotting

Mitochondrial and cytosolic fractions were prepared from cortical neurons seeded at 'high density' (210 000 $cells \cdot cm^{-2}$) in 6-well plates cultured for 6 days and intoxicated with CPT in the presence or absence of olesoxime or BDNF. Cells were scrapped in cold mitochondria buffer [210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.5, 0.04% digitonin, 1 mM DTT and complete protease inhibitor cocktail (Roche, Mannheim, Germany)] and centrifuged 5 min at 560 g at 4°C. Supernatant was collected and then centrifuged at 8000 g at 4°C for 30 min. The mitochondria-enriched pellet was then resuspended in mitochondria buffer. Total protein extracts were prepared by scrapping neurons in CelLytic mammalian lysis buffer (Sigma-Aldrich). Protein content of total, cytosolic or mitochondrial extracts was determined using the Micro BCA Protein Assay kit (Pierce, Rockford, IL, USA). Proteins were loaded on precast NuPAGE® 4–12% bis-tris SDS-polyacrylamide gels (Invitrogen), and transferred onto nitrocellulose (Pierce). Membranes were blocked for 1 h in 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.2% Tween 20 with 5% (w/v) dry skim milk powder and then incubated overnight with primary antibodies at 4°C. After washing, membranes were incubated for 1 h with appropriate HRP-conjugated secondary antibodies (Pierce) and then developed by an enhanced chemiluminescence (ECL) system according to manufacturer's instructions (SuperSignal® West Dura Chemiluminescent Substrate, Pierce). Autoradiography signals were quantified using ImageJ (NIH) software. A representative autoradiographic film is shown along with mean quantification of at least three independent experiments.

Primary antibodies and dilution used were: goat anti-p53 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti–p53-upregulated modulator of apoptotis (PUMA) α/β (1:500, Santa Cruz Biotechnology), mouse anti-cytochrome c (1:1000, BD Pharmingen, Franklin Lakes, NJ, USA), goat anti–apoptosis-inducing factor (AIF) (1:1000, Santa Cruz Biotechnology), mouse anti-COX IV (1:250, Santa Cruz Biotechnology), rabbit anti-TOM20 (1:1000, Santa Cruz Biotechnology), rabbit anti-enolase (1:200, Santa Cruz Biotechnology), mouse anti-actin (1:100, Sigma-Aldrich), mouse anti-AKT (1:500, Cell Signaling Technology, Beverly, MA, USA), rabbit anti-ERK1/2 (1:1000, Cell Signaling Technology), rabbit anti-phospho-AKT (Ser473) (1:1000, Cell Signaling Technology), rabbit anti-phospho- Erk1/2 (Thr202/Tyr204) (1:500, Cell Signaling Technology). Secondary antibodies used were: goat anti-mouse (1:50 000, Pierce), goat anti-rabbit (1:50 000, Pierce), and donkey anti-goat (1:50 000, Jackson ImmunoResearch Laboratories, West Grove, PA).

In situ mitochondrial respiration measurement

Mitochondrial oxygen consumption was measured using a XF-24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA, USA) as previously described (Yao *et al.*, 2009). Briefly, primary cortical neurons from E17 rat embryos were cultured into poly-ornithine and laminin coated Seahorse XF-24 plates at 'high density' (210 000 $cells \cdot cm^{-2}$). Neurons were grown in complete Neurobasal medium for 6 days before experiment. On the day of oxygen consumption analysis, cells were changed to unbuffered DMEM (DMEM base medium supplemented with 25 mM glucose, 2 mM sodium pyruvate, pH 7.4) and placed into the XF Analyzer calibrated at 37°C. After 10 min of equilibration, four baseline measurements of oxygen consumption rate (OCR) were taken before injection CPT (10 μM final concentration) or an equivalent amount of DMSO. Then OCR measurements were taken every 20 min. The OCR was automatically calculated and recorded by the Seahorse XF-24 software. In a second set of experiments, primary cortical neurons at DIV6 were first treated for 8 h with CPT alone or with olesoxime, and then processed for basal OCR measurement as described above. After the assays, plates were saved and cell survival was measured in each well using calcein-AM to confirm non-significant differences in cell numbers per well. Representative results of three independent experiments are shown.

Results

BDNF and olesoxime delayed CPT-induced cell death in cultured cortical neurons

As previously shown in newborn cortical neurons (Hetman *et al.*, 1999), CPT induced a dose-dependent cell death of E17 rat cortical neurons when applied for 24 h at DIV6, with 10 μM inducing around 50% cell death (see Supporting Information Figure S1). Next, we tested the survival-promoting potential of olesoxime and BDNF against 10 μM CPT using either low or high cell density conditions to further allow biochemistry. Both BDNF and olesoxime dose-dependently promoted survival of neurons intoxicated with CPT (Figure 1B–E). Maximal rescue with BDNF was obtained at a concentration of 10 ng·mL^{-1} both in 'low' and 'high' cell density conditions; higher concentrations (100 ng·mL^{-1}) providing similar rescue (Figure 1A). This concentration was next chosen as the maximal effective dose of BDNF for further analysis of its mechanism of action. At 'low' cell density, maximal rescue with olesoxime was obtained at concentrations ranging from 0.3 to 1 μM, while higher concentrations displayed some lower rescue probably because of cytotoxicity under 'low-density' conditions as observed on naïve non-intoxicated cells (see Supporting Information Figure S2). Since some cytotoxicity could already be observed at 1 μM, 0.3 μM was considered the maximal effective concentration for olesoxime when used in 'low' cell density conditions. Surprisingly, when higher cell density was used, higher concentrations of olesoxime were needed for activity (10 to 30 μM) indicating that the compound concentration per cell was important for its activity (Figure 1A). Very similar results were obtained when using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrrazolium bromide (MTT) reduction assay as a measure of functionally active neurons (data not shown).

When observed after 48 h of CPT intoxication, the neuroprotective effects of BDNF or olesoxime were still evident (Supporting Information Figure S3). Interestingly, when BDNF and olesoxime were combined at their maximal effective concentrations, no additive effect was observed, suggesting that both compounds share similar target and/or neuroprotective pathways (Figure 1F).

BDNF, but not olesoxime involved ERK1/2 survival pathways

Because BDNF protection against CPT toxicity is mediated primarily by activation of the ERK, and to a lesser extent through stimulation of the PI3K/Akt pathway (Hetman *et al.*, 1999), we investigated whether olesoxime could involve one or both of these pro-survival pathways. Cortical neurons were seeded at 'high density' in 6-well plates and treated for 1 h with DMSO or optimal concentrations of BDNF or olesoxime as determined earlier (see Figure 1 in 'high-density' model). As expected, BDNF induced a strong phosphorylation of ERK1/2 and Akt proteins while total ERK and total Akt were not affected (Figure 2A and Supporting Information Figure S4). In comparison, olesoxime induced no changes in phosphorylated nor in total ERK or Akt levels. Interestingly, when longer treatment with olesoxime was performed, a dose-dependent phosphorylation of ERK could be observed with a peak after 16 h (Figure 2B and Supporting Information Figure S4) while no changes in phosphorylated Akt levels were observed at later time points (data not shown).

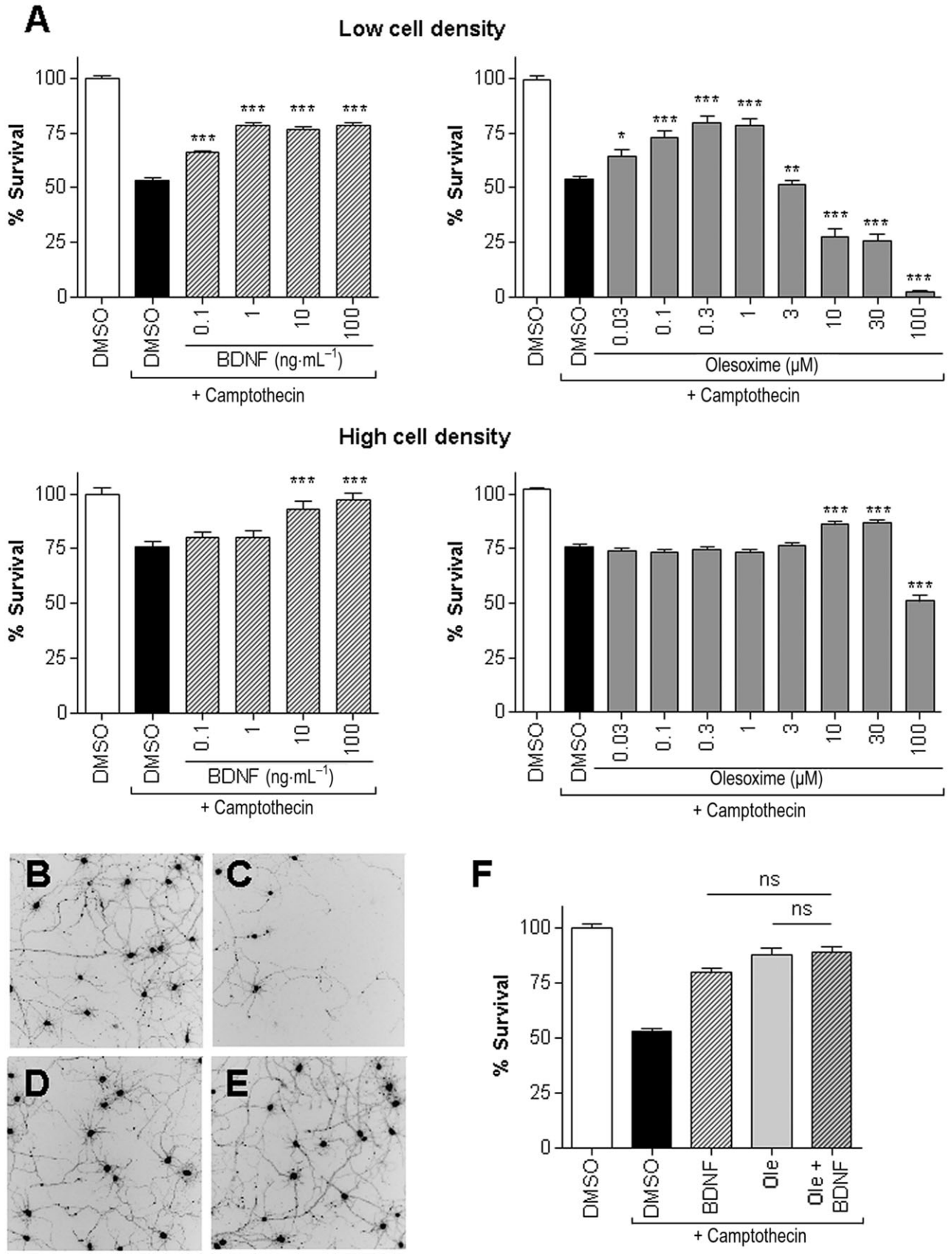
To define whether ERK activation could play a role in the neuroprotective mechanism of olesoxime, we tested whether an ERK inhibitor (U0126) would inhibit the effects of olesoxime to protect cortical neurons from CPT toxicity. While U0126 significantly inhibited olesoxime-induced ERK phosphorylation (Figure 2C), it did not reversed the neuroprotection afforded by olesoxime (Figure 2D). Altogether these data rule out a significant role of ERK signalling in olesoxime's neuroprotective mechanism of action.

BDNF and olesoxime do not prevent p53 activation and downstream transcription pathways

Because olesoxime did not engage conventional pro-survival pathways, we wondered whether it could interfere with CPT-induced pro-apoptotic pathways. In mouse postnatal cortical neurons, CPT induces p53 expression and transcriptional activation of the BH3-only protein PUMA, which results in Bax-dependent caspase-3 activation (Uo *et al.*, 2007). To investigate this in rat embryonic neurons, we cultured cortical neurons in 6-well plates at 'high' density to obtain sufficient material for biochemical analysis. We then prepared either total protein extracts or cytosolic and mitochondrial fractions from neurons treated with CPT alone or together with optimal concentrations of olesoxime or BDNF. As previously reported in mouse cortical neurons, we observed a significant increase in p53 expression in total protein extracts from rat embryonic cortical neurons following 8 h of CPT intoxication along with induction of PUMA mitochondrial expression (Figure 3). As observed in mouse cortical neurons (Uo *et al.*, 2007), we did not observed major changes in Bcl-2

Figure 1

Olesoxime and BDNF improved survival of primary cortical neurons intoxicated with CPT. (A) Primary cortical neurons were seeded in 96-well plates at either 'low' (6250 cells·cm^{-2}; upper panels) or 'high' (210 000 cells·cm^{-2}, lower panels) cell density for 6 days and then treated with DMSO, BDNF or olesoxime in the absence or presence of 10 μM CPT. Surviving neurons after 24 h intoxication were stained with calcein-AM and individually counted with Trophos' Plate RUNNER HD® when plated at 'low' density. When plated at 'high' density, the global fluorescence of surviving cells in each well was measured. Representative extended dose-responses with BDNF and olesoxime, identically replicated on two independent cultures, are shown (mean ± SEM, $n = 8$ replicates wells per treatment condition). (B–E) Representative images (colour-to-greyscale converted to maximize contrasts) of surviving neurons stained with calcein-AM and observed under fluorescent microscope (objective 20×) after 24 h treatment with DMSO (B, C), BDNF (D) or olesoxime (E) in the absence (B) or presence of CPT (C–E). (F) When combined at their maximal effective concentration, BDNF (10 ng·mL^{-1}) and olesoxime (0.3 μM) did not demonstrate additive effects in the 'low cell density' model. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with DMSO condition.


A
Low cell density
% Survival
100
75
50
25
0
DMSO
DMSO
0.1
1
10
100
BDNF (ng·mL−1)
+ Camptothecin
DMSO
DMSO
0.03
0.1
0.3
1
3
10
30
100
Olesoxime (μM)
+ Camptothecin
High cell density
B
C
D
E
F
ns
ns
DMSO
DMSO
BDNF
Ole
Ole + BDNF
+ Camptothecin

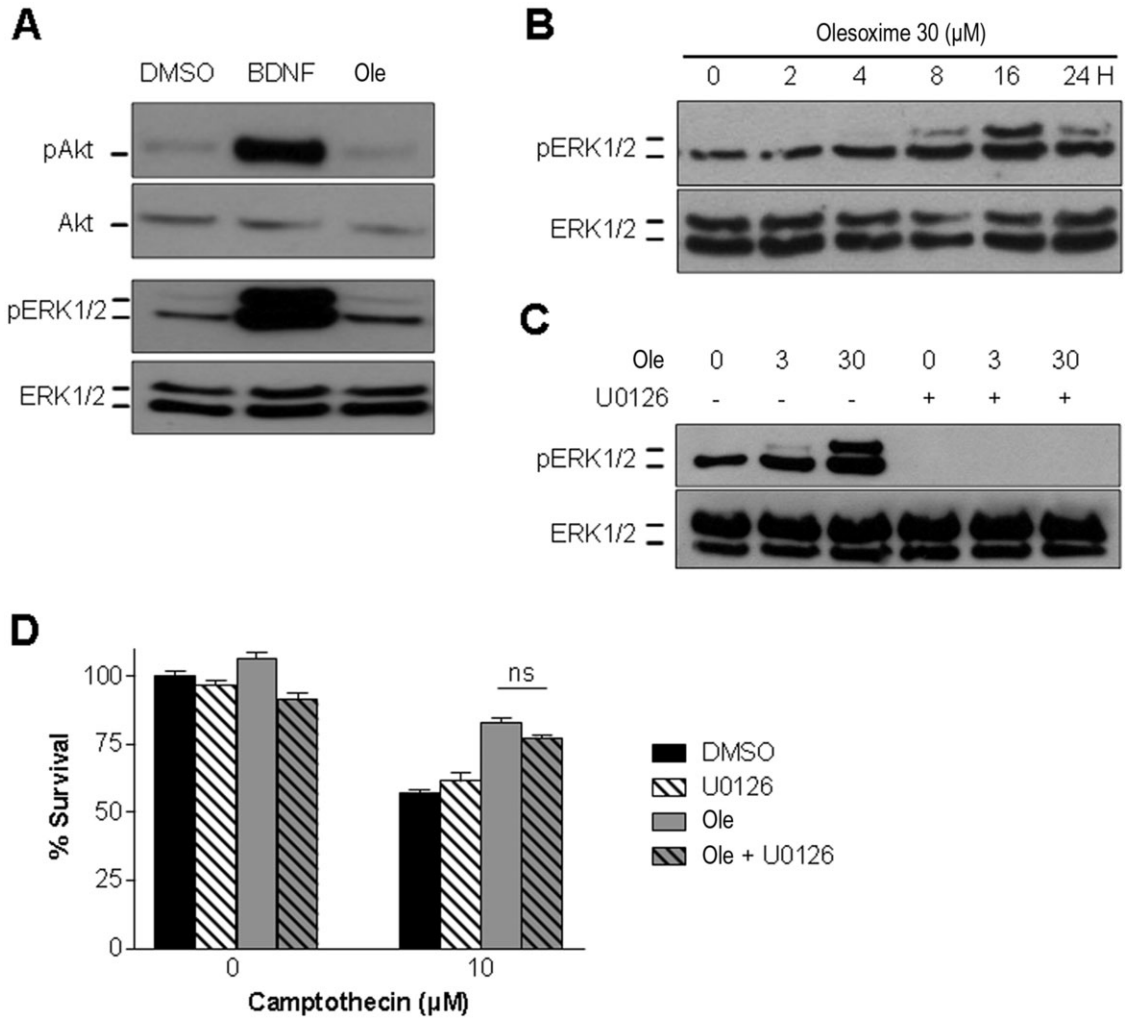


Figure 2

PI3K and ERK1/2 survival pathways. (A) Cortical neurons seeded in 6-well plates at 'high density' for 6 days were treated with DMSO, BDNF 10 ng·mL^{-1} or olesoxime 30 μM for 1 h. Total protein extracts were then loaded onto nitrocellulose membranes and blotted for total and phosphorylated forms of Akt and ERK1/2. Only BDNF induced a strong phosphorylation of both Akt and ERK1/2. (B) Time course of treatment with olesoxime 30 μM showed a later induction of ERK1/2 phosphorylation between 8 and 24 h. (C) Dose-dependent ERK1/2 activation by olesoxime was inhibited when co-administered for 16 h with the ERK inhibitor U0126 10 μM. Representative blots of at least three independent experiments are shown. Quantification of signals is provided in the Supporting Information. (D) Cortical neurons at 'low density' in 96-well plates were treated with DMSO, 0.3 μM olesoxime, 10 μM U0126 or a combination of olesoxime and U0126 in absence or in presence of 10 μM CPT for 16 h, and neuronal survival was measured as described in Figure 1. Inhibition of ERK activation did not significantly reduce the neuroprotective effect of olesoxime. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test (ns, $P > 0.05$).

or Bax protein levels following CPT intoxication (Supporting Information Figure S5). Importantly, we found that neither olesoxime nor BDNF prevented p53 or PUMA activation when co-administered with CPT (Figure 3). We conclude that both olesoxime and BDNF act downstream of p53 and PUMA induction.

BDNF and olesoxime reduced cytochrome c release and caspase activation

Translocation of cytochrome c from mitochondria to the cytoplasm and consequent caspase activation has been shown to be an important feature of CPT-induced apoptotic cell death (Stefanis *et al.*, 1999; Keramaris *et al.*, 2000; Uo *et al.*, 2007). We then asked whether, in the model of CPT-induced death, olesoxime prevented caspase activation, as we previously saw with low potassium-induced cell death in cerebellar granule cells (Bordet *et al.*, 2007). To answer this, we prepared cytosolic and mitochondrial fractions from cortical neurons treated with CPT alone or together with olesoxime or BDNF, and tested them for the presence of cytochrome c using Western blotting and the induction of caspase 3/7-like activity using a fluorogenic assay. We found that cytochrome c was significantly reduced in the mitochondrial fraction after CPT intoxication compared with untreated control cells while both BDNF and olesoxime reduced cytochrome c release from mitochondria (Figure 4A,B).

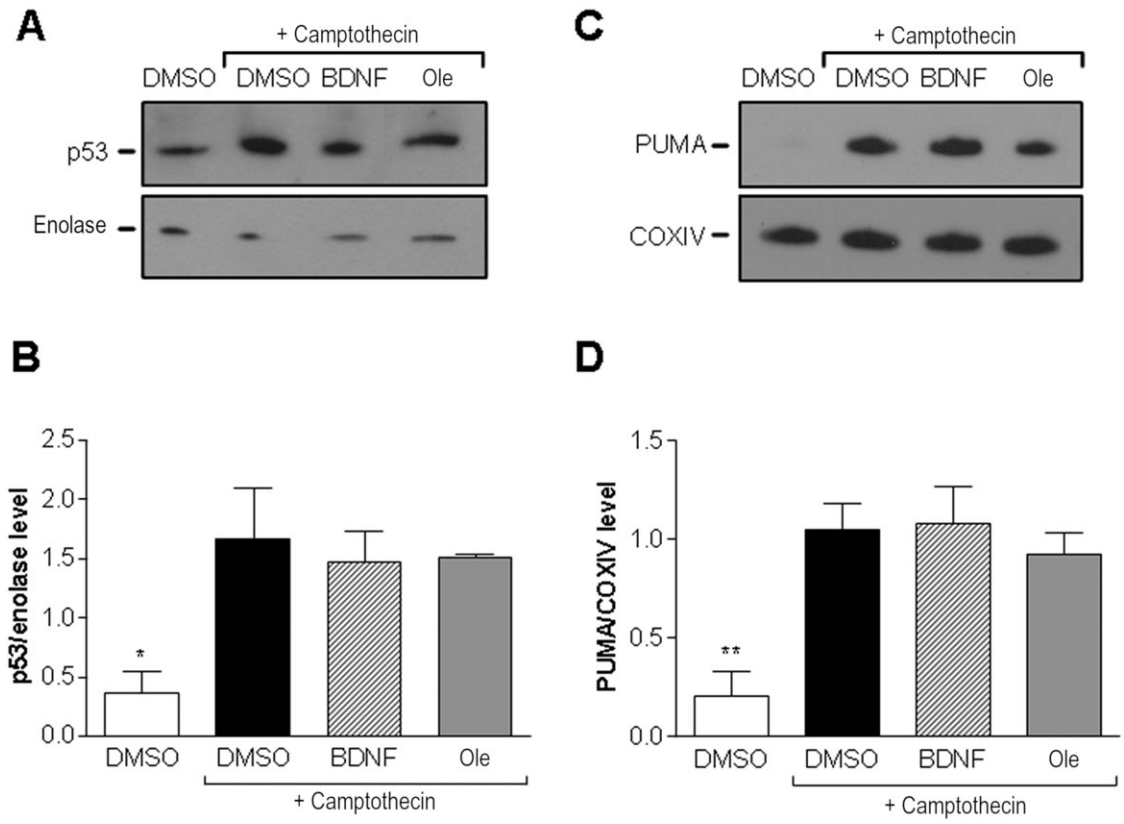


Figure 3

BDNF and olesoxime had no effect on p53 death pathway activation induced by CPT. Cortical neurons seeded at 'high density' in 6-well plates for 6 days were treated with DMSO, 10 ng·mL^{-1} BDNF or 30 μM olesoxime in combination with 10 μM CPT and compared with control condition (DMSO only). (A, B) After 7 h treatment, neurons were lysed and total proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with antibodies against p53 and enolase proteins. A representative experiment is shown in A. (B) Intensity of signals was quantified on three independent experiments using ImageJ. p53 levels are expressed relative to enolase protein levels (mean ± SEM, n = 3). (C, D) After 8 h treatment, mitochondrial enriched fraction was isolated and proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with antibodies against PUMA and COXIV proteins. A representative experiment is shown in C. (D) PUMA protein levels were quantified on three independent experiments using ImageJ and expressed relative to COXIV protein levels (mean ± SEM, n = 3). Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test. *P < 0.05 compared with DMSO condition.

Strong activation of caspase 3 has been reported in E16 mouse cortical neurons submitted to CPT (Keramaris *et al.*, 2000). Here we found similar activation of caspases-3/7 in E17 rat cortical neurons intoxicated with CPT along with a lower increase in activated caspase-9 (Figure 4C). Both BDNF and olesoxime significantly reduced the level of activated caspases-3/7 (Figure 4D). Interestingly, the level of activated caspases-3/7 was even more decreased by olesoxime than with BDNF. In parallel, we showed that olesoxime had no direct caspase inhibitory effect using recombinant proteins (Supporting Information Table S1). We conclude that both olesoxime and BDNF interfere with caspase-dependent death in CPT-treated cortical neurons by acting upstream of cytochrome c release.

BDNF and olesoxime provide additive cytoprotection to caspase inhibitors by preventing AIF release

Previous studies reported the involvement of caspase-independent death pathways induced by CPT when the broad-spectrum caspase inhibitor BAF (100 μM) was used (Stefanis *et al.*, 1999; Lang-Rollin *et al.*, 2003). To see whether BDNF or olesoxime could impact on this caspase-independent death pathway, we tested their pro-survival activity in the CPT model alone or in combination with BAF. Treating cortical neurons at 'low' density with CPT and 100 μM BAF resulted in similar survival as was observed with maximal effective concentrations of BDNF or olesoxime (Figure 5A). When optimal concentrations of BDNF or olesoxime were combined to BAF, additive effects were observed (Figure 5A) strongly supporting an additional effect of BDNF or olesoxime to inhibition of caspase-dependent death pathways.

The AIF was previously shown to be critically involved in the regulation of caspase-independent neuronal death mediated by CPT (Cregan *et al.*, 2002; Cheung *et al.*, 2005). Here we explored whether olesoxime would impact the release of AIF in this model. Cytosolic fractions from cortical neurons treated with CPT alone or together with olesoxime or BDNF were assessed for the presence of AIF using Western blotting.

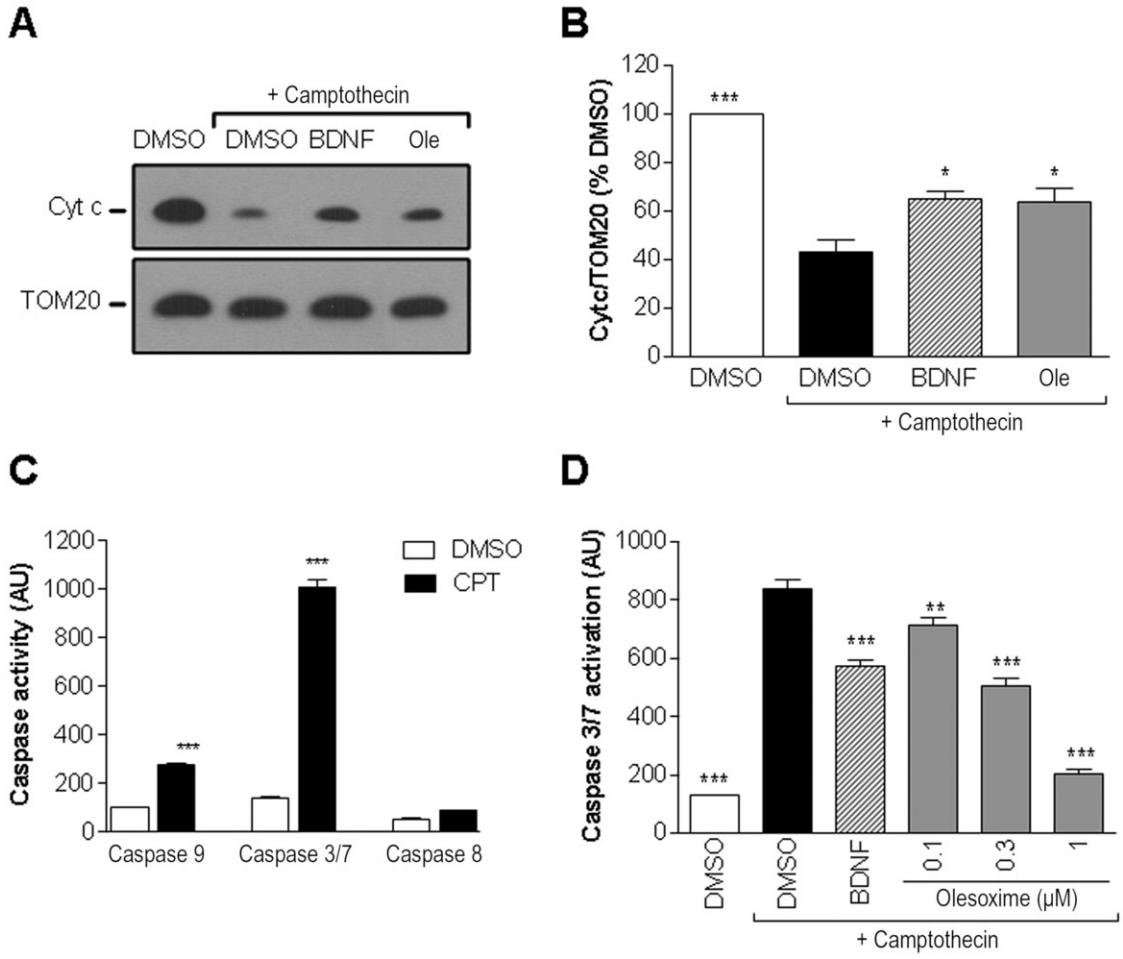


Figure 4

Olesoxime and BDNF reduced cytochrome c release and caspase activation. (A, B) Cortical neurons seeded at 'high density' in 6 well plates and cultured for 6 days were co-treated with 10 μM CPT and 30 μM olesoxime, 10 ng·mL^{-1} BDNF or vehicle (DMSO) and compared with non-intoxicated control condition (DMSO only). (A) After 24 h treatment, mitochondrial enriched fraction was isolated and proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with antibodies against cytochrome c (cyt c) and TOM20 proteins. Cytochrome c release could be evidenced in CPT conditions, while BDNF or olesoxime reduced such release. (B) Cytochrome c release was quantified in three independent experiments and expressed as mitochondrial cyt c protein levels relative to TOM20 (mean ± SEM, $n = 3$). (C) Caspases activation was measured in cortical neurons seeded at 'low density' in 96-well plates and treated for 24 h with 10 μM CPT. A strong activation of caspases-3/7, along with an increase in activated caspase-9, was detected. Representative results of three independent experiments are shown (mean ± SEM, $n = 8$ replicates/condition). (D) Olesoxime and BDNF effect on caspases-3/7 activation was measured using the same assay 24 h after co-treatment with 10 μM CPT. Representative results of three independent experiments are shown (mean ± SEM, $n = 8$ replicates/condition). Statistical analysis was performed by one-way ANOVA followed by Dunnett's Multiple Comparison Test. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with DMSO condition.

We found that CPT induced a huge increase in cytosolic AIF levels, which were dramatically reduced by olesoxime or BDNF treatment (Figure 5B,C). We conclude that olesoxime and BDNF also block caspase-independent death pathways upstream of AIF release from mitochondria.

Olesoxime preserves mitochondrial functions

Previous results strongly suggest that olesoxime preserves mitochondrial membrane integrity preventing pro-apoptotic factor release. To confirm this we measured the activity of the mitochondrial respiratory chain which is located into the inner mitochondrial membrane as a functional marker of membrane integrity. To do so, we measured mitochondrial respiration directly on cortical neurons using XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). Incubation with CPT produced a time-dependent decrease in OCR, which was statistically significant after 8 h ($P < 0.05$, two-way ANOVA; Figure 6A) while no cell death was observed at that time (data not shown). After 8 h intoxication with CPT, OCR was reduced by 55.2 ± 5.7% (mean ± SEM) while reduction in OCR was limited to 14.5 ± 5.3% when neurons were co-treated with olesoxime (Figure 6B), indicating a preserved mitochondrial function in the presence of olesoxime.

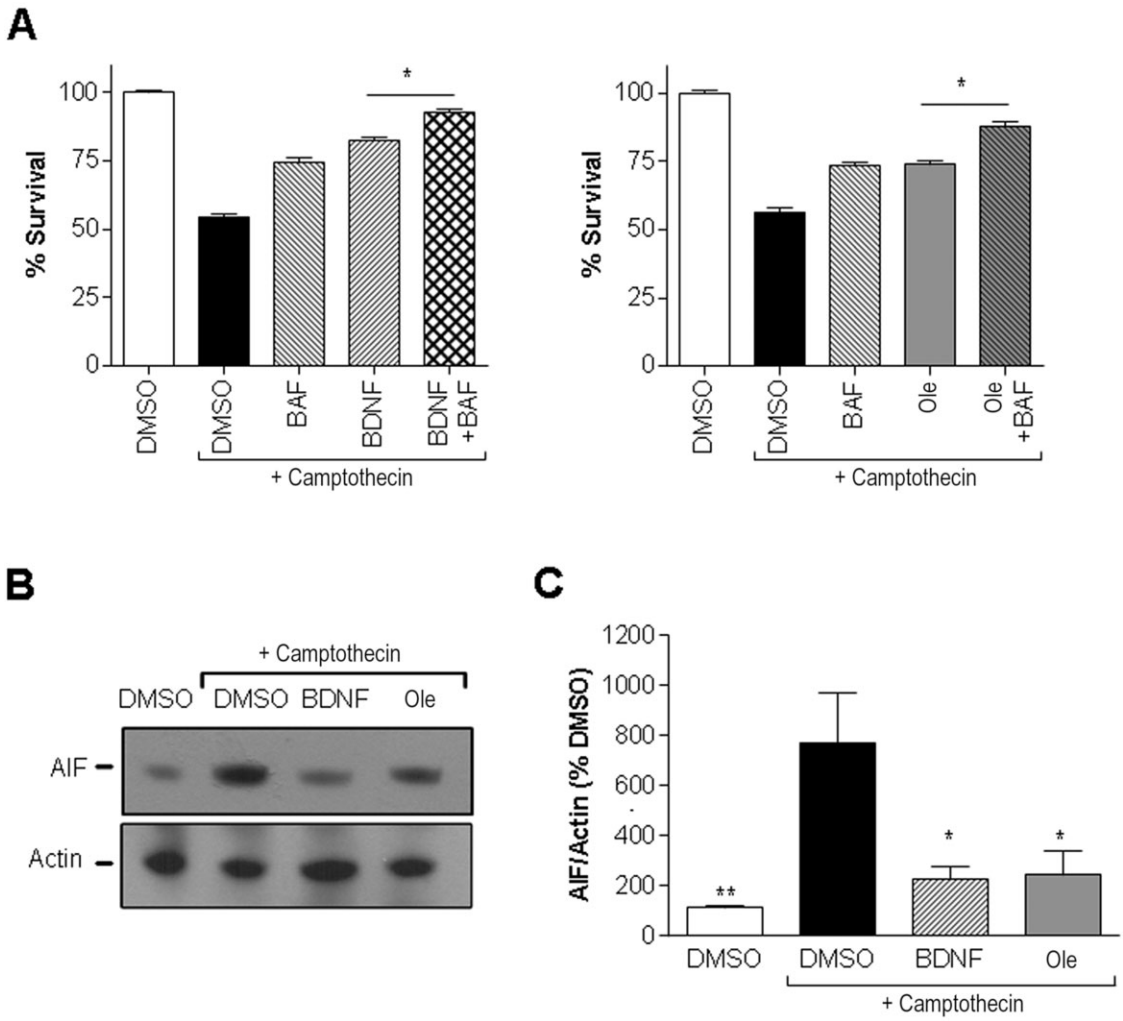


Figure 5

Olesoxime and BDNF displayed additive effect to caspase inhibitors by preventing AIF release induced by CPT intoxication. (A) Cortical neurons seeded at 'low density' in 96-well plates and cultured for 6 days were co-treated with 10 μM CPT and 10 ng·mL^{-1} BDNF, 0.3 μM olesoxime, 100 μM BAF or a combination of both compounds with BAF as indicated. Survival was measured after 24 h CPT intoxication and expressed as percentage of survival compared with control DMSO-treated neurons without campthothecin. *$P < 0.05$ one-way ANOVA followed by Bonferroni's multiple comparison test. (B) Cortical neurons seeded at 'high density' in 6-well plates and cultured for 6 days were co-treated with 10 μM CPT and 10 ng·mL^{-1} BDNF, 30 μM olesoxime or DMSO. Cytoplasmic-enriched fractions were isolated after 24 h intoxication and proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with antibodies against AIF and actin proteins. A representative blot is presented. (C) AIF levels relative to actin were quantified in three independent experiments using ImageJ. Data are mean ± SEM ($n = 3$). Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test. *$P < 0.05$, **$P < 0.01$ compared with DMSO condition.

Discussion

We used the model of CPT-induced toxicity of primary cortical neurons to investigate at the molecular and pathway level the mechanism of action of olesoxime. Olesoxime was originally identified by its ability to rescue trophic factor deprived primary motor neurons; however, due to the limited number of cells one can obtain from embryonic rodent spinal cord, it was not feasible to investigate its mechanism of action using cultured primary motor neurons. Here we show that olesoxime dose-dependently rescues cortical neurons from CPT toxicity. CPT-induced cell death involves DNA damage and activation of both caspase-dependent and caspase-independent death pathways that have been well described (Stefanis *et al.*, 1999); therefore, this system provides a more amenable system to investigate for biochemical analysis of olesoxime's mechanism of action to promote neuronal survival. Furthermore, BDNF, a trophic factor that olesoxime can replace to maintain motor neuron survival, rescues cortical neurons from CPT-induced death and its effects on survival and death pathways in this system have been well characterized (Hetman *et al.*, 1999). For this reason, we compared the activity of olesoxime to that of BDNF on the chain of events known to be involved in CPT-induced neuronal toxicity.

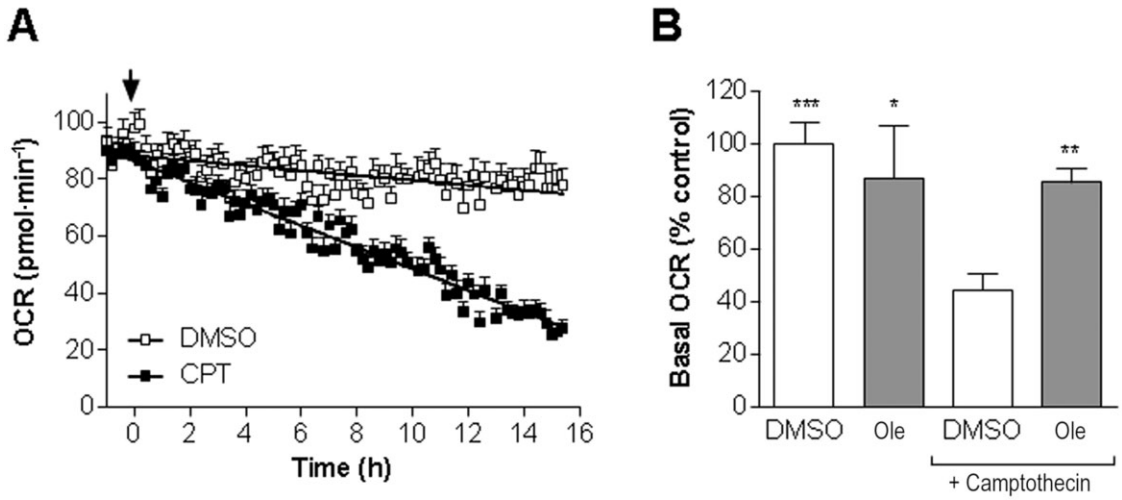


Figure 6

Olesoxime preserves mitochondrial respiratory chain function from CPT intoxication. (A) Cortical neurons were cultured at 'high density' for 6 days in Seahorse XF-24 plates. On the day of oxygen consumption analysis, cells were changed to unbuffered DMEM supplemented with 25 mM glucose, 2 mM sodium pyruvate (pH 7.4) and allowed to equilibrate into the XF Analyzer at 37°C for 10 min. Four baseline measurements of oxygen consumption rate (OCR) were then taken before injection of 10 μM CPT in DMSO or DMSO only (arrowhead). Then OCR measurements were taken every 20 min up to 16 h after injection. OCRs were automatically calculated and recorded by the Seahorse XF-24 software. Data are mean ± SEM of a representative experiment ($n = 10$ replicates/condition). (B) Basal OCR was measured after 8 h treatment with DMSO or 30 μM olesoxime (ole) with or without CPT, and expressed as a percentage of the control, DMSO only. Representative results of three independent experiments are shown (mean ± SEM; $n = 6$ replicates/condition). Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with DMSO.

First, we showed that the survival-promoting effects of olesoxime and BDNF were not additive suggesting they engage similar and/or convergent cell death or survival pathways. Indeed, we found that both olesoxime and BDNF prevent cytochrome c release from mitochondria and consequent caspase activation. Additionally, in the presence of BAF, a pan-caspase inhibitor, both olesoxime and BDNF provided additional protection against CPT-induced death beyond attenuation of caspase-independent death pathways. Consistent with this observation, both olesoxime and BDNF largely prevented AIF release from mitochondria. Altogether, these studies point to mitochondria as an important target in both olesoxime's and BDNF's neuroprotective mechanism of actions.

Apoptosis induced by CPT is mainly driven through p53-mediated induction of pro-apoptotic Bcl-2 family proteins, their translocation to mitochondria and Bax activation that results in mitochondrial membrane permeablization, depolarization and the release of cytochrome c and AIF (Xiang *et al.*, 1998; Keramaris *et al.*, 2000; Morris *et al.*, 2001; Cregan *et al.*, 2002; Uo *et al.*, 2007). Here we showed the level of p53 and PUMA, a pro-apoptotic BH3-family protein, are both increased in cortical neurons by CPT treatment but neither BDNF nor olesoxime prevented their induction.

The neuroprotective effects of BDNF are mediated by TrkB receptors, a receptor tyrosine kinase that leads to downstream activation of Akt and MAPK/ERK-mediated survival signalling, which regulates heterodimerization between Bax and various Bcl-2 family proteins to prevent mitochondrial permeabilization and caspase activation (Allsopp *et al.*, 1993; 1995; Perez-Navarro *et al.*, 2005; Lim *et al.*, 2008). Hetman *et al.* previously showed that the ERK inhibitor PD98059 abolished the neuroprotection afforded by BDNF in CPT-treated cortical neurons while PI3-kinase inhibition had almost no effect (Hetman *et al.*, 1999). These results were further confirmed in many other models mimicking glutamate-mediated excitotoxicity (Almeida *et al.*, 2005; Zhu *et al.*, 2005; Markham *et al.*, 2012) or hypoxic-ischaemic injury (Han and Holtzman, 2000). To see if olesoxime has similar effects as BDNF, we investigated Akt and MAPK/ERK activation in olesoxime-treated cortical neurons. However, unlike BDNF, which rapidly resulted in phosphorylation and activation of Akt and ERK1/2 in cortical neurons, olesoxime treatment did not induce either Akt or EKR1/2 phosphorylation with a time course consistent with its survival-promoting effects. Surprisingly, a late and transient increase in phosphorylated ERK1/2 was observed that might be related to a delayed effect of olesoxime, which has not been investigated. Since the ERK inhibitor, U0126, did not significantly reduce olesoxime's neuroprotective effects, we conclude that the ERK pathway is not critically involved in its mechanism of action. Therefore, even though olesoxime treatment can substitute for trophic factors, including BDNF, to promote neuronal survival, it does not activate the signalling PK pathways that mediate the activity of trophic factors.

The mitochondrial VDAC plays a key role in the control of mitochondrial membrane permeabilization through the interaction with pro-apoptotic and anti-apoptotic Bcl-2 family members (Shimizu *et al.*, 1999; Cheng *et al.*, 2003; Arbel and Shoshan-Barmatz, 2010). It is proposed that Bcl-xL and Bax bind directly to the VDAC and antagonistically modulate its activity regulating cytochrome c release and

mitochondrial membrane loss (Shimizu *et al.*, 1999). Olesoxime, is a cholesterol-like compound that binds to VDAC and concentrates at the mitochondria because it is also a substrate for the mitochondrial cholesterol transporter, TSPO (Bordet *et al.*, 2007; 2010). We hypothesize that its accumulation at the outer mitochondrial membrane modulates VDAC and Bax protein–protein interaction either directly or indirectly through the modulation of mitochondrial membrane fluidity (Dr G. Eckert, pers. comm.). Here we showed that olesoxime's activity is dependent on its intracellular concentration, strongly arguing for a mechanical mechanism of action rather than for the catalytic activation of a survival pathway. Olesoxime not only preserved the physical integrity of the mitochondrial membrane, but also its function, preventing both pro-apoptotic factor release and mitochondrial respiratory failure. Therefore, by separate mechanisms, olesoxime and BDNF both prevent mitochondrial permeabilization and release of pro-apoptotic factors triggered by CPT in cortical neurons and, we suppose, this also explains how olesoxime prevents trophic factor insufficiency in motor neurons.

Although the therapeutic potential of neurotrophins has generated much excitement over the past twenty years, adverse side-effect profiles have limited the clinical usefulness of neurotrophic factors themselves (Price *et al.*, 2007). Indeed, given the important role of Akt and MAPK/ERK signals in the control of biological functions including cell proliferation, survival, metabolism, cell migration and metastasis, targeting one of these pathways is expected to raise concerns about potential adverse side effects. Olesoxime, by mimicking neurotrophin pro-survival activities without impacting Akt and MAPK/ERK signalling, may have a better therapeutic profile and provide a similar pro-survival benefit in many neurodegenerative diseases where neurotrophins were considered as a therapeutic solution such as AD, PD and HD.

Acknowledgement

We are grateful to Dr B. Buisson and E.M. Steidl for first development of the CPT intoxication model at Trophos, to the Trophos' *In Vivo* Pharmacology Department for Animal Housing, and to the MitoTarget Consortium for helpful comment throughout the project. This work was supported by the European Union under the Seventh Framework Program for RTD – Project MitoTarget – Grant Agreement HEALTH-F2-2008-223388.

Conflict of interest

All authors are/were employees of Trophos, which developed the olesoxime drug mentioned in this paper.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Camptothecin induces rat cortical neuron death. At DIV6, E17 cortical neurons seeded at 'low density' in 96 well plates were treated with 0.1 to 100 μM camptothecin or with an equal volume of vehicle (0.5% DMSO). Neuronal survival was measured 24 h later by automatic counting of fluorescent calcein-AM-positive neurons per well using Trophos' Plate RUNNER HD®. Results are expressed as percentage of survival compared to control DMSO-treated neurons without campthothecin and reported as mean ± SEM ($n = 8$ replicate wells per concentration). A representative experiment out of three independent experiments is shown.

Figure S2 BDNF and olesoxime effects on survival of cortical neurons. A dose response of BDNF or olesoxime was performed on primary cortical neurons seeded either at (A) 'low density' (6250 cells·cm^{-2}) or (B) 'high density' (210 000 cells·cm^{-2}). At DIV6, cortical neurons were treated with BDNF, olesoxime, or with an equal volume of vehicle (0.5% DMSO). Surviving neurons after 24 h intoxication were stained with calcein-AM and individually counted with Plate Runner when plated at 'low' density (A). When plated at 'high' density, the global fluorescence of surviving cells in each well was measured (B). Survival is expressed as percentage of survival compared to DMSO-treated control cells and reported as the mean ± SEM ($n = 8$ replicates wells per treatment condition). A representative out of three independent experiments is shown. Statistical analysis was performed by one way ANOVA followed by Dunnett's Multiple Comparison Test. *$P < 0.05$, ***$P < 0.001$ compared to DMSO condition.

Figure S3 Long term survival effect of BDNF and olesoxime in the camptothecin model. Cortical neurons seeded at 'low density' in 96 well plates and cultured for 6 days were treated with DMSO, 10 ng·mL^{-1} BDNF or 0.1 to 1 μM olesoxime and intoxicated with 10 μM camptothecin (CPT) for 24 or 48 h. DMSO-treated neurons (0.5% final concentration) and not intoxicated served as control condition. Surviving neurons were stained with calcein-AM and automatically counted using Trophos Plate Runner®. Data are mean ± SEM ($n = 8$ replicate wells per concentration). A representative out of two independent experiments is shown. Statistical analysis was performed by one way ANOVA followed by Dunnett's Multiple Comparison Test. ***$P < 0.001$ compared to DMSO condition.

Figure S4 Quantification of BDNF or olesoxime effects on PI3K and ERK1/2 survival pathways. Cortical neurons seeded in 6 well plates at 'high density' for 6 days were treated with DMSO, BDNF 10 ng·mL^{-1} or olesoxime 30 μM (ole) for 1 h (A), with olesoxime 30 μM for up to 16 h (B), or with a combination of olesoxime (ole) plus or minus the ERK inhibitor U0126 10 μM for 16 h (C). Total protein extracts were then loaded onto nitrocellulose membranes and blotted for total and phosphorylated forms of Akt and ERK1/2. Total and phosphorylated forms of ERK1/2 or Akt were quantified from three to five independent experiments (including representative experiments presented in Figure 2) using NIH ImageJ software and reported as the mean (±SEM) ratio of phosphorylated/total forms relative to DMSO (densitometric

index). Statistical analysis was performed by one way ANOVA followed by Dunnett's Multiple Comparison Test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to DMSO condition.

Figure S5 Camptothecin-induced changes in Bcl-2 protein family members. Cortical neurons seeded at 'high density' in 6 well plates and cultured for 6 days were treated with camptothecin for 2 to 24 h or with DMSO only (Ctrl). Mitochondrial enriched fractions were isolated and proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated with antibodies against PUMA α/β, Bcl-2 and Bax proteins. (A) Representative blots are shown. A time-dependent increase in PUMA could be observed while Bcl-2 or Bax protein levels at the mitochondria were not drastically modified during camptothecin intoxication. (B) Puma, (C) Bcl-2, or (D) Bax protein levels were quantified from three to four independent experiments using ImageJ software and reported as the mean ± SEM. Differences in expression did not reach statistical significance when compared to control (one-way ANOVA, $P > 0.05$).

Table S1 In vitro caspases assays with olesoxime. Potential direct caspase inhibitory effect of olesoxime was tested using in vitro enzymes assays (Cerep). Human recombinant form of caspase-3, -7, -8 or -9 were produced in E. coli and their activity was tested in presence or in absence of 10 μM olesoxime in DMSO. Specific fluorescent substrates were used: benzyloxycarbonyl-Asp-Glu-Val-Asp-AFC at 3.6 μM and 30 μM, for caspase-3 and caspase-7, respectively; benzyloxycarbonyl-Ile-Glu-Thr-Asp-AFC (10 μM) for caspase-8; and Acetyl-Leu-Glu-His-Asp-AFC (25 μM) for caspase-9. Specific caspase inhibitors were used as reference compounds: Ac-DEVD-CHO for caspase-3 and caspase-7, Ac-IETD-CHO for caspase-8, and Ac-LEHD-CHO for caspase-9. The results are expressed as a percent of control specific activity ((measured specific activity/control specific activity) × 100) obtained in the presence of olesoxime (performed in duplicate). Inhibitions lower than 20% were not considered significant and mostly attributable to variability of the signal around the control level.