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MINI-REVIEW

Prerequisites for ubiquinone analogs to prevent mitochondrial permeability transition-induced cell death

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Abstract The permeability transition pore (PTP) is a mitochondrial inner membrane channel involved in cell death. The inhibition of PTP opening has been proved to be an effective strategy to prevent cell death induced by oxidative stress. Several ubiquinone analogs are known to powerfully inhibit PTP opening with an effect depending on the studied cell line. Here, we have studied the effects of ubiquinone 0 (Ub_0), ubiquinone 5 (Ub_5) and ubiquinone 10 (Ub_{10}) on PTP regulation, H_2O_2 production and cell viability in U937 cells. We found that Ub_0 induced both PTP opening and H_2O_2 production. Ub_5 did not regulate PTP opening yet induced H_2O_2 production. Ub_{10} potently inhibited PTP opening yet induced H_2O_2 production. Both Ub_0 and Ub_5 induced cell death, whereas Ub_{10} was not toxic. Moreover, Ub_{10} prevented tert-butyl hydroperoxide-induced PTP opening and subsequent cell death. We conclude that PTP-inhibitor ubiquinone analogs are able to prevent PTP opening-induced cell death only if they are not toxic per se, which is the case when they have no or low pro-oxidant activity.

Keywords Ubiquinone · Permeability transition pore · Cell death · Mitochondria · H_2O_2 · U937 · tert-butyl hydroperoxide

Abbreviations

PTP	Permeability transition pore
CsA	Cyclosporin A
CRC	Ca^{2+} retention capacity
Ub_0	Ubiquinone 0
Ub_5	Ubiquinone 5
Ub_{10}	Ubiquinone 10
DUb	Decylubiquinone
tbH	tert-butyl hydroperoxide

Introduction

Mitochondria are essential to cell survival, not only through energy metabolism, but also because they play a key role in cell death. In response to various stresses, apoptotic factors are released from the intermembrane space to cytosol, which allows the execution of cell death programs (Giacomello et al. 2007; Kushnareva and Newmeyer 2010; Tait and Green 2010).

The mitochondrial permeability transition designs an abrupt change in the inner mitochondrial membrane, which becomes non selectively permeable to molecules smaller than 1500 Da (Zoratti and Szabo 1995). This results in a drastic ATP synthesis inhibition through the collapse of the proton-motive force, and in the release of mitochondrial NADH (Dumas et al. 2009). The permeability transition also leads to an increase in reactive oxygen species (ROS) production and an inhibition of respiratory chain Complex I (Batandier et al. 2004; Zorov et al. 2000). The mitochondrial

Deceased: Prof Xavier Leverve (1950–2010) in memoriam.

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permeability transition is due to the opening of an inner membrane channel called Permeability Transition Pore (PTP). The molecular nature of the pore has yet to be determined. However, based on the antiapoptotic effect of PTP inhibition, permeability transition has been involved in oxidative stress-induced cell death (Baines et al. 2005; Crompton 2000; Ichas and Mazat 1998; Kroemer and Reed 2000; Nakagawa et al. 2005; Schinzel et al. 2005), including ischemia reperfusion injury in man (Piot et al. 2008).

Ubiquinone analogs represent a recently recognized family of PTP regulators (Devun et al. 2010; Fontaine and Bernardi 1999; Fontaine et al. 1998; Walter et al. 2000, 2002). Regardless of their redox status or partition coefficient, ubiquinone analogs are classified in three functional groups. Whether they favor or inhibit PTP opening or are neutral but compete with active quinones, they are called PTP-activatory, PTP-inhibitory or PTP-inactive quinones, respectively. As compared to the other classes of PTP regulators, ubiquinone analogs display noticeable tissue specificity. For example, we have recently shown that Ub_0 inhibits and induces PTP opening in rat hepatocytes and MH1C1 cells, respectively (Devun et al. 2010). As a consequence, PTP-regulation by ubiquinone analogs cannot be safely extrapolated from results obtained with liver mitochondria.

Ubiquinone analogs have been reported to display either anti or pro-oxidant properties (Devun et al. 2010). They have been shown to prevent oxidative stress-induced cell death (Armstrong et al. 2003; Kelso et al. 2002; Yerushalmi et al. 2001) by a mechanism that may involve their antioxidant properties. On the contrary, they have also been shown to generate structural and functional damages (Bellomo et al. 1990) by a mechanism that may involve ROS production. None of these studies have questioned a role for PTP in these models of cell death regulated by ubiquinone analogs.

To determine whether ubiquinone analogs may prevent cell death because they inhibit PTP opening, we have studied the effect of three ubiquinone analogs on a well-characterized model of cell death induced by PTP opening (i.e., tBH-induced cell death in U937 cells) (Chauvin et al. 2001). We found that Ub_{10} inhibited PTP opening and tBH-induced cell death in U937 cells, whereas Ub_0 and Ub_5 did not inhibit PTP opening and failed to prevent tBH-induced cell death.

Materials

Cells. U937 cells obtained from a lymphoblast taken from a histiocytic lymphoma (purchased from ATCC ECACC 85011440) were maintained in exponential growth phase in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. *Ubiquinones*. Ub_0 (coenzyme Q_0 or 2,3 dimethoxy-5-methyl-1,4-benzoquinone), Ub_5 (coenzyme Q_1 or 2,3 dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone) and Ub_{10} (coenzyme Q_2 or 2,3 dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone) were purchased from Sigma, and diluted in DMSO.

Calcium Retention Capacity measurement. U937 cells maintained in exponential growth phase were harvested and washed with PBS. Cells were permeabilized immediately before use by incubation under stirring at 25 °C in a medium containing 250 mM sucrose, 10 μM EDTA, 50 μg/ml digitonin, 1 mM Pi-Tris, 10 mM Tris-MOPS, and 5 mM succinate (pH 7.4). After 2 min, the tested ubiquinone was added. The measurement of extra-mitochondrial calcium concentration was carried out fluorimetrically at 25 °C with a PTI Quantamaster spectrofluorometer equipped with magnetic stirring and thermostatic control in the presence of 1 μM calcium green 5N (excitation and emission wavelengths were set at 506 and 532 nm respectively). Calcium loading was performed by repetitive additions of 2.5 μM calcium every 2 min, until PTP opening occurred.

Calcein staining. U937 cells incubated with 10 μM Ub_{10} or vehicle for 20 min were stained with calcein plus cobalt as previously described (Petronilli et al. 1999). Cells on glass coverslips were studied by time-lapse laser confocal microscopy at 37 °C in a humidified atmosphere (95% air, 5% CO_2) using a microscope equipped with a perfusion chamber (POC Chamber, LaCom, Erbach, Germany) and an incubation system (O_2–CO_2–°C, PeCom, Erbach, Germany). Images were collected with a Leica TCS SP2 AOBS inverted laser scanning confocal microscope using a 63X water immersion objective (HCX PL APO 63.0 X 1.20W Corr). Laser excitation was 488 nm. Fluorescence emission adjusted with AOBS was 506–541 nm.

Cell death protocols

Short exposure: Cells were treated for 1 h with different concentrations of Ub_0, washed with PBS and replaced in complete RPMI medium. Cell death measurement was assessed after 6 and 24 h. *Long exposure:* Cells were incubated in the presence of 10 μM of Ub_5 or Ub_{10} for a continuous exposure. Cell death measurement was regularly assessed from start until after 72 h.

tbH-induced cell death model: Cells pre-treated or not for 20 min with 10 μM Ub_{10} were exposed to 500 μM tBH for 60 min. Cells were then washed with PBS and incubated in Petri dishes in a complete RPMI medium at 37 °C for 24 h. Cytotoxicity was evaluated by annexin staining. Annexin V-positive cells were quantified by flow cytometry using a FACScan flow cytometer (Becton-Dickinson). Cells (1×10^6/ml) were exposed to 5% *v*/*v* annexin V-FluoProbes Alexa 488 for 15 min at room temperature.

ROS production measurement

U937 cells were permeabilized immediately before use by incubation under stirring at 25 °C in a medium containing 125 mM KCl, 20 mM Tris, 1 mM EGTA, 5 mM glutamate-Tris, 2.5 mM malate-Tris (pH 7.2) plus 50 μg/ml digitonin. H_2O_2 production rate was measured fluorimetrically using 2 μM amplex red in the presence of 10 UI/ml horseradish peroxidase (excitation and emission wavelengths set at 560 and 584 nm respectively). The calibration of the signal was obtained by adding a known amount of H_2O_2.

Chemicals. Annexin and Calcium Green-5N were obtained from Molecular Probes. All other chemicals were purchased from Sigma.

Statistics. Results are expressed as mean±S.E.M. and statistically significant differences were assessed by paired Student's *t* test or ANOVA (StatView®). Significance was defined as $p<0.05$.

Results

Effects of Ub_0, Ub_5 and Ub_{10} on PTP regulation in U937 cells Ca^{2+} is the single most important factor for PTP opening. Ca^{2+} retention capacity (CRC) represents the minimum Ca^{2+} load required to induce PTP opening in an entire population of mitochondria. "PTP-inhibitors" and so-called "PTP-inducers" refer to factors that increase and decrease the amount of Ca^{2+} required to induce PTP opening (i.e., CRC). As shown in Fig. 1, Ub_0 dramatically reduced while Ub_5 did not affect the CRC of permeabilized U937 cells. On the contrary, Ub_{10} increased the CRC of permeabilized U937 cells in a dose-dependent manner. These results indicate that Ub_0 induced, Ub_5 did not regulate and Ub_{10} inhibited PTP opening in permeabilized U937 cells. Note that the CRC in the presence of a saturating amount of CsA was 12.5±1.1 nmol.10^6 cells (n=3). Therefore, over 10 μM, Ub_{10} was more potent than CsA for PTP inhibition.

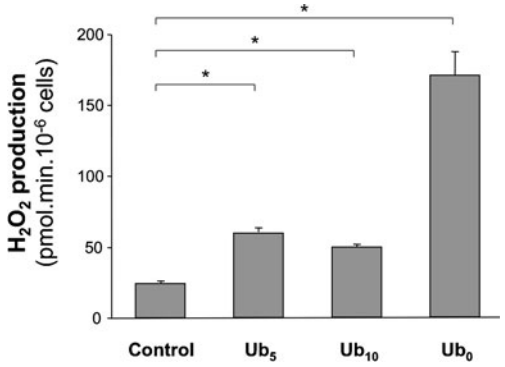

Fig. 2 Effect of Ub_0, Ub_5 and Ub_{10} on H_2O_2 production. U937 cells were incubated in a medium containing 125 mM KCl, 20 mM Tris, 1 mM EGTA, 5 mM glutamate-Tris, 2.5 mM malate-Tris, 50 μg/ml digitonin, 2 μM amplex and 10 UI/ml horseradish peroxidase and 10 μM of Ub_0, Ub_5, Ub_{10} or vehicle. The final volume was 2 ml, pH 7.2, 25 °C. The results are mean±S.E.M. of at least 3 independent experiments. *, $p\leq0.05$, paired Student's *t* test

Effect of Ub_0, Ub_5 and Ub_{10} on H_2O_2 production in U937 cells Ubiquinone analogs have been reported to either reduce or increase reactive oxygen species (ROS) formation (Seung et al. 1998; Shivaram et al. 1998). We have recently shown that ubiquinone analogs regulate ROS production in different ways according to the cell type, with effects that do not correlate with the way they regulate PTP opening (Devun et al. 2010). Therefore, we next measured H_2O_2 production in U937 cells in the absence or presence of Ub_0, Ub_5 or Ub_{10}. As shown in Fig. 2, Ub_0, Ub_5 and Ub_{10} increased ROS production in U937 cells, Ub_0 being more potent than Ub_5 and Ub_{10}.

Corresponding effect of Ub_0, Ub_5 and Ub_{10} on cell death Intact cells were then incubated in the presence of Ub_0, Ub_5 or Ub_{10} in order to assess their toxicity to U937 cells. As shown

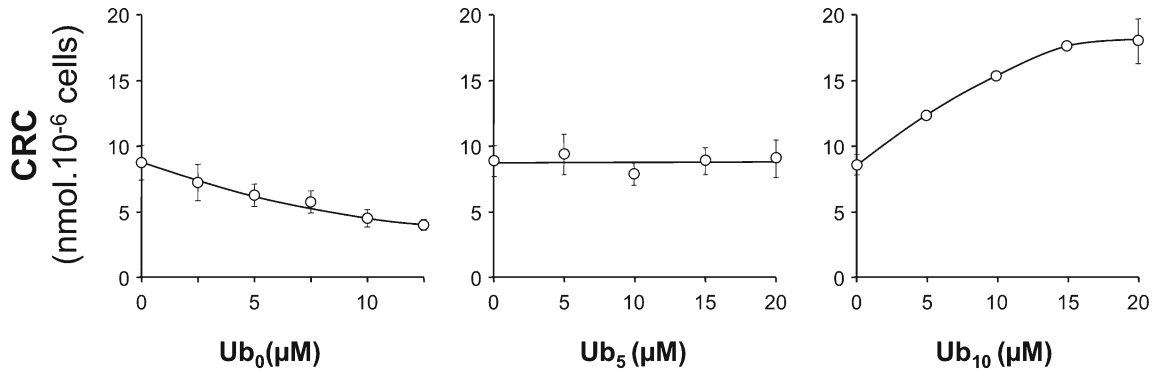

Fig. 1 Effects of Ub_0, Ub_5 and Ub_{10} on the Ca^{2+} retention capacity of U937 cells. The incubation medium contained 250 mM sucrose, 1 mM Pi-Tris, 10 mM Tris-MOPS, 5 mM succinate-Tris, 50 μM digitonin and 1 mM Calcium Green-5N. The final volume was 2 ml, pH 7.4, 25 °C. Experiments started with the addition of 5.10^6 cells followed by the addition of the indicated concentrations of ubiquinone analogs. After 2 min of incubation, calcium pulses were added every 2 min until pore opening. Each point represents the mean±S.E.M. of four independent experiments

in Fig. 3, Ub_0 (PTP-activator and pro-oxidant) induced a clear dose-dependent increase in mortality after a 1-h transient exposure. On the contrary, a 1-h transient exposure to Ub_5 (PTP-inactive and pro-oxidant) or Ub_{10} (PTP-inhibiting and pro-oxidant) did not induce significant toxicity after 6 or 24 h (data not shown). U937 cells were then incubated continuously in the presence of Ub_5 and Ub_{10}. Despite its pro-oxidant activity, PTP-inhibiting Ub_{10} remained non-toxic whereas PTP-inactive but pro-oxidant Ub_5 exhibited a considerable toxicity after a 24-h exposure (Fig. 3). These results (a) confirm that PTP opening leads to cell death, (b) suggest that Ub_5 induces cell death by a pro-oxidant mechanism, and (c) indicate that the PTP-inhibitor Ub_{10} might be used to prevent PTP opening-induced cell death despite its pro-oxidant activity.

Ub_{10} protects U937 cells against tbH-induced cell death In order to check whether Ub_{10} prevents PTP opening-cell death, U937 cells were exposed to tbH in the presence or absence of Ub_{10}. As previously shown (Chauvin et al. 2001), tbH induced PTP opening, as assessed by calcein decompartmentation (Fig. 4, panel a) and led to a dramatic increase in cell mortality (Fig. 4, panel b). As expected, tbH-induced cell death was prevented by CsA (not shown, but see (Chauvin et al. 2001)). Ub_{10}, which did not display any antioxidant activity in U937 cells (see Fig. 2), prevented tbH-induced PTP opening (Fig. 4, panel a) and tbH-induced cell death (Fig. 4, panel b), whereas 10 μM N-Acetyl-Cystein failed to prevent tbH-induced cell death in U937 cells (data not shown).

Discussion

In this work we have shown that in U937 cells, Ub_{10} (a) inhibits PTP opening, (b) increases H_2O_2 production, and (c) prevents tbH-induced cell death. To the best of our knowledge, this is the first demonstration that an ubiquinone analog can prevent cell death because it inhibits PTP opening, independently of any putative antioxidant property. Moreover, we have confirmed that the regulation of PTP opening and ROS production by ubiquinone analogs changes depending on the studied cell line, which precludes any extrapolation from results obtained with liver mitochondria.

In rat liver mitochondria, Ub_0 and Ub_{10} potently inhibit PTP opening while Ub_5 is inactive. Although Ub_{10} and Ub_5 behave in U937 cells as in rat liver mitochondria, Ub_0 favors PTP opening in U937 cells. In a recent work, we have reported that Ub_0 induced PTP opening in MH1C1 cells, whereas it inhibited PTP opening in Clone-9 cells (Devun et al. 2010). We also found that Ub_5 was inactive in MH1C1 cells, whereas it induced PTP opening in Clone-9 cells. This unique feature allowed us to induce cell death selectively in cells in which the analog used induced PTP opening while sparing the other cells (Devun et al. 2010).

Surprisingly, PTP-inactive or PTP-inhibiting quinones can also be cytotoxic. This was the case for Ub_5 in U937 cells (see Fig. 3d) and for Ub_0 in Clone-9 cell (Devun et al. 2010). In both cases, the ubiquinone analog displayed a pro-oxidant activity in the cell line in which it induced cell death (see Fig. 3d and (Devun et al. 2010)). Note however that Ub_{10} was pro-oxidant but a PTP-inhibitor in U937 cells and

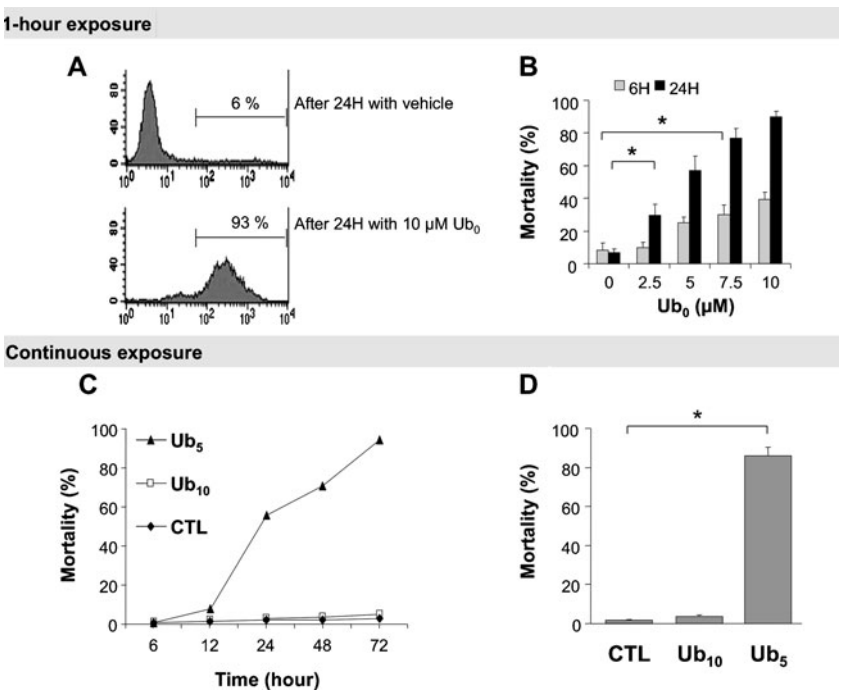

Fig. 3 Effects of Ub_0, Ub_5 and Ub_{10} on U937 cells viability. U937 cells were incubated for 1 h in the presence or absence of Ub_0 (panels **a** & **b**), or continuously incubated in the presence or absence of Ub_5 or Ub_{10} (Panels **c** & **d**). Cell viability was measured by annexin V staining measured by cytometry. The results in panels **b** and **d** are mean±S.E.M. of at least 4 independent experiments. *, $p \leq 0.05$, paired Student's *t* test

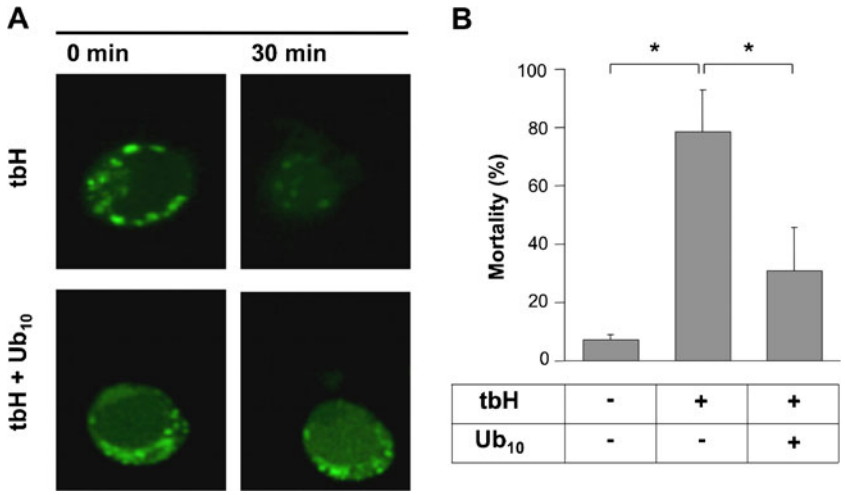

Fig. 4 Effect of Ub_{10} on tbH-induced PTP opening and cell death. Panel **a**: U937 cells coloaded with 0.25 μM calcein-AM and 8 mM $CoCl_2$ in the absence or presence of 10 μM Ub_{10} were exposed to 1 mM tbH. The fluorescence of calcein was imaged every min for 30 min. Panel **b**: U937 cells incubated in the presence of 10 μM Ub_{10} or vehicles, were exposed to tBH (500 μM) for 60 min. Cytotoxicity was assessed by annexin V staining measured by cytometry. Results are mean±S.E.M. of at least 3 independent experiments. *, $p \leq 0.05$, repeated measures ANOVA

did not display any toxicity in this cell line (Fig. 3c and d). We hypothesize that the pro-oxidant activity of Ub_{10} in U937 is offset by its potent PTP-inhibitory effect.

It has been shown that short chain quinones could induce an oxidative stress, depending on the site of interaction with complex I and on their physical characteristics (Fato et al. 2008). Other mechanisms in the toxicity of ubiquinone analogs may involve the alkylation of critical sulfhydryl groups (Bellomo et al. 1990), DNA damage (D'Odorico et al. 1997), arylation (Seung et al. 1998) or the formation of quinoproteins (Miyazaki and Asanuma 2009).

Therefore, to prevent PTP-opening cell death, an ubiquinone analog must inhibit PTP opening without being toxic per se. This was the case for Ub_{10} in U937 cells. On the other hand, despite the fact that Ub_0 has been shown to be the most potent PTP inhibitor found so far in rat liver mitochondria (Walter et al. 2000), Ub_0 has not proved to prevent cell death (Armstrong et al. 2003). At least two non-mutually exclusive explanations can be proposed: either Ub_0 may be toxic per se (by PTP-dependent or PTP-independent mechanisms), or Ub_0 may not inhibit PTP opening in the studied cell lines.

In other experimental models, ubiquinones or ubiquinone analogs have been reported to prevent cell death. Decylubiquinone has been shown to prevent glutathion depression-induced cell death in HL 60 cells (Armstrong et al. 2003), idebenone to protect hepatocytes during hydrophobic bile acid toxicity (Shivaram et al. 1998; Yerushalmi et al. 2001), and coenzyme Q_{10} (ubiquinone 50) to block apoptosis in neuronal PC12 cells exposed to ceramide or ethanol (Kagan et al. 1999). In these studies, the effect of these quinones on PTP regulation has not been tested, and the protective effect has been attributed to antioxidant activity. Moreover, note that idebenone (hydroxy-decyl-ubiquinone) has been reported to induce PTP opening in rat liver mitochondria (Walter et al. 2000).

Conclusion

PTP regulation by ubiquinone analogs is a widespread phenomenon. However, the effect of a given ubiquinone analog on PTP regulation, ROS production and cell viability in a particular cell line or tissue cannot be extrapolated from results obtained with other cell lines or tissues.

Ubiquinone analogs can (a) inhibit PTP opening and stimulate ROS production (Ub_0 in Clone-9, Ub_{10} in U937), (b) favor PTP opening and inhibit ROS production (Ub_0 in MH1C1 cells), (c) favor both PTP opening and ROS production (Ub_5 in Clone-9, Ub_0 in U937), (d) inhibit both PTP opening and ROS production (DUb in Clone-9 and MH1C1 cells), (e) increase ROS production with no obvious effect on PTP regulation (Ub_{10} in Clone-9, Ub_5 in U937), (f) decrease ROS production with no obvious effect on PTP regulation (Ub_5 and Ub_{10} in MH1C1 cells).

At least two non mutually exclusive mechanisms can account for ubiquinone analogs toxicity: a PTP-inducing effect and a pro-oxidant effect. PTP-inhibitor ubiquinone analogs are able to prevent PTP opening-induced cell death only if they are not toxic per se. This is the case when they have no pro-oxidant activity or when their pro-oxidant activity is thwarted by a potent PTP-inhibitory effect. On the one hand, these data make PTP regulation more complex. On the other hand, they open new perspectives for strategies aiming at preventing PTP opening-induced cell death in a subpopulation of cells.

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