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Cytotoxicity of the novel spin trapping compound 5-ethoxycarbonyl-3,5-dimethyl-pyrroline *N*-oxide (3,5-EDPO) and its derivatives

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Abstract—ESR spin trapping allows detection of superoxide radicals. Novel spin traps forming more stable superoxide adducts ($t_{1/2}$ ca. 12–55 min) were tested for their toxicity to cultured cells. The following toxicity ranking was obtained: 4,5-DPPO > 4-BEMPO ~ 3-BEMPO > *trans*-3,5-EDPO > 3,5-DPPO ~ 4,5-DiPPO ~ 4,5-EDPO > *cis*-3,5-EDPO ~ 3,5-DiPPO > DEPMPO. In conclusion, 4,5-EDPO, *cis*-3,5-EDPO and 3,5-DiPPO can be recommended for further investigation of superoxide in biological systems.

Atmospheric oxygen can be univalently reduced to superoxide radicals in a number of enzymatic and non-enzymatic reactions. Superoxide can dismutate spontaneously or catalysed by SOD giving hydrogen peroxide which, in turn, reacts with transition metals or other redox equivalents producing hydroxyl radicals. Thus, superoxide radicals play a special role and represent a precursor of other reactive oxygen species. For this reason special attention of researchers has been drawn to superoxide.[1–5]

This radical has been shown to be involved in ageing[6,7] as well as in the onset and development of inflammation, stroke, cancer, autoimmune and cardiovascular diseases.[8–13] Growing evidence supports the idea that superoxide and/or hydrogen peroxide also possesses important signalling functions regulating cell proliferation, differentiation and migration.[14] Hydrogen peroxide can also regulate the activity of signalling proteins such as protein tyrosine kinases,[15] protein tyrosine phosphatases[16] and peroxiredoxins.[17]

Direct or indirect methods enable the detection of superoxide radicals.[18,19] During indirect detection oxidation or reduction of the detection molecule takes place leading to fluorescence, chemiluminescence or changes in visible absorbance spectra. A disadvantage of indirect methods is often given due to the lack of specificity as well as due to difficulties in the identification of radicals. Direct detection of the unpaired electrons from free radicals is enabled by the electron spin resonance technique. Application of spin traps stabilizes short lived radicals thus allowing the

Abbreviations: LDH, lactate dehydrogenase; MEM, Minimal Essential Medium; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DMPO, 5,5-dimethylpyrroline *N*-oxide; EMPO, 5-(ethoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide; 3,5-EDPO, 5-(ethoxycarbonyl)-3,5-dimethyl-1-pyrroline *N*-oxide; 4,5-EDPO, 5-(ethoxycarbonyl)-4,5-dimethyl-1-pyrroline *N*-oxide; 3,5-DiPPO, 3,5-dimethyl-5-(*iso*-propoxycarbonyl)-1-pyrroline *N*-oxide; 4,5-DiPPO, 4,5-dimethyl-5-(*iso*-propoxycarbonyl)-1-pyrroline *N*-oxide; 3,5-DPPO, 3,5-dimethyl-5-(propoxycarbonyl)-1-pyrroline *N*-oxide; 4,5-DPPO, 4,5-dimethyl-5-(propoxycarbonyl)-1-pyrroline *N*-oxide; 3-BEMPO, 5-butyl-5-(ethoxycarbonyl)-3-methyl-1-pyrroline *N*-oxide; 4-BEMPO, 5-butyl-5-(ethoxycarbonyl)-4-methyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; $O_2^{\cdot -}$, superoxide anion radical.

Keywords: Free radicals; ESR; Spin traps; Oxidative stress; Cytotoxicity; Superoxide.

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measurement of these species at biologically relevant temperatures. Spin traps with specific properties are necessary for this method.

Spin traps such as DMPO, EMPO, BMPO and DEPMPO are commercially available for superoxide detection. The half-life of their superoxide adducts is 45 s,[20] 8.6 min,[1,21,22] 23 min and about 15 min, respectively.[23,24] Since superoxide formation rates in biological systems are rather low, longer accumulation times of superoxide adducts are required. Consequently, the low stability of the superoxide adducts limits the sensitivity of spin trap application.

The aim of the current experiments was to identify an efficient novel spin trap(s) with high stability of superoxide adduct and with low toxicity to the cells.

A number of novel derivatives of the spin trap EDPO with half-lives of superoxide adducts up to 55 min have been synthesized in our laboratory .[4,5] Superoxide adduct half-lives as well as *n*-octanol/buffer partition coefficients of the spin traps are summarized in Table 1. Detection of superoxide radicals in biological systems is of great interest. Thus, toxicity of the spin traps represents an important factor of their application. The current study aims to investigate the toxicity of the novel EDPO derivatives in cultured human cell lines and in primary rat hepatocytes.

The structure of the investigated spin traps is shown in Figure 1. First, the new spin traps were analysed with respect to their toxicity to cultured human colon carcinoma cells SW480. Their effect on the cell number is shown in Figure 2. The concentration of the spin traps was chosen between 10 and 100 mM, in accordance with previous experiments where superoxide radicals were successfully detected in enzymatic model systems such as xanthine/xanthine oxidase.[19]

As Figure 2a and b show, SW480 cell number decreased in a dose-dependent manner for all spin traps tested. However, the rate of decrease in cell number was dependent on the nature of the spin trap. 4,5-DPPO was the most toxic spin trap with considerable cell loss already at 10 mM and about 95% cell loss at 100 mM (Fig. 2b). 3-BEMPO and 4-BEMPO exhibited strong toxicity above 50 mM concentration. 3,5-DPPO, 3,5-EDPO, 4,5-EDPO and 3,5-DiPPO were less toxic to these cells. No significant cell loss was observed with 10 mM 3,5-EDPO and 3,5-DPPO. Only moderate cell loss (<25%) was detected at 50 mM of 3,5-DPPO, 3,5-EDPO, 4,5-EDPO and 3,5-DiPPO (Fig. 2a). LD_{50} values for all investigated spin traps are summarized in Table 1.

3,5-EDPO: $R^1 = C_2H_5$ $R^2 = CH_3$ $R^3 = CH_3$ $R^4 = H$
4,5-EDPO: $R^1 = C_2H_5$ $R^2 = CH_3$ $R^3 = H$ $R^4 = CH_3$
3,5-DPPO: $R^1 = C_3H_7$ $R^2 = CH_3$ $R^3 = CH_3$ $R^4 = H$
4,5-DPPO: $R^1 = C_3H_7$ $R^2 = CH_3$ $R^3 = H$ $R^4 = CH_3$
3,5-DiPPO: $R^1 = iso\text{-}C_3H_7$ $R^2 = CH_3$ $R^3 = CH_3$ $R^4 = H$
4,5-DiPPO: $R^1 = iso\text{-}C_3H_7$ $R^2 = CH_3$ $R^3 = H$ $R^4 = CH_3$
3-BEMPO: $R^1 = C_2H_5$ $R^2 = C_4H_9$ $R^3 = CH_3$ $R^4 = H$
4-BEMPO: $R^1 = C_2H_5$ $R^2 = C_4H_9$ $R^3 = H$ $R^4 = CH_3$

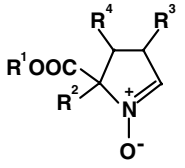

Figure 1. The structure of investigated novel spin traps.

In order to identify the type of cell death (necrosis or apoptosis) LDH release into the medium was measured and apoptosis staining was performed. The results of LDH measurements in cellular supernatants are represented in Figure 2c and d and confirmed the measurements of cell numbers. Again, more toxic spin traps caused higher LDH release.

Staining for apoptotic nuclei did not reveal any difference between controls and spin trap treated cultures (data not shown). Thus, the toxic spin traps 3-BEMPO, 4-BEMPO and 4,5-DPPO caused cell death mostly by necrosis.

Table 1. Half-lives of superoxide adducts, *n*-octanol/buffer partition coefficient and toxicity of investigated spin traps to human SW480 colon carcinoma cells

Spin trap	LD_{50}, mM	$t_{1/2}$, min	*n*-Octanol/buffer partition coefficient	Ref.
EMPO	136	8.6	0.15	1
3-BEMPO	24	47.2	10.84	5
4-BEMPO	21	36.2	10.33	5
3,5-DPPO	78	47.3	1.66	4
4,5-EDPO	142	44.6	0.44	4
4,5-DiPPO	55	42.8	1.03	4
3,5-DiPPO	88	55.0	1.12	4
cis-3,5-EDPO	85[a]	11.5	0.45	4
trans-3,5-EDPO		44.2	0.46	4
DEPMPO	143	13	0.06	23,32

LD_{50} of spin traps was determined from dose–response experiments as the concentration corresponding to 50% cell death after 24 h incubation. LDH release per cell in % of non-treated control was measured after 24 h incubation with 50 mM of the spin trap as described in Materials and methods.
[a] A mixture of 30% *cis*-3,5-EDPO and 70% *trans*-3,5-EDPO.

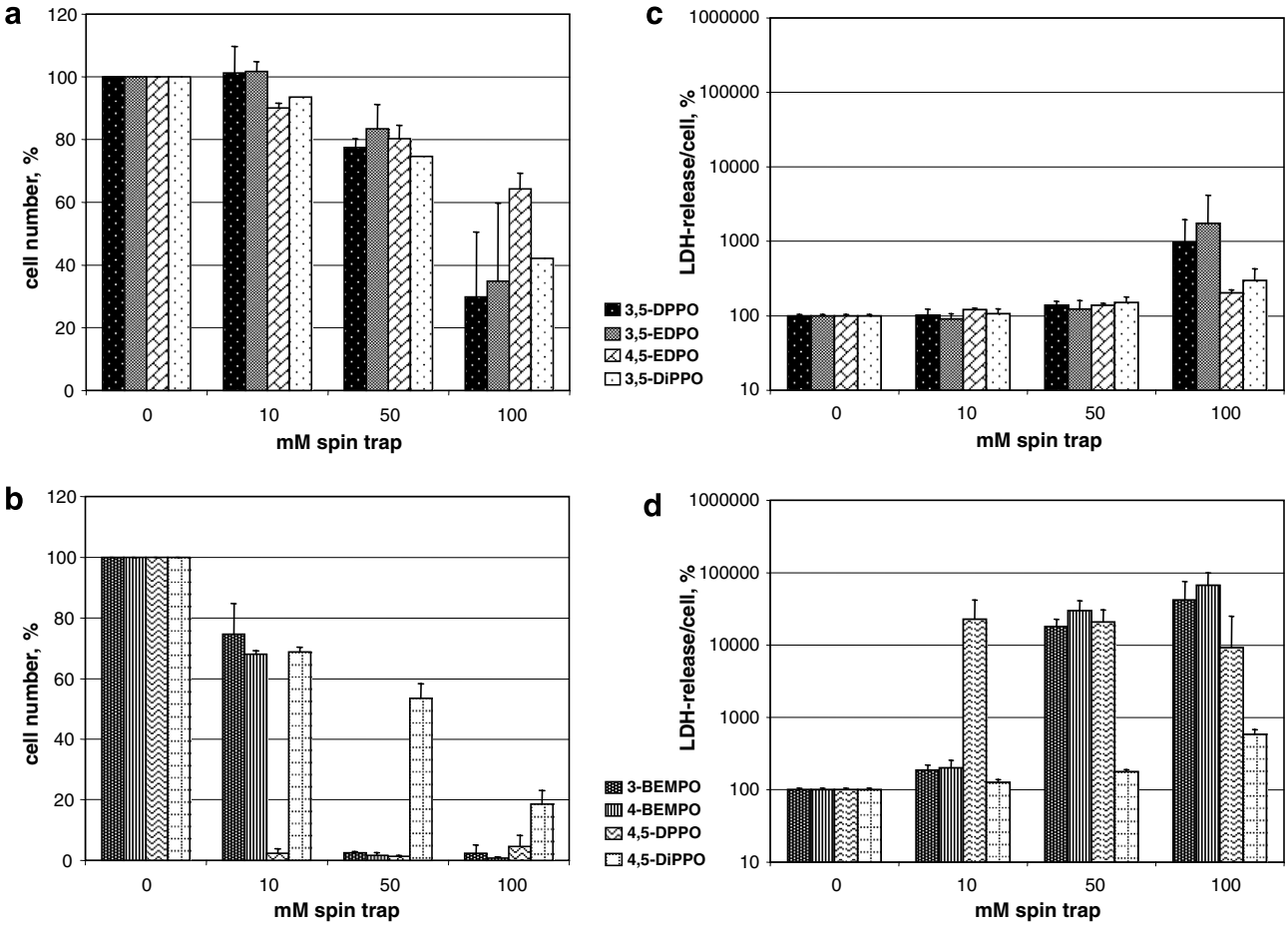

Figure 2. Effect of spin trap on cell number and LDH release of SW480 colon carcinoma cells.

Fifty millimolar of 3,5-DPPO, 3,5-EDPO, 4,5-EDPO and 3,5-DiPPO caused no significant differences of LDH release with respect to the controls (Fig. 2c). LDH increase remained moderate even at 100 mM 4,5-EDPO and 3 ,5-DiPPO.

In order to identify cell type specific differences, toxicological properties of the spin traps were also investigated using cultured human hepatocarcinoma HepG2 cells and primary rat hepatocytes.[33]

Because of their high toxicity 3-BEMPO, 4-BEMPO and 4,5-DPPO were not further investigated despite the high stability of their superoxide adducts.

Figure 3 shows LD_{50} (Fig. 3a) and LDH release (Fig. 3b) in HepG2 cells determined from the dose–response experiments performed in a similar way as described for SW480 cells. LD_{50} was defined as the concentration corresponding to 50% cell death after 24 h incubation. Only the spin traps shown to be less toxic to the colon cells were tested. The concentration of LDH released into the culture supernatants at 50 mM concentration of each spin trap confirmed toxicity as shown by LD_{50} concentrations. In general, the toxicity of the spin traps was moderate, LD_{50} values lying between 47 and 182 mM and resembling those obtained with the colon cell line. However, a different

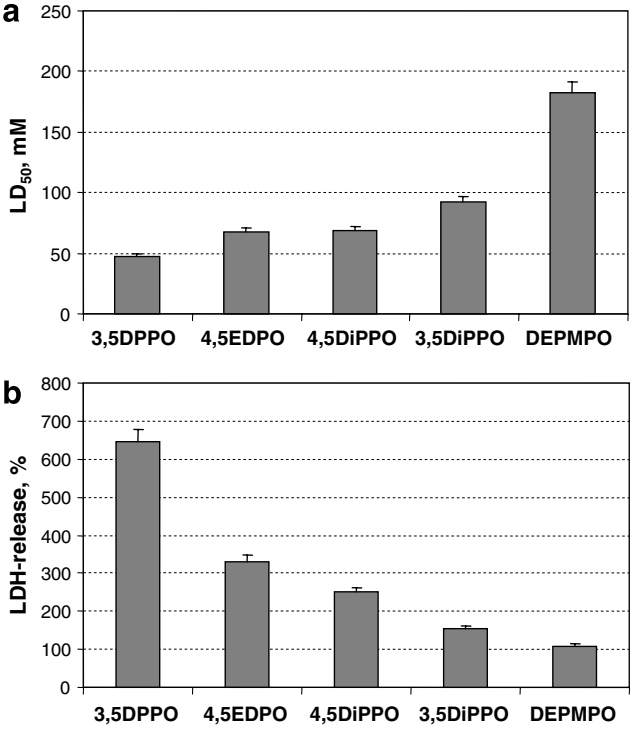

Figure 3. Toxicity of different novel EDPO derivatives to human HepG2 hepatocarcinoma cells.

toxicity ranking was found. For instance, among the spin traps tested, 4,5-EDPO possessed the lowest toxicity to colon cells, but to liver cells it was more toxic than 4,5-DiPPO, 3,5-DiPPO and *cis*-3,5-EDPO. 3,5-DPPO had the lowest LD_{50} in this experiment (47 mM) whereas *cis*-3,5-EDPO was similarly well tolerated as DEPMPO, exhibiting LD_{50} values of 110 mM.

cis- and *trans*-isomers of 3-5-EDPO were separated and compared for their toxicity to HepG2 cells (Fig. 4). *cis*-3,5-EDPO was found to be less toxic than *trans*-3,5-EDPO, as could be judged from both, concentration dependent decrease of the cell number and increase of LDH release.

Trying to simulate in vivo exposition to the spin traps, primary rat hepatocytes were also incubated with these compounds (Fig. 5). In this experiment we tested only the spin traps which were found to be less toxic in both cultured colon and liver carcinoma cells: 4,5-EDPO, 3,5-EDPO and 3,5-DiPPO. DEPMPO was investigated under the same conditions, since its low toxicity to both cultured cells and whole animals is well documented.[25]

The number of viable primary hepatocytes decreased by about 60% with 4,5-EDPO, 3,5-EDPO and 3,5-DiPPO, but remained unchanged with DEPMPO (Fig. 5a). Increased LDH release was observed with 3,5-EDPO, 4,5-EDPO, 3,5-DiPPO and 3,5-EDPO (Fig. 5b) which implicates cell death via necrosis.

The current study investigates cytotoxic effects of novel derivatives of the spin trap 3,5-EDPO in three different cell types. The ideal spin trap should be suitable for future applications in vivo. Cancer is one of the most important disorders in which radicals are involved. Cancer cells usually possess higher detoxification activity as normal cells, but still have the metabolic competence similar to that in the organ of origin. Metabolic competence is, in turn, responsible for the detoxification process and determines the sensitivity of the cells to toxic compounds. Thus, in our case we have tested the toxicity to the cell lines stemming from colon and liver and the information obtained could be used for an assessment of the spin trap toxicity to the respective organ. The fact that some novel spin traps are toxic to colon carcinoma cells makes these compounds unsuitable for detection of radical production in both cultured cells

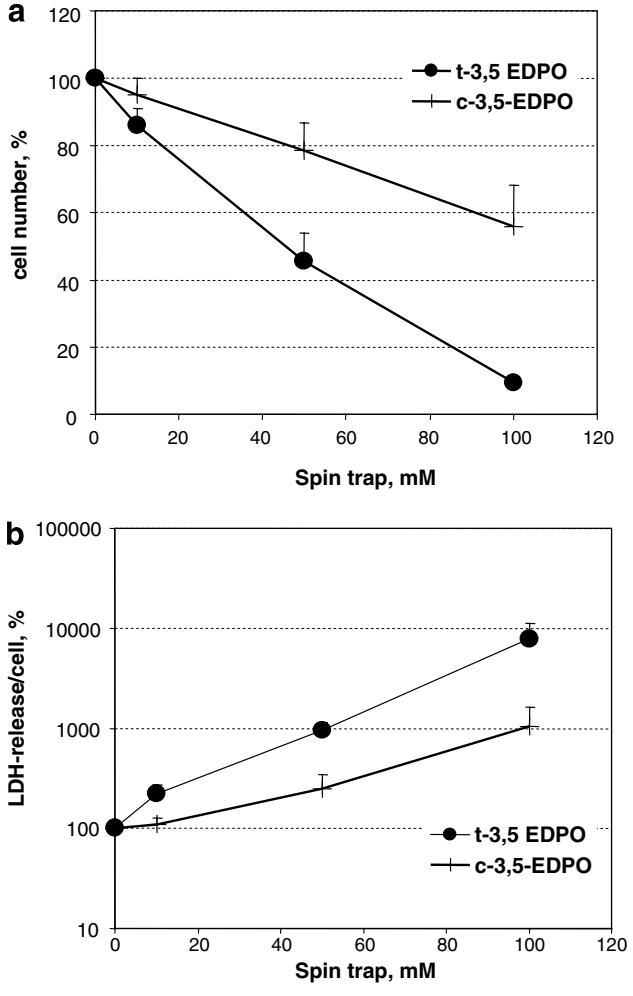

Figure 4. Comparison of *trans*-3,5-EDPO and *cis*-3,5-EDPO toxicity to human HepG2 hepatocarcinoma cells.

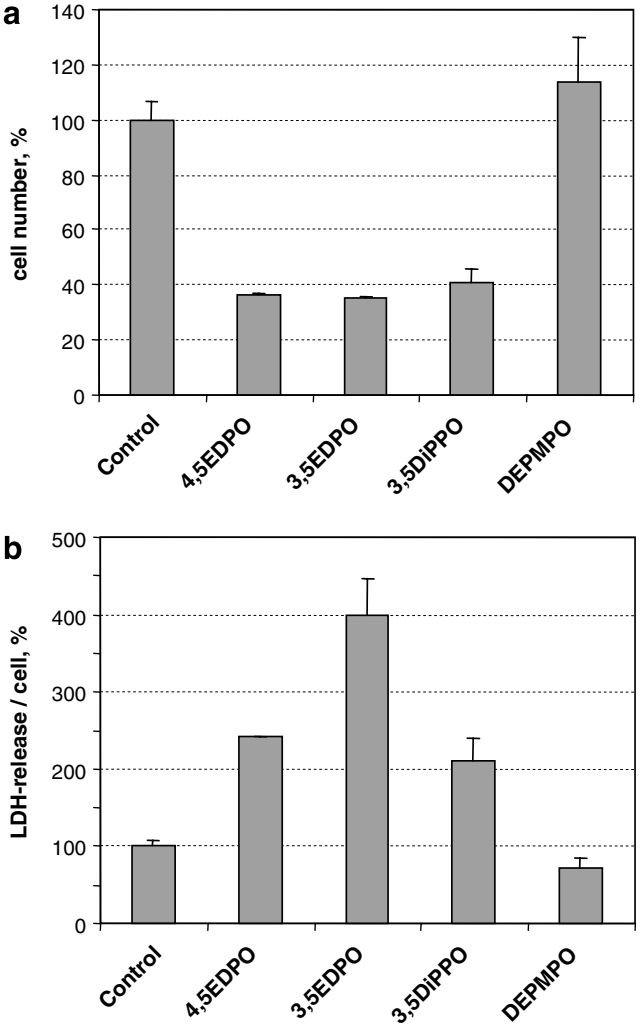

Figure 5. Influence of spin traps on cultured primary rat hepatocytes.

(in vitro) or in animal models of colon cancer (in vivo). Primary cells are closer to the situation in vivo than cultured cancer cell lines. Primary liver cells were chosen for verification of the results with the least toxic spin traps since liver possesses the highest detoxification activity in the body.

It has been shown that cytotoxicity strongly depends on the structure of the spin trap. Our findings confirm results obtained by other authors [26]. The spin traps DEPMPO and EMPO, having superoxide spin adducts with half-lives around 14 and 8.6 min, respectively, had already been studied in different biological systems exhibiting relatively small toxic effects .[27–30] The commonly used spin traps DMPO and PBN are also showing low or moderate toxicity in vivo,[31] but are forming rather unstable superoxide adducts.

Although the spin traps 3-BEMPO, 4-BEMPO and 4,5-DPPO form very stable superoxide adducts $t_{1/2} = 30–55$ min,[4,5] , they exhibit high toxicity even at concentrations as low as 10 mM. This fact makes them unsuitable for radical detection in living cells. The novel spin traps 3,5-DPPO, 4,5-EDPO, 4,5-DiPPO, 3,5-DiPPO and 3,5-EDPO were developed and synthesized by our group [4,5] and their toxic effects were investigated in this paper for the first time.

An inverse correlation was observed between LD_{50} values of the spin trap and LDH release. In general, the spin traps with higher lipophilicity are expected to be more toxic, although our data show that the structure of the spin trap is also important. Different toxicities of *cis*- and *trans*-3,5-EDPO isomers found in the current study confirm this thesis. Clarification of the reason for such different toxicity was not the topic of the current study, inhibition of detoxifying enzymes, however, could be one of the mechanisms.

Primary rat hepatocytes were more sensitive to 4,5-EDPO, 3,5-EDPO and 3,5-DiPPO than other cell lines used in this study. Only DEPMPO was not toxic to these cells. Since the new spin traps possess much higher superoxide adduct half lives, they would provide more quantitative measurements with higher sensitivity. Shorter period of incubation time can compensate for their slightly higher toxicity to the primary cells.

Shorter incubation times of the spin traps with cultured cells are expected to exhibit lower toxicity. So, the spin trap BMPO was found to be less toxic in a 6 h incubation[26] than in a 24 h incubation.[3] Different cell types were used in these studies, however.

The following ranking of the spin traps according to their toxicity can be made (beginning with the highest): 4,5-DPPO > 4-BEMPO > 3-BEMPO > 4,5-DiPPO ~ 3,5-DPPO > *cis*-3,5-EDPO > 4,5-EDPO ~ 3,5-DiPPO > DEPMPO.

The least toxic spin traps were DEPMPO, 3,5-DiPPO, 4,5-EDPO and *cis*-3,5-EDPO. Low toxicity and high stability of 3,5-DiPPO, 4,5-EDPO and *cis*-3,5-EDPO superoxide adducts make these spin traps good candidates for superoxide detection in living cells. Simpler ESR-spectra of superoxide adducts and a threefold higher adduct stability as compared to DEPMPO mark the advantages of these novel spin traps.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.07.054.

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