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Cytotoxicity of novel derivatives of the spin trap EMPO

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Abstract—Free radicals are involved in different regulatory and pathological processes. The formation of superoxide in living cells or whole organisms is of major interest. ESR spin trapping allows identification of the radicals if proper spin traps are available. Our study investigates the toxicity of novel derivatives of the spin trap EMPO to cultured human lung carcinoma cells (A549), breast carcinoma cells (SKBR3), colon carcinoma cells (SW480) as well as to human fibroblasts (F2000). A dose-dependent decrease of the cell number was observed for all spin traps. At 100 mM BuMPO, *t*-BuMPO and *s*-BuMPO caused pronounced cell loss (>90%) and increased LDH-release, while DEPMPO, EMPO, PrMPO and *i*-PrMPO caused only moderate cell loss (<60%) without any effect on the LDH-release after 24 h. At 10 mM and 50 mM the latter agents even decreased LDH-release. 10 mM and 50 mM of *i*-PrMPO as well as 10 mM PrMPO increased intracellular GSH content acting like antioxidants, whereas 50 mM *s*-BuMPO and PrMPO decreased GSH content by 67% and 38%, respectively. Staining for apoptotic nuclei did not reveal any differences between controls and treated cultures indicating necrotic cell death possibly due to membrane toxicity. The following toxicity ranking was obtained: *t*-BuMPO > BuMPO > *s*-BuMPO > PrMPO > *i*-PrMPO ~ DEPMPO ~ EMPO. The least toxic compounds were DEPMPO (LD_{50} = 143 mM for SW480, 117 mM for A549 or 277 mM for F2000) and *i*-PrMPO (LD_{50} = 114 mM for SKBR3), the most toxic one was *t*-BuMPO (LD_{50} = 5–6 mM for all cell types). In conclusion, up to 50 mM *i*-PrMPO ($t_{1/2}$ = 18.8 min) and up to 10 mM *s*-BuMPO ($t_{1/2}$ = 26.3 min) can be recommended for further investigation of superoxide in biological systems.

The interest of researchers in free radicals has been growing since these species are involved in the onset and development of ageing[1,2] and of many diseases such as inflammation, stroke, cancer, autoimmune and cardiovascular diseases.[3–6] A great variety of methods are used for direct or indirect detection of free radicals.[7] The principle of indirect measurements is based on oxidation or reduction of the detecting dye which leads to fluorescence, chemiluminescence or changes in absorbance spectra. However, application of indirect methods is often restricted by the lack of specificity as well as by the difficulties in the identification of radicals involved.

Electron spin resonance makes a direct detection of free radicals possible due to their unpaired electrons. Application of spin traps stabilizes short-lived radicals thus allowing the measurement of these species at room temperature. For this purpose properly designed spin traps are necessary.

A number of different reactive oxygen species can be expected in biological systems. The most common are superoxide, hydroxyl, alkoxyl and peroxyl radicals as well as singlet oxygen and hydrogen peroxide. Frequently superoxide is the primary radical from which other radicals are generated. The reason for that is the presence of various enzymatic one-electron donors.[7]

Until now, only a few spin traps such as DMPO, EMPO and DEPMPO were used for superoxide detection. The half-life of their superoxide adducts is 45 s,[8] 8.6 min[9–11]

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; BuMPO, 5-(*n*-butoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide; *s*-BuMPO 5-(*sec*-butoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide; *t*-BuMPO, 5-(*tert*-butoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide; *i*-PrMPO, 5-(*iso*-propoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide; PrMPO, 5-(propoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; LO·, lipoxyl radical; $O_2^{\cdot -}$, superoxide anion radical.

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

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Table 1. Half-lives of superoxide adducts and *n*-octanol/buffer partition coefficient for investigated spin traps

Spin trap	$t_{1/2}$ (min)	*n*-Octanol/buffer partition coefficient	References
EMPO	8.6	0.15	11
PrMPO	4.6	0.5	11
BuMPO	1.1	1.32	11
i-PrMPO	18.8	0.2	15
s-BuMPO	26.3	1.06	15
t-BuMPO	15.7	0.8	15
DEPMPO	13	0.06	12,13

and about 15 min, respectively.[12–14] 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.

A number of novel derivatives of the spin trap EMPO with half-lives of superoxide adducts up to 26 min have been synthesized in our laboratory.[15] Superoxide adduct half-lives as well as *n*-octanol/buffer partition coefficients of the spin traps are summarized in Table 1. Since superoxide radical formation in biological systems like in living cells or whole organisms is of major interest, biological activities of the spin traps, especially their toxicity, represent an important factor of application. The current study aimed to investigate the toxicity of the novel EMPO derivatives in different human tumour cell lines and in cultured human fibroblasts.

EMPO, PrMPO and BuMPO were synthesized as described in Ref. 11. *i*-PrMPO, *s*-BuMPO and *t*-BuMPO were synthesized as described in Ref. 15. DEPMPO was synthesized as described in Refs. 13 and 14.

Human lung carcinoma cell line A549, human breast carcinoma cell line SKBR3 and human colon carcinoma cell line SW480 were obtained from the American Type Culture Collection, human fibroblasts F2000 were obtained from the Fa. Flow, UK.[16] The cell lines were kept under standard tissue culture conditions. A549, SW480 and F2000 were cultured using medium (MEM) containing 10% foetal calf serum (FCS). SKBR3 were cultured in Dulbecco's Minimal Essential Medium containing 10% foetal calf serum (FCS).

Cells were seeded into 24-well plates. Spin traps were diluted into Minimal Essential Medium containing 1 mg/mL BSA from aqueous stocks, sterile filtered and added to the culture 48 h after plating. After 24 h supernatants were collected, centrifuged at 3000 rpm/4 °C and analyzed for LDH-release as described below. Cell number was determined by neutral red uptake as described below.

For determination of cell number neutral red (50 μg/mL) uptake from serum-free MEM during two hours was measured from triplicate cultures. The dye was taken up into the lysosomes of viable cells and dissolved with 1% acetic acid in 70% ethanol. Thereafter, fixed cultures were stained with Hoechst 33258 (8 μg/mL in PBS). Apoptosis was identified using morphological criteria.[17]

LDH release into the medium was measured using an enzyme detection kit obtained from Roche Diagnostics according to the manufacturer's instructions. Samples were analyzed in duplicate. Triton X 100 treated cultures were used as a positive control.

For GSH measurements cells were incubated with an indicated concentration of spin trap for 24 h, washed twice with PBS and harvested using rubber policeman. Cell pellet was collected by centrifugation at 4 °C and sonicated in 100 μl of 50 mM PBS (pH 7.2) containing 1 mM DTPA. After 15 min centrifugation at 10,000*g*, 4 °C, the supernatant was collected and frozen at −20 °C until GSH measurements. GSH content was determined using Glutathione Assay Kit from Cayman Chemical, USA, according to manufacturer's instruction.

All experiments were performed at least three times. Unless otherwise indicated, data are expressed as means + SD, and statistical differences were determined using ANOVA with significance considered as $p < 0.05$.

The first screening analysis of the spin traps was performed on colon carcinoma cells SW480. Their effect on cell growth is shown in Figures 1A and C. The concentration of the spin traps between 10 and 100 mM was chosen according to the requirements for successful detection of free radicals in enzymatic model systems (xanthine/xanthine oxidase). The spin traps were added to the treatment medium and incubated with cells for 24 h. As Figures 1A and C 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. Based on the dose–response relationship observed two groups of spin traps could be defined: (1) those which cause pronounced cell loss (>90%) at 100 mM concentration and (2) those which cause only moderate cell loss (<60%) at 100 mM concentration. BuMPO, *t*-BuMPO and *s*-BuMPO belong to the first group. As little as 10 mM of *t*-BuMPO reduced the cell number by 90%. At 100 mM of *s*-BuMPO, BuMPO, or *t*-BuMPO cell survival was lower than 10%. DEPMPO, EMPO, PrMPO and *i*-PrMPO belong to the second group. No significant cell loss was observed applying 10 mM of these spin traps. 10–15% of cells died using 50 mM, whereas at 100 mM the cell loss reached only a value between 30% and 55% (Fig. 1C).

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 Figures 1B and D. Again, the spin traps could be divided into two groups depending on whether they are able to increase LDH-release with respect to the controls. As can be seen, incubation with *t*-BuMPO at all concentrations as well as with *s*-BuMPO at 100 mM induced a strong increase in LDH-release

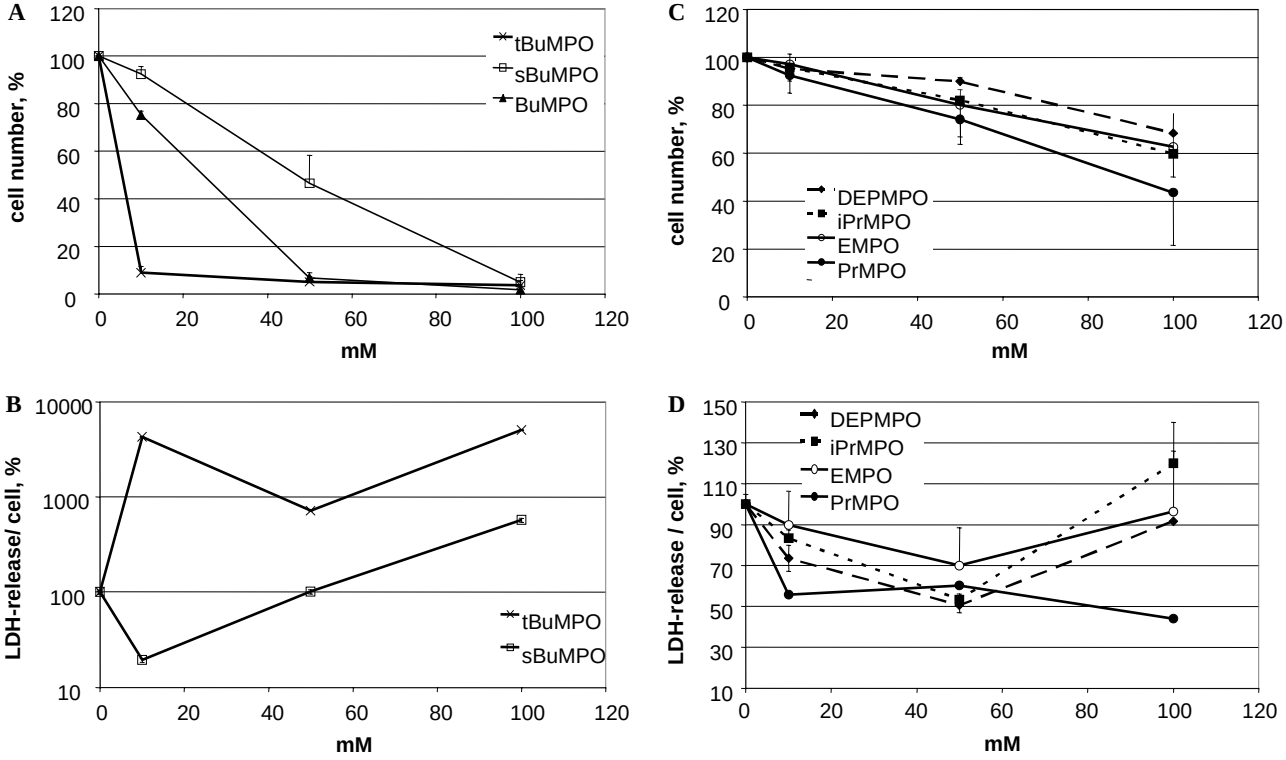

Figure 1. Effect of spin trap concentration on cell number (A, C) and LDH-release (B, D) of SW480 colon carcinoma cells. 7×10^4 cells per well were seeded into 24-well plates. Spin traps were diluted into MEM containing 1 mg/mL BSA, sterile filtered and added to the culture 48 h later. After 24 h, LDH-release and cell number were determined from triplicate cultures as described in the text.

(Fig. 1B). Staining for apoptotic nuclei did not reveal any difference between controls and spin trap treated cultures (data not shown). Thus, the toxic spin traps *t*-BuMPO and *s*-BuMPO caused cell death by necrosis.

LDH measurements in supernatants containing BuMPO could not be performed since this spin trap interferes with the LDH detection method (data not shown). In contrast, DEPMPO, EMPO, *i*-PrMPO and PrMPO even reduced LDH-release at low concentrations (10 and 50 mM), whereas no significant difference from the controls was found at 100 mM (Fig. 1D).

To identify cell type specific differences of sensitivity, spin traps were tested with three additional cell lines. Figure 2A shows LD_{50} of spin traps in A549 lung carcinoma cells determined from dose–response experiments as described for SW480 cells as the concentration corresponding to 50% cell death after 24 h incubation. The results resembled those obtained with the colon cell line: *t*-BuMPO had the lowest LD_{50} (5.16 mM), whereas *i*-PrMPO, DEPMPO and EMPO were less toxic exhibiting LD_{50} values between 89 and 117 mM. The concentration of LDH released into the culture supernatants at 50 mM concentration of each spin trap confirms toxicity as shown by LD_{50} concentrations (Fig. 2B): *t*-BuMPO was the most toxic spin trap with a more than 40-fold LDH induction, whereas LDH-release after incubation with *i*-PrMPO, DEPMPO and EMPO remained unchanged or were even slightly below the control values indicating some protective effect.

Similar data were obtained with other cells such as SW480 human colon carcinoma cells, SKBR3 human breast carcinoma cells or non-malignant human fibroblasts F2000 (see Table 2).

Searching for a compromise between low spin trap toxicity and high stability of its superoxide adducts, *i*-PrMPO and *s*-BuMPO were chosen for further analysis. In the following experiment, the highest non-toxic concentration of spin trap for the cell types under investigation was determined. The results are shown in Figure 3. As the data of cell number and LDH-release in Figures 3A and B show, the spin trap *s*-BuMPO was tolerated by SKBR3, F2000, A549 and SW480 cells if only 10 mM concentration was used. Higher concentrations either affected cell growth or increased LDH-release. The spin trap *i*-PrMPO was less toxic (Figures 3C and D). The cell number after 24 h of incubation dropped to below 60% only if a concentration of 100 mM was used. In the presence of 50 mM *i*-PrMPO, around 60–80% of the cells were still present after 24 h.

In order to investigate more subtle effects of the spin traps on cellular metabolism, *s*-BuMPO, PMPO and *i*-PMPO were tested for their influence on intracellular GSH levels (Fig. 4). SW480 were treated with the spin traps under the same conditions as for the experiments shown in Figure 1. The results are shown in Figure 4. 50 mM of *s*-BuMPO and of PrMPO reduced glutathione content by 67% and 38%, respectively. *i*-PMPO at both

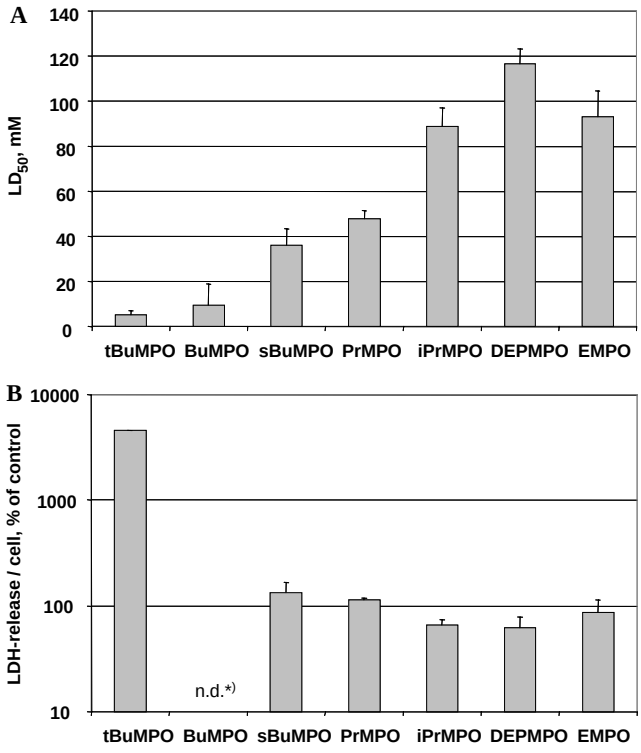

Figure 2. Toxicity of different novel EMPO derivatives to A549 lung carcinoma cells. 10^4 cells per well were seeded into 24-well plates and grown for 48 h. Spin traps were diluted into MEM containing 1 mg/mL BSA, sterile filtered and added to the culture. After 24 h, LDH-release (B) and cell number (A) were determined from triplicate cultures as described in the text. LD_{50} of spin traps was determined from dose–response experiments as described for SW480 cells (Fig. 1) as the concentration corresponding to 50% cell death after 24 h incubation. n.d.*—LDH concentration could not be determined because of interference between the spin trap with LDH detection method.

10 mM and 50 mM as well as PrMPO at 10 mM increased intracellular GSH level.

The current study investigates cytotoxic effects of novel derivatives of the spin trap EMPO in four different cell types. It has been shown that cytotoxicity strongly depends on the structure of the spin trap. Our findings confirm results obtained by other authors.[18] The spin traps DEPMPO and EMPO, having superoxide spin adducts with half-lives of around 14 and 8.6 min, respectively, had already been studied in different biological systems exhibiting relatively small toxic effects.[19–22] Widely used PBN and DMPO are also well tolerated in vivo[23] but form unstable superoxide adducts. Although the spin trap *t*-BuMPO forms a rather stable superoxide adduct ($t_{1/2}$ = 16–22 min),[15,24] it exhibits, however, a high toxicity to all cell types investigated even at concentrations as low as 10 mM. This toxic effect of *t*-BuMPO was also demonstrated for CHO and 9 L tumour cells.[18] The novel spin traps *s*-BuMPO, BuMPO, *i*-PrMPO and PrMPO were developed and synthesized by our group[15] and their toxic effects were investigated in this paper for the first time.

Interestingly, concentrations of spin traps which did not significantly reduce cell numbers (10 mM *s*-BuMPO and up to 50 mM DEPMPO, PrMPO, *i*-PrMPO, or EMPO, Fig. 1) caused a reduction of LDH release below the control value. Since cultured cells are exposed to hyperoxic conditions in comparison to the situation in tissue, some baseline of cellular damage occurs due to the formation of oxygen-derived free radicals. Spin traps scavange free radicals thus acting as antioxidants. These results were confirmed by analysis of GSH level (Fig. 4) which was higher than control in samples incubated with 10 mM *s*-BuMPO, PrMPO and *i*-PrMPO as well as 50 mM *i*-PrMPO. Consequently, the observed reduction in LDH release could be explained by the protective antioxidative activity of these spin traps. Similar antioxidative protecting effects of spin traps DMPO,[25,26] PBN,[27–31] and TEMPOL[32] were observed in other systems.

For all investigated cell lines an inverse correlation was found between LD_{50} values of the spin trap and LDH-release (Fig. 2 and Table 2). In general, the spin traps with higher lipophilicity were also more toxic, although our data show that the structure of the spin trap is also important. Together with apoptosis staining data, which did not show any difference between spin trap treated cultures and controls, this finding indicates a necrotic cell death most likely due to the loss of membrane integrity. Consequently, the toxicity of spin traps could be expected to be similar in different cell types as shown in our study.

Table 2. Toxicity of novel EMPO derivatives to the different cell lines

	t-BuMPO	BuMPO	*s*-BuMPO	PrMPO	*i*-PrMPO	DEPMPO	EMPO
SW480							
LD_{50} (mM)	6	25	47	90	122	143	136
LDH release (%)	720	nd[a]	101	60	54	51	70
SKBR3							
LD_{50} (mM)	5	7	29	66	114	89	77
LDH release (%)	1280	nd[a]	1404	91	78	70	89
F2000							
LD_{50} (mM)	5	8	37	84	77	277	122
LDH release (%)	670	nd[a]	1211	105	84	90	90

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 the text.
[a] LDH concentration could not be determined because of interference between the spin trap with the LDH detection method.

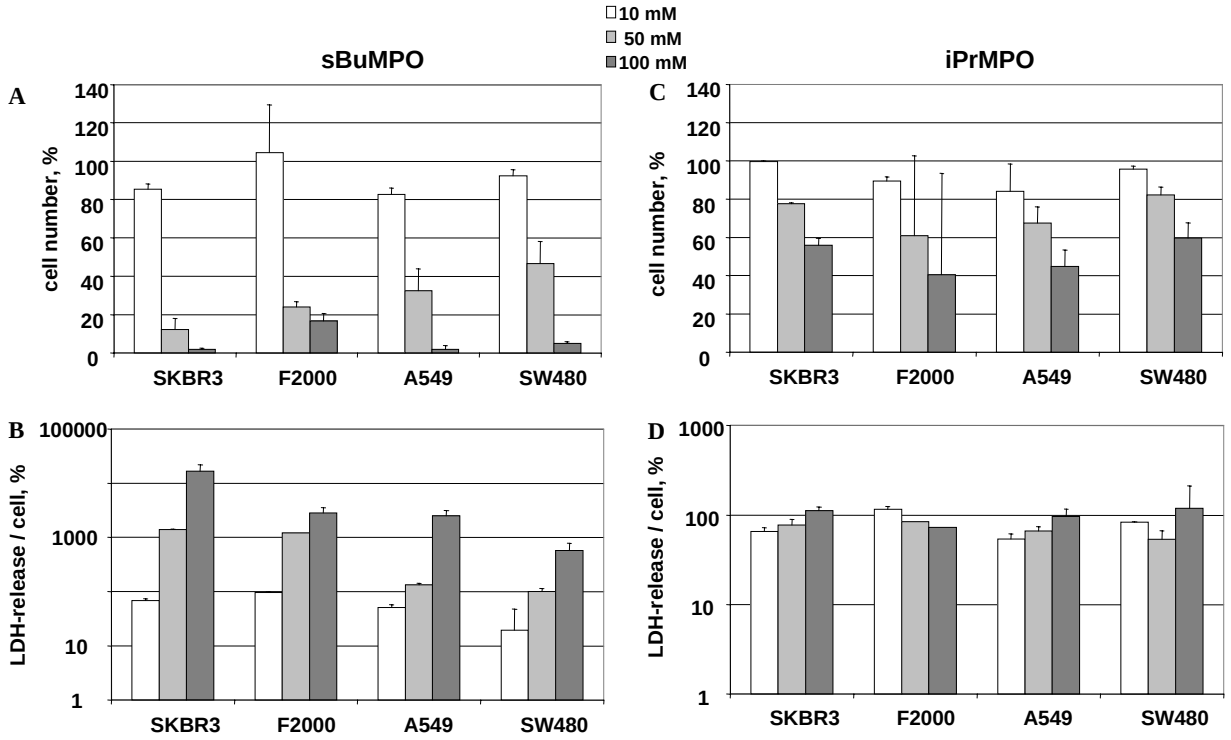

Figure 3. Comparison of *s*-BuMPO (A, B) and *i*-PrMPO (C, D) toxicity to the different cell types. Cells were seeded into 24-well plates and grown for 48 h. Spin traps were diluted into MEM containing 1 mg/mL BSA, sterile filtered and added to the culture. LDH-release and cell number were determined after 24 h from triplicate cultures as described in the text.

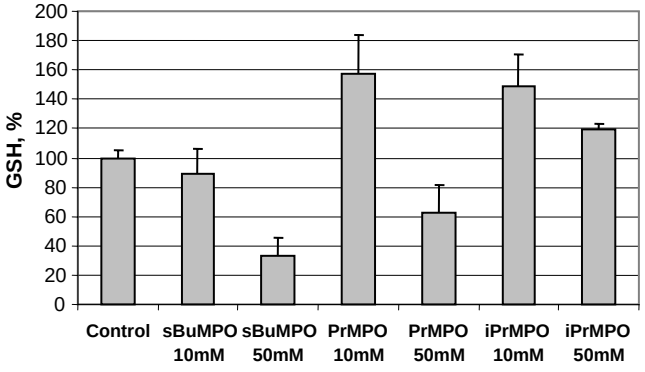

Figure 4. Influence of spin traps on intracellular GSH content of SW480. Cells were seeded into 24-well plates and grown for 48 h. Spin traps were diluted into MEM containing 1 mg/mL BSA, sterile filtered and added to the culture. GSH content in cell lysate was determined after 24 h of incubation as described in the text.

From the LD_{50} and LDH release data (Fig. 2 and Table 2) the following ranking of the spin traps according to their toxicity can be made (beginning with the highest): *t*-BuMPO > BuMPO > *s*-BuMPO > PrMPO > *i*-PrMPO ~ DEPMPO ~ EMPO.

The two least toxic spin traps were DEPMPO (for SW480, F2000 and A549) and *i*-PrMPO (for SKBR3). Low toxicity and high stability of their superoxide adducts (around 15 and 18.8 min, respectively) make these two spin traps good candidates for superoxide detection in living cells. Simpler ESR-spectra of superoxide adducts and slightly higher adduct stability as compared to DEPMPO mark the advantages of the novel spin trap *i*-PrMPO. It should be considered, however, that low toxic *i*-PrMPO scavanges free radicals thus acting like an antioxidant and increasing the GSH level (Fig. 4).

The toxicity of EMPO was of the same order of magnitude as those of *i*-PrMPO and DEPMPO. However, the stability of EMPO superoxide adduct (8.6 min) is lower than those of DEPMPO and *i*-PrMPO.[12,15] The most toxic spin trap for all cell lines investigated was *t*-BuMPO which cannot be recommended for superoxide measurements in cellular systems despite the fact that its half-life of superoxide adduct is high (15.7 min) and comparable with that of the best commercially available spin trap DEPMPO (around 15 min). The spin trap BuMPO is not suitable for superoxide detection in cells either. It forms rather unstable superoxide adducts (1.1 min) and is almost as toxic as *t*-BuMPO for all cell lines investigated. The spin trap *s*-BuMPO with the highest superoxide adduct half-life of 26.3 min is suitable for the detection of superoxide radicals as long as its concentration remains below 50 mM. At 10 mM *s*-BuMPO did not disturb redox balance of the cell (Fig. 4). The restrictions due to its toxicity at higher concentrations are compensated by a significantly higher superoxide adduct stability, which allows for longer incubation times.

Although PrMPO was tolerated moderately well by the cells (LD_{50} of 48–90 mM), at 50 mM it disturbs cellular metabolism reducing intracellular GSH content (Fig. 4). The stability of its superoxide adduct is rather low

(4.6 min) compared to those of other spin traps investigated in this paper. PrMPO might be useful for superoxide detection only in those cases where particular properties (e.g., partition coefficient) are required that exclude the use of otherwise better suited spin traps like *i*-PrMPO, *s*-BuMPO, DEPMPO or EMPO.

Comparison of the two spin traps, *i*-PrMPO and *s*-BuMPO, having the longest half-lives of superoxide adducts combined with a rather low toxicity, revealed greater differences among themselves than between the different cell lines (Fig. 3). This confirms the suggestion that toxicity is mainly defined by the structure of the spin trap itself rather than by the cell type. Although the spin trap *s*-BuMPO has a higher superoxide adduct stability than *i*-PrMPO, it is also more toxic and hence can only be used at lower concentrations than *i*-PrMPO. Thus, 10 mM *s*-BuMPO and 50 mM *i*-PrMPO can be recommended for further investigations. Which of these spin traps allows a better overall performance in cellular systems and whether they offer a substantial improvement in comparison to DEPMPO will be investigated in future studies.

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