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Assaying the Antioxidant and Radical Scavenging Properties of Aliphatic Mono- and Di-*N*-oxides in Superoxide Dismutase-Deficient Yeast and in a Chemiluminescence Test

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ABSTRACT. The antioxidative action of amphiphilic mono-(alkanoylamino) ethyldimethylamine-*N*-oxides (EDA), di-*N*-oxides 1,1-bis{[2-(*N*,*N*-dimethylamino)ethyl]amido}alkane-di-*N*-oxides (MEDA) and 1,1-bis {[3-(*N*,*N*-dimethylamino)propyl]amido}alkane-di-*N*-oxides (MPDA) with a 12- and 14-membered acyl chain against *tert*-butylhydroperoxide (TBHP)-produced peroxyl and paraquat (PQ)-generated superoxide radicals was determined in superoxide dismutase-deficient mutants of *Saccharomyces cerevisiae*, and, in parallel, in a chemical assay based on chemiluminescence changes caused in a luminol system by peroxyl radicals generated from the azo-compound 2,2´-azobis(2-amidinopropane dihydrochloride) (AAPH). At 30 μmol/L, the shorter-chain compounds did not affect strain survival while longer-chain ones, in some cases, lowered the survival of *sod2* and *sod1 sod2* cells. Whether nontoxic or medium-toxic, all *N*-oxides protected the *sod* strains against the toxic effect of PQ and TBHP, the protection being stronger with the di-*N*-oxides. The survival was lowered only by 14-MPDA in the TBHP-exposed *sod2* mutant. Membrane lipids isolated from all strains were protected against TBHP-induced peroxidation by both mono- and di-*N*-oxides, the protection being dependent on the alkyl chain length. Mono-*N*-oxides were again less active than di-*N*-oxides with the same alkyl chains, the antiperoxidative activity being also dependent on lipids isolated from the individual mutants. In the chemiluminescence assay, the IC_{50} value of the *N*-oxides for scavenging of radicals generated from AAPH generally decreased (*i.e*. the scavenging efficiency increased) with increasing chain length and was the highest in MEDA.

Abbreviations

AAPH	2,2´-azobis(2-amidinopropane) dihydrochloride
IC_{50}	antioxidant concentration that reduces luminol luminescence to 50 %
EDA	2-(alkanoylamino)ethyldimethylamine-*N*-oxides; *see* Fig. 1
MEDA	1,1-bis{[2-(*N*,*N*-dimethylamino)ethyl]amido}alkane-di-*N*-oxides; *see* Fig. 1
MPDA	1,1-bis{[3-(*N*,*N*-dimethylamino)propyl]amido}alkane-di-*N*-oxides; *see* Fig. 1
PDA	3-(alkanoylamino)propyldimethylamine-*N*-oxides; *see* Fig. 1
PQ	paraquat
ROS	reactive oxygen species
SOD	superoxide dismutase (EC 1.15.1.1)
SUV	small unilamellar vesicles
TBA	thiobarbituric acid
TBHP	*tert*-butylhydroperoxide
TBRS	thiobarbituric acid-reactive substances
TMA-DPH	*N*,*N*,*N*-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium 4-toluenesulfonate

The antioxidative properties of nitroxides have made them potentially useful in preventing health problems caused by oxidative stress (Mitchell *et al.* 2001), oxidative damage of foodstuffs (Maurya *et al*. 2006) and cosmetics (Damiani *et al*. 2006). The chemical mechanisms of antioxidative activity and structure–activity relationship of nitroxides are being intensely investigated (Damiani *et al*. 2005). Nitroxides can act as multifunctional antioxidants that remove superoxide anions as superoxide dismutase mimics (Offer *et al.* 2000), trap carbon-centered radicals (Blough 1988) or terminate radical chain reactions (Nilsson *et al*. 1989). They also attenuate oxidative damage in various experimental models such as cultured cells (Meto-

diewa *et al*. 2000). Their effects depend on their structure, *e.g.*, ring size and ring substituents (Goldstein *et al*. 2006). The wide range of oxidation–reduction properties of nitroxides stems from their ability to act both as electron acceptors and donors (Krishna and Samuni 1994; Metodiewa *et al*. 2000).

In vitro investigations of mono- and dinitroxides indicate that nitroxides with two NO groups exert a stronger action against lipid peroxidation than those with a single group (Damiani *et al.* 2005). Recently, the investigations have focused on other compounds with the NO group – the *N*-oxides, which have been in clinical use for 15 years as bioreductive prodrugs because of their propensity to undergo one- or four-electron reductions under hypoxic conditions and produce further cytotoxic events that make them very useful as, *e.g.*, antitumor drugs (Anderson *et al*. 2005). Preliminary experiments with trapping of superoxide radical and radicals derived from peroxidized linoleic acid by different *N*-oxide spin traps have recently been reported (Stolze *et al*. 2004). Studies of the interaction between newly synthesized *N*-oxides and liposomal or erythrocyte membranes revealed a strong protective effect of these compounds dependent on the length of the alkyl chain in their molecules and the number of *N*-oxide groups (Kleszczynska *et al.* 2005; Krasowska *et al.* 2006).

Here we report on the antioxidant properties of amphiphilic mono- and di-*N*-oxides against peroxyl and superoxide radicals in live cells of *Saccharomyces cerevisiae* and compare the results of this biological assay with the data provided by a chemical assay based on chemiluminescence changes in a luminol system caused by peroxyl radicals generated from the azo-compound AAPH.

MATERIALS AND METHODS

Yeast strains. Mutant strains with deleted *SOD1* (JS-C50), *SOD2* (JS-C55*)* and both *SOD1 SOD2* (JS-C128) genes were constructed on the genetic background of parental *S. cerevisiae* strain US50-18C (JS-C66) (*MATα PDR1-3 ura3 his1*) (Krasowska *et al.* 2006) as described by Balzi *et al*. (1987). The yeast strains were grown in YEPD medium (in %: bactopeptone 2, glucose 2, yeast extract 1) for 20 h at 30 °C.

Reagents. All amine-*N*-oxides under study were synthesized in our laboratory (Fig. 1). EDA were prepared by acylation of N^1,N^1-dimethylethane-1,2-diamine with long-chain acyl chlorides to 2-(alkanoylamino)-ethyldimethylamines followed by oxidation with hydrogen peroxide. MEDA were prepared by alkylation of ethyl malonate with long-chain 1-bromoalkanes to ethyl 2-alkylmalonates followed by transamidation with N^1,N^1-dimethylethane-1,2-diamine to 1,1-bis{[2-(*N*,*N*-dimethylamino)ethyl]amido}alkanes, and oxidation with hydrogen peroxide. MPDA were prepared by transamidation of ethyl 2-alkylmalonates with N^1,N^1-dimethylpropane-1,3-diamine to 1,1-bis{[3-(*N*,*N*-dimethylamino)propyl]amido}alkanes, and oxidation with hydrogen peroxide. The structure and purity of the compounds were verified using ^{1}H-NMR spectra (*Bruker* Avance DRX 300 instrument) in $CDCl_3$ solution with tetramethylsilane as internal standard.

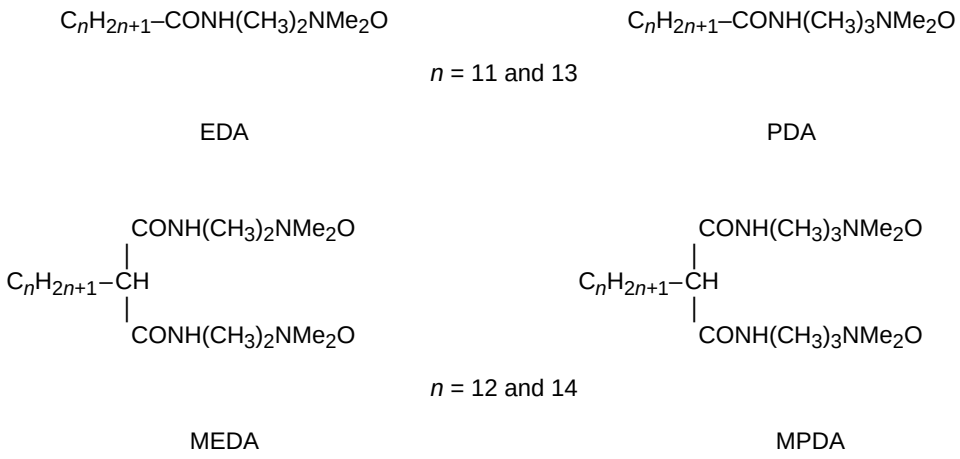

Fig. 1. Structures of tested alkane mono- and di-*N*-oxides.

The fluorescent dye TMA-DPH was purchased from *Molecular Probes* (USA). Lecithin was obtained from *Lipid Products* (UK). The oxidation inducers PQ and TBHP were purchased from *Fluka* (Poland) and *Sigma-Aldrich* (Poland), respectively. TBA and trichloroacetic acid were obtained from *Fluka* (Poland). AAPH was obtained from *Polyscience* (USA), luminol from *Aldrich* (Poland).

Isolation of plasma membrane lipids was done according to Dufour *et al.* (1988) (for *Schizosaccharomyces pombe*) with modifications by Krasowska *et al*. (2002).

Lipid peroxidation was measured in lipids isolated from plasma membranes of *S. cerevisiae* mutants. The oxidants, PQ and TBHP (both at a final concentration of 100 μmol/L), were allowed to induce the oxidation of a lipid containing the antioxidants. The end products of lipid peroxidation, *i.e*. malonaldehyde and other TBRS, were quantified by a method adapted from Aust (1994).

In all experiments, 1 mL samples containing 1.5 μg of lipids and 30 μmol/L antioxidants were incubated for 15 min at 30 °C. Oxidation inducers were added to a concentration of 100 μmol/L and the samples were incubated for another 15 min at the same temperature. Then 2 mL of reagent A (15 % trichloroacetic acid and 0.37 % TBA in 0.25 mol/L HCl) were added and the mixture was thoroughly blended. Test tubes containing the samples were heated for 15 min at 100 °C, cooled under tap water, and centrifuged (2000 ***g***, 10 min). A_{535} was measured on a *Hach* Odyssey spectrophotometer against a reference blank containing the TBA reagent and distilled water.

Antioxidant activity tests. Cell survival after exposure to 10 μmol/L pro-oxidants PQ and TBHP and additionally treated with 30 μmol/L *N*-oxides as antioxidants was determined in 96-well microplates (Krasowska *et al*. 2003) in 100 μL YPG medium (in %: glucose 2, yeast extract 1, bactopeptone 1) ("surface–volume ratio" = 0.28). After a 20-h incubation at 30 °C, A_{600} was measured on an ASYS UVM340 microplate reader (Krasowska *et al*. 2006). EDA and chemically equivalent compounds with two *N*-oxide groups, MEDA and MPDA were tested for their ability to reduce oxygen sensitivity and rescue the growth defects caused by SOD deletions in *S. cerevisiae*. Amine-*N*-oxide concentration was 30 μmol/L since, in a related group of *N*-oxides, this concentration was determined to be nontoxic (Krasowska *et al.* 2006).

The chemiluminescence test (plus IC_{50} values obtained in the chemiluminescence test for the action of mono- and di-*N*-oxides against AAPH-generated peroxyl radicals) (done according to Krasowska *et al*. 2001) was performed in a final volume of 250 μL in 96-well microplates in 0.1 mol/L Tris buffer (pH 9). Luminol was used as a light source after excitation by peroxyl radicals from AAPH. Twenty-five μL freshly prepared AAPH in distilled water was pipetted into the sample, and 100 μL of luminol solution (1 mmol/L stock solution of luminol in 0.1 mol/L NaOH diluted 4× in distilled water) was automatically injected into the sample at the beginning of measurement. The tested compounds were automatically injected 60 s after luminol injection. Photons were counted on EG&G *Berthold* LB96P microplate luminometer at 30 °C.

Estimation of antioxidant localization in the lipid phase was done by determining the effect of the antioxidants on the fluorescence of the probe TMA-DPH incorporated into SUV produced from yeast plasma membrane lipids and 0.6 mmol/L lecithin. The SUV were prepared according to Lentz (1988) with modifications (Jemioła-Rzemińska *et al*. 1996).

The effects of the antioxidants on the fluorescent probe variously immersed in the SUV membrane were assayed by measuring the fluorescence quenching of the probe (Krasowska *et al*. 2006).

RESULTS AND DISCUSSION

Protection of live yeast cells from oxidative stress by N-*oxides.* At 30 μmol/L, 12-EDA, 12-MEDA and 12-MPDA had no appreciable negative effect on the strain survival and 12-MPDA even seemed to enhance the growth of the *sod1* and *sod2* mutants (Table I). In contrast, their counterparts with 14-membered aliphatic chains lowered, in some cases, cell survival. 14-EDA reduced the survival of all strains by 10–20 %, 14-MEDA affected the *sod1* and *sod1 sod2* mutants, and 14-MPDA inhibited the growth of *sod1* and *sod2* mutants by up to 10 % and suppressed the growth of *sod1 sod2* strain by 70 %.

Table I. Survival (%)[a] of *S. cerevisiae* JS-C66 parent strain and its *sod* mutants in the presence of 10 μmol/L pro-oxidants PQ and TBHP and in the presence of 30 μmol/L amine-*N*-oxides

Strain	Control	PQ[b]	TBHP[b]	12-EDA	14-EDA	12-MEDA	14-MEDA	12-MPDA	14-MPDA
JS-C66	100 ± 2.3	100 ± 8	70 ± 15	99 ± 2.4	85 ± 8.4	99 ± 10.5	95 ± 5.5	108 ± 2.8	102 ± 1.6
JS-C50	100 ± 5.6	40 ± 12	35 ± 12	91 ± 6.2	74 ± 15.7	95 ± 2.8	82 ± 3.7	127 ± 2.9	81 ± 7.9
JS-C55	100 ± 5.0	76 ± 12	50 ± 4	96 ± 1.8	70 ± 7.0	97 ± 4.5	99 ± 4.3	110 ± 3.4	89 ± 10
JS-C128	100 ± 7.6	37 ± 12	15 ± 3	82 ± 23	77 ± 11.7	92 ± 8.7	77 ± 7.0	94 ± 2.1	33 ± 2.8

[a]Values from three experiments; (±) – confidence intervals, $\alpha = 0.05$. [b]Data from Krasowska *et al*. (2006).

Exposure to the superoxide generator PQ and hydroperoxide producing TBHP suppressed the survival of *S. cerevisiae sod* mutants by 30–90 %, depending on the SOD deletion. At 10 μmol/L, TBHP exhibited a somewhat stronger toxicity than PQ, both pro-oxidants being more toxic for *sod1* and *sod1 sod2* mutants than for *sod2* cells (Krasowska *et al*. 2006).

Despite their own mild growth-suppressing effects in some cases (*see* Table I), the *N*-oxides protected to a different extent the SOD-deficient yeast strains against the toxic effects of PQ and TBHP (Table II). The protection of the parent strain JS-C66 against growth inhibition caused by PQ was negligible because its survival was not affected by 10 μmol/L PQ. The *N*-oxide-induced increase of survival of the *sod1* mutant JS-C50 exposed to PQ was 5–50 %, with the *sod2* mutant JS-C55 15–60 % and with the *sod1 sod2* strain JS-C128 0–60 %.

Table II. Survival (%)[a] of *S. cerevisiae* strains exposed to paraquat and TBHP (both at 10 μmol/L) and treated with 30 μmol/L EDA, MEDA and MPDA relative to the survival of cells exposed to paraquat and TBHP alone

Strain	12-EDA	14-EDA	12-MEDA	14-MEDA	12-MPDA	14-MPDA
Paraquat						
JS-C66	+1.7 ± 4.2	+0.9 ± 5.9	–4.5 ± 6.1	–4.0 ± 5.0	–1.5 ± 4.5	–1.0 ± 4.4
JS-C50	+12 ± 4.9	+5.0 ± 10.7	+40 ± 14.8	+23 ± 6.1	+53 ± 7.2	+29 ± 9.7
JS-C55	+15 ± 8.4	+23 ± 11.9	+63 ± 6.8	+37 ± 13.7	+33 ± 9.7	+14 ± 4.4
JS-C128	+17 ± 5.6	+19 ± 5.4	+62 ± 8.7	+7.0 ± 7.1	+64 ± 6.9	+0.3 ± 7.7
TBHP						
JS-C66	+24 ± 2.9	+26 ± 3.8	+33 ± 2.4	+27 ± 5.9	+26 ± 2.6	+14 ± 7.8
JS-C50	+59 ± 6.3	+51 ± 1.5	+64 ± 4.6	+64 ± 16.3	+56 ± 6.9	+23 ± 17.0
JS-C55	+20 ± 4.2	+17 ± 2.6	+18 ± 2.8	–2.9 ± 12.0	–5.9 ± 2.1	–50 ± 9.0
JS-C128	+85 ± 3.9	+77 ± 2.6	+79 ± 7.5	+60 ± 7.7	+48 ± 3.4	+52 ± 10.8

[a]*See* footnote *a* to Table I; survival stimulation (+) or inhibition (–).

The protection afforded by the *N*-oxides to TBHP-exposed cells was as a rule higher than with the PQ-exposed ones. The survival of the parent JS-C66 strain by individual *N*-oxides was enhanced, depending on their structure, by 14–30 %, that of the *sod1* mutant JS-C50 by 20–60 %, and that of the *sod1 sod2* mutant JS-C128 by about 50–90 %. With the *sod2* mutant JS-C55, in which the increase in survival caused by 14-EDA and 12-MEDA was some 17 to 18 %, 14-MEDA and 12-MPDA afforded no protection and 14-MPDA showed a 50 % decrease in survival in relation to the level observed with TBHP alone, attesting to a considerable adverse effect of 14-MPDA on *sod2* mutant cells. Also with all other strains, the protection by 14-MPDA was the lowest.

Protection against membrane lipid peroxidation by N-*oxides* in vitro. The peroxidation of lipids isolated from plasma membranes of *sod* mutants after TBHP treatment was markedly dependent on the character of SOD deletion. Lipids from the *sod1 sod2* mutant were the most sensitive to peroxidation and all mutants were 15–30-times more sensitive than the parental strain JS-C66 (Krasowska *et al*. 2006). The compounds with one *N*-oxide group (EDA and PDA) were more active when their alkyl chains were longer (14-EDA and 14-PDA; Krasowska *et al.* 2006) whereas the opposite was true for di-*N*-oxides with extended hydrophilic parts (MEDA and MPDA), in which homologues with shorter alkyl substituent were more active (Table III). Generally, the mono-*N*-oxides were less active than di-*N*-oxides with the same alkyl chains.

Table III. Inhibition by 30 μmol/L *N*-oxides (in %) of TBHP (100 μmol/L)-induced peroxidation of plasma membrane lipids isolated from individual strains

Strain	12-EDA	14-EDA	12-MEDA	14-MEDA	12-MPDA	14-MPDA
JS-C66	15.0 ± 1.0	35.2 ± 6.1	67 ± 2.1	23 ± 5.7	70.5 ± 2.8	50 ± 4.5
JS-C50	20.0 ± 2.4	37.3 ± 3.7	78 ± 3.9	46 ± 6.3	79.1 ± 1.3	45 ± 6.1
JS-C55	14.0 ± 1.2	22.1 ± 5.2	67 ± 5.8	40 ± 7.8	56.4 ± 5.7	28 ± 8.5
JS-C128	13.2 ± 4.6	24.2 ± 7.5	39 ± 6.5	19 ± 4.7	86.8 ± 3.6	68 ± 6.6

[a]*See* footnote *a* to Table I.

The antiperoxidative activity of di-*N*-oxides appeared to depend on the lipid composition of individual mutants. While the prevention of peroxidation of lipids from the *sod1* and *sod2* mutants in both the MEDA and MPDA was about equal, with the *sod1 sod2* mutant the latter was considerably more active than the former.

Incorporation of N-*oxides into membranes*. Like previously found with PDA (Krasowska *et al.* 2006), in liposomes reconstituted from the membranes of individual strains mono-*N*-oxides of EDA caused a concentration-dependent decrease in the polarization coefficient of the membrane-matrix-localized TMA-DPH probe in the case of lipids from the *sod2* (JS-C55) and *sod1 sod2* (JS-C128) mutants (Fig. 2), indicating an interaction with the lipids. Di-*N*-oxides MEDA and MPDA with bulky hydrophilic parts caused no appreciable change of the probe polarization coefficient (*data not shown*).

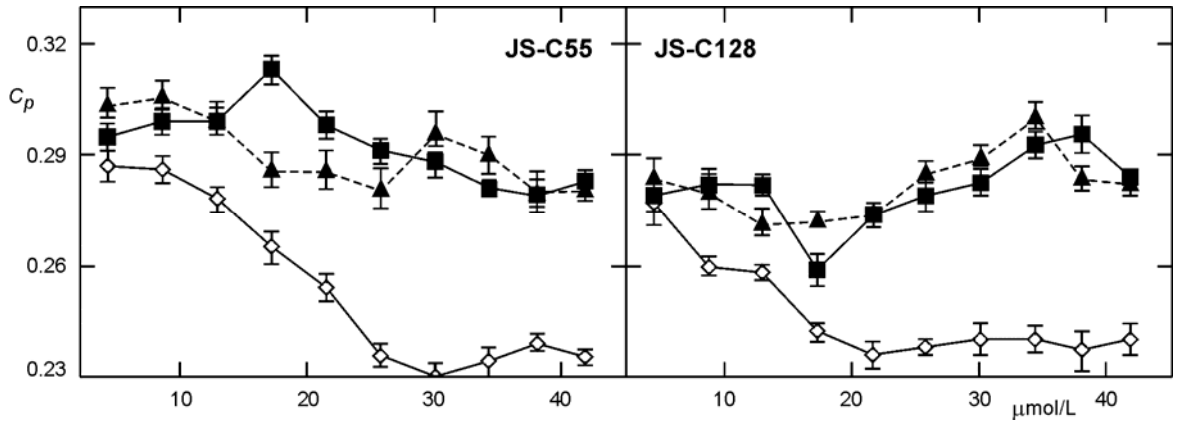

Fig. 2. Polarization coefficients (C_p ± confidence intervals) of TMA-DPH fluorescence in SUV reconstituted from the membranes of the tested strains as affected by 12-EDA (*empty diamonds*), 12-MEDA (*full triangles*), and 12-MPDA (*full squares*) (all at concentrations in μmol/L).

Chemiluminescence quenching as a tool for assessing antioxidant action of N-*oxides*. Summarizing the data of the chemiluminescence assay (Table IV) and comparing them with similar data for the PDA-type compounds (Krasowska *et al*. 2006) showed that with increasing length of the alkyl chain in the *N*-oxide molecule the IC_{50} value tends to decrease (*i.e*. the radical-quenching action tends to increase with increasing alkyl chain length, which is considered to play an important role in biological systems by affecting the ability of the given compound to be incorporated into the plasma membrane).

Table IV. IC_{50} values (μmol/L)[a] obtained in the chemiluminescence test for the action of mono- and di-*N*-oxides against AAPH-generated peroxyl radicals

N-Oxide	IC_{50}	*N*-Oxide	IC_{50}
11-PDA[b]	6040 ± 0.95	14-EDA	>640
13-PDA[b]	43 ± 0.35	12-MEDA	6.0 ± 0.43
15-PDA[b]	730 ± 0.70	14-MEDA	0.12 ± 0.60
12-EDA	>700	12-MPDA	1100 ± 0.50
		14-MPDA	600 ± 0.90

[a]*See* footnote *a* to Table I. [b]Data from Krasowska *et al*. (2006).

In the chemiluminescence test, di-*N*-oxides were more effective against peroxyl radicals from AAPH than mono-*N*-oxides, the most potent by far being the MEDA. This is in keeping with their protective action against the adverse effects of PQ and TBHP on yeast survival and also with the antiperoxidative effect on isolated yeast plasma membrane lipids.

Krasowska *et al.* (2006) showed that mono-*N*-oxides from the group of PDA act as efficient SOD mimics and inhibitors of lipid peroxidation in yeast. Other compounds with NO group, such as nitroxides with one or two nitroxide functions, have also been studied for comparative purposes (Damiani *et al*. 2005).

The magnitude of the antioxidative action of individual compounds was found to vary with the length of the alkyl chain and the structure of the hydrophilic part of mono-*N*-oxides (EDA and also PDA; *cf*. Krasowska *et al*. 2006) or di-*N*-oxides MEDA and MPDA. As seen from fluorescence polarization data, compounds with long alkyl chains and single *N*-oxide groupings are probably incorporated into the plasma membrane while similar di-*N*-oxides with bulky hydrophilic parts of molecules are not. The stimulation of survival of the yeast strains provided by the di-*N*-oxides thus appears to be mostly due to the neutralization of pro-oxidants exterior to the membrane.

This assumption is supported by the finding that, in tests on live cells, di-*N*-oxides were more effective than mono-*N*-oxides against the superoxide anion inducer PQ. Superoxide anion does not penetrate into the lipid bilayer and PQ exerts its activity rather on the surface of cells. Despite the low ability of di-*N*-oxides to incorporate into the lipid bilayer as compared with mono-*N*-oxides, their activity against TBHP are of similar magnitude (*see* Table II). This can reflect the presence of two N→O groups in the molecule of di-*N*-oxides, which gives rise to a higher antioxidative power.

Combinations of the pro-oxidants with the *N*-oxides showed, in some cases, interesting effects on the cells, *e.g*., marked survival suppression was exhibited by 14-MPDA in TBHP-exposed *sod1 sod2* mutant. Should the two compounds act additively, then the combination TBHP + 14-MPDA should suppress the growth of the strain to ≈5 % of the control because of the marked growth inhibition by TBHP. In fact, the survival was some 30 % of the control because the growth-suppressing effect of the pro-oxidant was reduced by 50 % by the *N*-oxide. On the other hand, the growth of the *sod2* mutant treated as above should be about 45 % while it was in fact some 25 % of the control, *i.e*. the combination was more lethal than expected.

Similarly, PQ alone caused 60–70 % decrease in the growth of the *sod1 sod2* mutant, and so did 14-MPDA alone. In the presence of their combination, however, the growth inhibition was 37 ± 12 %, although it should have been much lower provided that their adverse effects added up.

The test with yeast *sod* mutants appears to be eminently suitable for assessing the action of various antioxidants and revealing their actual effects, harmful and/or protective, when used in combination with certain pro-oxidants. For instance, the 14-MPDA appears to be unsuitable for use as antioxidant and oxidative-stress protectant of live cells but can provide considerable protection against free radicals and other ROS in systems *in vitro*.

Additional indication about the relationships between the structure and activity of *N*-oxides was obtained by the chemiluminescence test in which the samples were composed of chemical compounds only and luminescence of luminol induced by peroxyl radicals derived from AAPH was quenched by the antioxidants. Although the data from this test cannot be directly correlated with the results of the survival test with *S. cerevisiae*, there are some similarities. The IC_{50} value for di-*N*-oxides, especially MEDA, was much higher than for mono-*N*-oxides of both PDA and EDA types, and, at the same time, nearly all *N*-oxides with longer aliphatic chain appeared to be more potent than short-chain ones.

In experiments with peroxidation of lipids from the yeast plasma membrane, all tested *N*-oxides were active against TBHP (*cf*. Table III). Like in both the survival test and the chemiluminescence assay, di-*N*-oxides were more active than mono-*N*-oxides; homologues with shorter alkyl chains were more active in the case of di-*N*-oxides and less active in the case mono-*N*-oxides. An appropriate selection of a suitable *N*-oxide with optimum antioxidative properties based on different techniques is therefore very important for potential applications. Di-*N*-oxides, such as MPDA, seem to be suited for protection of cells or substances against surface-acting pro-oxidants, while mono-*N*-oxides could provide protection against intramolecular damage, such as lipid peroxidation.

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REFERENCES

ANDERSON R.F., SHINDE S.S., HAY M.P., DENNY W.A.: Potentation of the cytotoxicity of the anticancer agent triapazamine by benzotriazine *N*-oxides: the role of redox equlibra. *J.Am.Chem.Soc.* **128**, 245–249 (2005).

AUST S.D.: Thiobarbituric acid assay reactants. *Meth.Enzymol*. **1**, 225–223 (1994).

BALZI E., CHEN W., ULASZEWSKI S., CAPIEAUX E., GOFFEAU A.: The multidrug resistance gene PDR1 from *Saccharomyces cerevisiae*. *J.Biol.Chem.* **262**, 16871–16879 (1987).

BLOUGH N.V.: Electron paramagnetic resonance measurements of photochemical radical production in hemic substances. 1. Effect of O_2 and charge on radical scavenging by nitroxides. *Environ.Sci.Technol.* **22**, 77–82 (1988).

DAMIANI E., CASTAGNA R., ASTOLFI P., GRECI L.: Aromatic and aliphatic mono- and di-nitroxides: a study on their radical scavenging abilities. *Free Radical Res*. **39**, 325–336 (2005).

DAMIANI E., ASTOLFI P., CIONNA L., IPPOLITI F., GRECI L.: Synthesis and application of a novel sunscreen-antioxidant. *Free Radical Res*. **40**, 485–494 (2006).

DUFOUR J.P., AMORY A., GOFFEAU A.: Plasma membrane ATPase from the yeast *Schizosaccharomyces pombe*. *Meth.Enzymol*. **157**, 513–528 (1988).

GOLDSTEIN S., SAMUNI A., HIDEG K., MERENYL G.: Structure–activity relationship of cyclic nitroxides as SOD mimics and scavengers of nitrogen dioxide and carbonate radicals. *J.Phys.Chem*. **110**, 3679–3685 (2006).

JEMIOŁA-RZEMIŃSKA M., KRUK J., SKOWRONEK M., STRZAŁKA K.: Location of ubiquinone homologues in liposome membranes studied by fluorescence anisotropy of diphenyl-hexatriene and trimethylammonium-diphenyl-hexatriene. *Chem.Phys.Lipids* **79**, 55–63 (1996).

KLESZCZYNSKA H., BONARSKA D., PRUCHNIK H., BIELECKI K., PIASECKI A., LUCZYNSKI J., SARAPUK J.: Antioxidative activity of new *N*-oxides of tertiary amines: membrane model and chromogen studies. *Z.Naturforsch.* **60**, 567–571 (2005).

KRASOWSKA A., DZIADKOWIEC D., ŁUKASZEWICZ M., WOJTOWICZ K., SIGLER K.: Effect of antioxidants on *Saccharomyces cerevisiae* mutants deficient in superoxide dismutases. *Folia Microbiol*. **48**, 754–760 (2003).

KRASOWSKA A., CHMIELEWSKA L., GAPA D., PRESCHA A., VÁCHOVÁ L., SIGLER K.: Viability and formation of conjugated dienes in plasma membrane lipids of *S. cerevisiae*, *S. pombe*, *R. glutinis*, and *C. albicans* exposed to hydrophilic, amphiphilic and hydrophobic pro-oxidants. *Folia Microbiol.* **47**, 145–151 (2002).

KRASOWSKA A., PIASECKI A., POLINCEUSZ A., PRESCHA A., SIGLER K.: Amphiphilic amine *N*-oxides with aliphatic alkyl chain act as efficient SOD mimics, antioxidants and lipid peroxidation blockers in yeast. *Folia Microbiol*. **51**, 99–107 (2006).

KRASOWSKA A., ROSIAK D., SZKAPIAK K., OŚWIĘCIMSKA M., WITEK S., ŁUKASZEWICZ M.: The antioxidant activity of BHT and new phenolic compounds PPA and PYA measured by chemiluminescence. *Cell Mol.Biol.Lett*. **6**, 71–81 (2001).

KRISHNA M.C., SAMUNI A.: Nitroxides as antioxidants. *Meth.Enzymol*. **234**, 580–589 (1994).

LENTZ B.R.: *Spectroscopic Membrane Probes* (L.M. Loew, Ed.). CRC Press, Boca Raton (USA) 1988.

MAURYA D.K., DEVASAGAYAM T.P., NAIR C.K.: Some novel approaches for radioprotection and the beneficial effect of natural products. *Indian J.Exp.Biol.***44**, 93–114 (2006).

METODIEWA D., KOCHMAN A., GEBICKA L., SKOLIMOWSKI J.: Evidence for peroxidatic oxidation of substituted piperidine nitroxides, acting as apoptosis inducers in Yoshida sarcoma cells *in vivo*. *Anticancer Res.* **20**, 2421–2426 (2000).

MITCHELL J.B., KRISHNA M.C., KUPPUSAMY P., COOK J.A., RUSSO A.: Protection against oxidative stress by nitroxides. *Exp.Biol.Med.* **226**, 620–621 (2001).

NILSSON U.A., OLSSON L.I., CARLIN G., BYLUND F.A.: Inhibition of lipid peroxidation by spin labels. Relationships between structure and function. *J.Biol.Chem.* **264**, 11131–11135 (1989).

OFFER T., RUSSO A., SAMUNI A.: The pro-oxidative activity of SOD and nitroxide SOD mimics. *FASEB J.* **14**, 1215–1223 (2000).

STOLZE K., ROHR-UDILOVA N., ROSENAU T., STADTMULLER R., NOHL H.: Very stable superoxide radical adducts of 5-ethoxycarbonyl-3,5-dimethyl-pyrroline *N*-oxide (3,5-EDPO) and its derivatives. *Biochem.Pharmacol*. **69**, 1351–1361 (2004).