

# Divergent effects of quinolinic aminoxylys on mitochondrial ultrastructure and localisation in osteosarcoma 143 B cells

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*In the present study we have shown that quinolinic aromatic aminoxylys are very efficient in protecting lipids of endoplasmic reticulum membranes against hydroperoxide-induced oxidation. The efficacy of these aminoxylys as protectors of lipids was much higher than the water-soluble 4-OH-TEMPO. We have also shown that QAL causes distinct changes of the morphology of mitochondria: from filamentous to granular enlarged structure via the folding of the former. QAL induces also perinuclear clustering of mitochondria. C-QAL as well as 4-OH-TEMPO treated cells revealed filamentous and scattered pattern of mitochondria. Antioxidant activity of QAL as well as morphological changes of mitochondrial raise the possibility that this drug can affect cell physiology via changes of mitochondrial function.*

**key words:** QAL, C-QAL, 4-OH-TEMPO, oxidative stress, mitochondrial ultrastructure

## INTRODUCTION

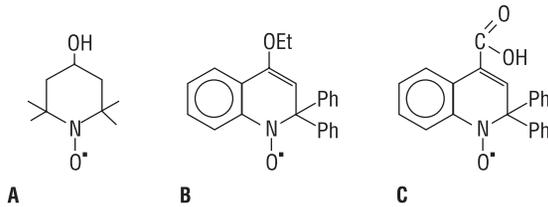
It is generally accepted that harmful reactive oxygen species (ROS) are constantly formed during cellular metabolism and the integrity of the cell system mostly depends on their removal by antioxidant defences [10]. Recently, stable aminoxylys radicals have been used successfully as general protectors of biological systems against oxidative stress. Antioxidant reactivity of aliphatic piperidine aminoxylys has been ascribed to their efficient trapping of carbon-centred radicals [6] as well as to their superoxide dismutase (SOD) mimic activity [16]. On the other hand lipid soluble quinolinic aminoxylys revealed far more extended reactivity since they react not only with C-centred radicals [5] but also with O-centred radicals, namely alkoxyyl [9] and peroxy radicals [4].

Some studies revealed the potentiality of synthetic antioxidants to change the morphology of cells exposed on them due to proapoptotic signalling [18]. Until now data concerning aromatic aminoxyyl effects on cell biology and morphology are largely unknown.

## MATERIAL AND METHODS

### Cell culture

143 B human osteosarcoma cells (ATCC CRL8303) were grown at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in Dulbecco's modified Eagles medium (Nissui Co. Ltd, Tokyo, Japan) containing 1 mM pyruvate supplemented with 10% foetal bovine serum and 50 µg/ml kanamycin. The cells were kindly provided by Dr M. Tanaka, Department of Gene Therapy, Gifu



**Figure 1.** Structures of aminoxyls: **A.** 4-OH-TEMPO; **B.** QAL; **C.** c-QAL.

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### Treatment of cells with chemicals

Cells were cultured in the presence of chemicals specified below: 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (4-OH-TEMPO) — the final concentration 100  $\mu$ M; 1,2-dihydro-2,2-diphenyl-4-ethoxyquinoline-1-oxyl (QAL) — the final concentration 100  $\mu$ M, 1,2-dihydro-2,2-diphenyl-4-carboxyquinoline-1-oxyl (c-QAL) — the final concentration 100  $\mu$ M. All reagents were prepared as 1000x-concentrated stock solutions dissolved in DMSO and stored at  $-20^{\circ}\text{C}$ . Aminoxyls were synthesised in our laboratories (Fig. 1).

### Staining and visualisation of mitochondria by confocal microscopy

Cells growing on  $10 \times 10$  mm-glass coverslips under various experimental conditions were incubated with 100 nM Mito Tracker Red CMXRos (CMXRos) (Molecular Probes, Inc.) for 30 minutes at  $37^{\circ}\text{C}$  in the atmosphere of 5%  $\text{CO}_2$ . Cells were then fixed with a fixative containing 2% glutaraldehyde and 2% formaldehyde in PBS for 30 minutes at room temperature, washed in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature followed by 4 washes in PBS. Coverslips with cells were placed on glass slides in a Perma Fluor mounting solution (Immunon, Pittsburg, PA), and analyzed by a confocal scanning system MicroRadiance (Bio-Rad, UK), equipped with an Argon ion laser and mounted on a light microscope Eclipse 600 (Nikon, Japan), using the software LaserSharp 2000 (Bio-Rad, UK) as described before [13].

### Isolation of microsomal membranes

Cells after washing several times in KCl were homogenised in 0.15 M KCl and 25 mM Tris-HCl pH 7.4. The homogenate was then centrifuged at 10,000 g and the obtained supernatant was centrifuged at 100,000 g for 1 h. The microsomes were resuspended in 0.15 M KCl. Protein concentration was determined

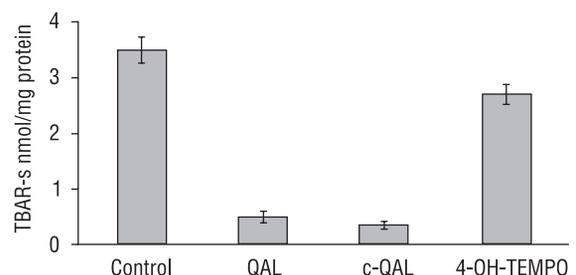
according to the Lowry method [15] using BSA as a standard.

### Microsomal lipid oxidation

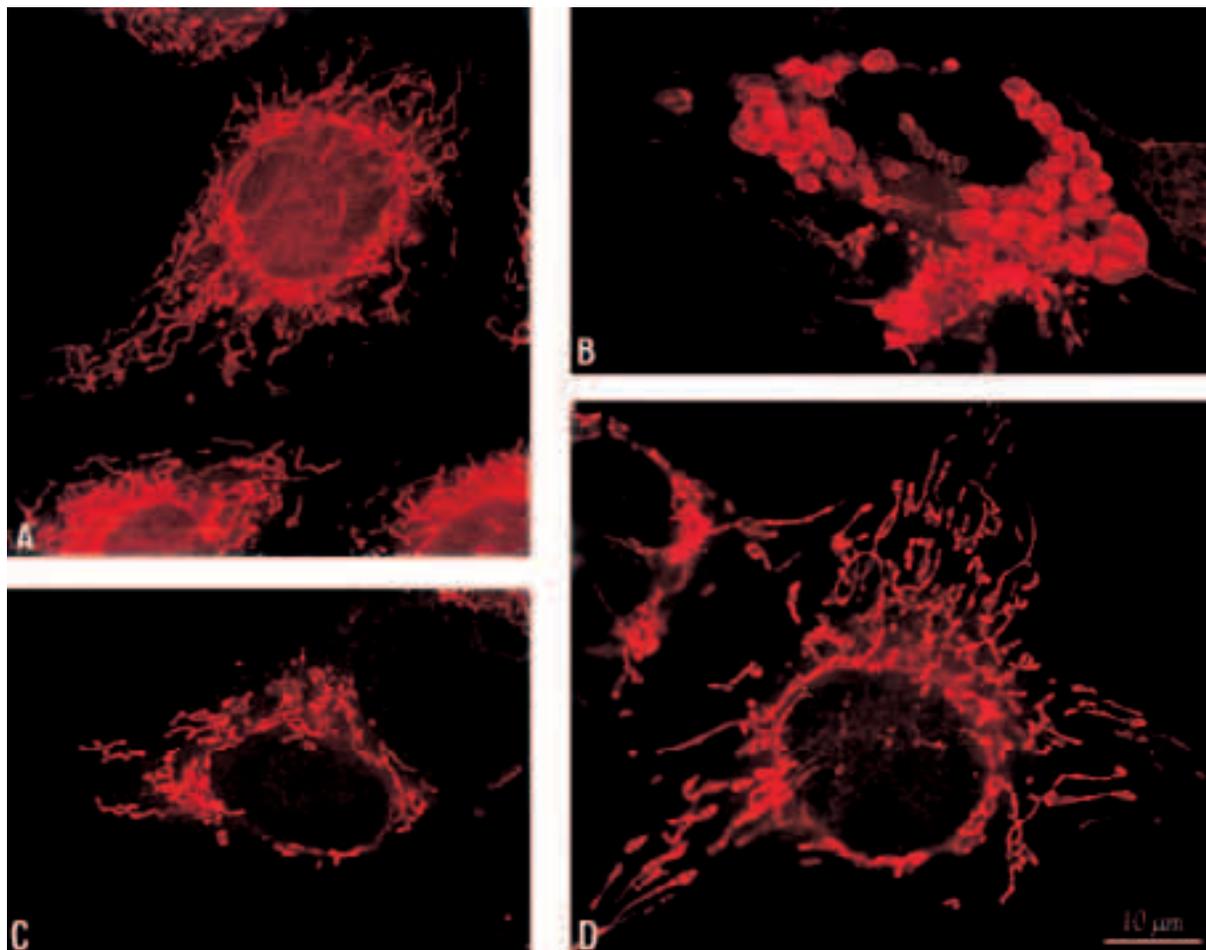
Microsomal membranes (1.5 mg of protein suspended in 2.0 ml of phosphate buffer pH 7.4) were treated with 2.5 mM  $\text{Bu}^t\text{OOH}$  for 30 min. Aminoxyls were added to the samples as ethanol solution before addition of  $\text{Bu}^t\text{OOH}$ . The control samples were treated with  $\text{Bu}^t\text{OOH}$ , and instead of aminoxyls, appropriate amounts of ethanol were added. The extent of TBAR-s production was measured by the method of Buege and Aust with 0.1 mM butylated hydroxytoluene (BHT) to prevent eventual peroxidation of microsomal lipids during assay [3].

## RESULTS

Aminoxyls, when added as ethanolic solution to microsomes, inhibited lipid peroxidation as assessed by the number of TBAR-s produced (Fig. 2). At 100  $\mu$ M concentration QAL and C-QAL inhibited lipid peroxidation almost completely. On the other hand efficacy of 4-OH-TEMPO was much lower in comparison with the lipid soluble aromatic aminoxyls QAL and C-QAL. Incubation of osteosarcoma cells with QAL resulted in distinct changes in the shape and intracellular redistribution of mitochondria. Mitochondria become initially rounded in shape, being not only granular but also distinctly enlarged (Fig. 3). C-QAL and 4-OH-TEMPO treated cells behaved like control ones i.e. mitochondria were filamentous and finally scattered throughout the cytosol (Fig. 3). QAL, being an inducer of morphological changes of aminoxyl-treated cells, revealed another interesting peculiarity. It can be easily noticed that mitochondria in QAL-treated cells display different intracellular localisation, being clustered perinuclearly (Fig. 3).



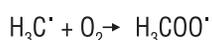
**Figure 2.** Effects of aminoxyls on TBAR-s formation in microsomes isolated from human osteosarcoma cells 143B. The incubation mixture contained 1.5 mg of microsomal protein 2.0 ml phosphate buffer pH 7.4 and 2.5 mM  $\text{Bu}^t\text{OOH}$ . After 30 min incubation at  $25^{\circ}\text{C}$  0.5 ml of the mixture was assayed for TBAR-s determination. Aminoxyls were added as ethanol solution and appropriate amounts of ethanol were added to control samples ( $n = 5$ ).



**Figure 3.** Morphological changes of mitochondria in 143B cells. Cells were treated with aminoxyls dissolved in DMSO during 24 h. Appropriate amounts of DMSO were added to control samples. **A.** Control cells; **B.** 100  $\mu\text{M}$  QAL; **C.** 100  $\mu\text{M}$  c-QAL; **D.** 100  $\mu\text{M}$  4-OH-TEMPO.

## DISCUSSION

Our results prove that quinolinic aromatic aminoxyls are extremely efficient antioxidants protecting endoplasmic reticulum phospholipids from radicals arising from  $\text{Bu}^t\text{OOH}$  decomposition which provide mainly methyl carbon centred radicals  $\cdot\text{CH}_3$  [1, 11] from which peroxy species initiating lipid peroxidation can be formed according to the equation:



Methyl peroxy radicals can be regarded as the main destructive species in  $\text{Bu}^t\text{OOH}$  dependent cytotoxicity [17]. Aromatic aminoxyls possess the ability to react with peroxy radicals [4] while piperidine ring dependent 4-OH-TEMPO can intercept only carbon centred radicals [2].

Mitochondria are both the major ATP producer and also the main intracellular source of ROS of the mammalian cells [14]. Several structural character-

istics of mitochondria can be changed during apoptosis [12] and antioxidants can now be regarded as one of the most efficient apoptosis inducers [18]. Taking into account this assumption we inspected morphology of mitochondria and their localisation inside the cell after exposition on aminoxyl antioxidants. After 24 h of treatment of osteosarcoma 143B cells with 100  $\mu\text{M}$  QAL the spatial redistribution of mitochondria evolved in the majority of the treated cells from scattered in non-exposed control cells on highly asymmetric perinuclear clustered distribution can be seen (Fig. 3). Mitochondria in the control cells, as well as exposed on c-QAL while stained with fluorescent dye CMX Ros revealed thin, filamentous structures under the confocal laser microscope. Exposition of cells on QAL results in distinct changes in shape of mitochondria into the population of enlarged granular structures. QAL induced modifications of the spatial redistribution of mitochondria as well as their morphology from

control filamentous to enlarged granular ones seem to be topics of special interest. Our results provide the first evidence that some antioxidants can act as tumour necrosis factor (TNF) mimic in spite of mitochondrial ultrastructure and redistribution. It has already been shown that TNF initiates hyperphosphorylation of kinesin light chain [8] and induces perinuclear clustering of granular mitochondria via 55 kDa receptor [7].

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