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Novel pharmacokinetic measurement using electron paramagnetic resonance spectroscopy and simulation of *in vivo* decay of various nitroxyl spin probes in mouse blood

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Pharmacokinetics of nitroxyl spin probes in mouse blood

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ABBREVIATIONS

EPR, electron paramagnetic resonance

TEMPO, 2,2,6,6-tetramethylpiperidine-N-oxyl

TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl

TEMPONE, 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl

amino-TEMPO, 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl

carboxy-TEMPO, 4-carboxy-2,2,6,6-tetramethylpiperidine-N-oxyl

CAT-1, 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-N-oxyl iodide

carbamoyl-PROXYL, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl

carboxy-PROXYL, 3-carboxy-2,2,5,5-tetramethylpyrrolidine-N-oxyl

MTD, maximum tolerated dose

Oxo63, tri[8-carboxy-2,2,6,6-tetrakis(2-hydroxymethyl)benzo[1,2-*d*:4,5-*d'*]bis(1,3)dithio-4-yl]methyl radical

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ABSTRACT

A novel approach to measure the time course of paramagnetic spin probe concentration in the circulating blood of a living mouse using X-band (9.4 GHz) electron paramagnetic resonance (EPR) spectrometer is described. Using this technique, the pharmacokinetics of several nitroxyl spin probes were examined. The decay profiles were also independently simulated using pharmacokinetic properties as well as redox-mediated factors responsible in converting the nitroxyl radicals to the corresponding hydroxylamines. Finally, suitability of nitroxyl radicals as the probes of *in vivo* redox status and for radioprotection was described. The studies indicate that the six-member piperidine nitroxyls are suitable for estimating redox status in the circulation while the five-member pyrrolidine nitroxyl radicals are suited for tissue redox status determination. For selective protection against radiation of normal tissues rather than cancer/tumor, efficient re-oxidation of the hydroxylamine in normal tissue is preferable. Simulation results showed that, for carbamoyl-PROXYL only administration of the radical form might give radioprotection and not the hydroxylamine. However, the hydroxylamine form of TEMPOL, i.e. TEMPOL-H, may give similar radioprotection as the radical form due to efficient re-oxidation *in vivo*.

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Nitroxyl radicals have been widely used as spin probes for low frequency *in vivo* EPR experiments to estimate the biological redox status in living experimental animals (Berliner and Wan, 1989; Kuppusamy et al., 2002; Ilangovan et al., 2002; Yamada et al., 2002; Kasazaki et al., 2003). When a nitroxyl spin probe is administered to a living animal, *in vivo* EPR signal intensities of the probe show characteristic signal intensity profiles as a function of time in the animal depending on the organ investigated. Generally, *in vivo* EPR signal decay rates are obtained based on a suitable region of its decay curve to estimate redox status in the animal (Yamada et al., 2002).

Several nitroxyl radicals have been used in studies that exhibit EPR signal decay profiles depending on the spin probe used. The decay constant of a spin probe depends on the route of administration (i.e. intravenously, intraperitoneally, and etc.) and methodology of the analysis (i.e. 1-compartment model, 2-compartment model, and etc.) (Kocherginsky and Swartz, 1995). Moreover, species, strains and gender of experimental animal was also found to affect the EPR signal decay profiles (Kocherginsky and Swartz, 1995; Matsumoto et al., 2000).

The following general observations can be summarized from earlier studies. The *in vivo* EPR signal decay rates of the spin probes are enhanced by reactive oxygen species (Utsumi et al., 1993; Sano et al., 1998; Phumala et al., 1999; Han et al., 2001), such as hydroxyl radical and superoxide, which reduce nitroxyl radical in presence of H atom donor, such as NAD(P)H or GSH (Samuni et al., 1988; Krishna et al., 1992; Samuni et al., 2002; Takeshita et al., 2002). In contrast, the decay rates are decreased due to re-oxidation of the hydroxylamine to the nitroxyl radical under the oxidative atmosphere such as exposure to hyperoxia or generation of hydrogen peroxide (Quaresima et al., 1993; Matsumoto et al., 2000). Under reducing conditions as in hypoxic tumors, the decay rate constants of nitroxyl radicals were found to increase because re-

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oxidation may be less efficient in such conditions (Minetti et al., 1991; Ilangovan et al., 2002; Kuppusamy et al., 2002).

The signal decay rate also depends on several kinetic factors such as the distribution of the spin probe from the blood to the tissues and vice versa, urinary excretion through the kidney, fecal excretion through liver and bile, and entrapment into specific tissues/organs. To estimate redox information independent of clearance, the pharmacokinetic analysis of nitroxyl spin probes is therefore needed. Several groups have reported the comparison of pharmacokinetics of several nitroxyl spin probes in experimental animals (Komarov et al., 1994; Hahn et al., 1998; Takechi et al., 1997).

Blood and/or plasma concentration of a drug in the paramagnetic form is a most common index to estimate its *in vivo* pharmacokinetics. In case of rats, the time course of EPR signal intensity of the spin probes in the blood can be obtained in real time by the monitoring the circulating blood using X-band EPR (Takechi et al., 1997). However in mice, the small blood volume and difficulty of surgical procedures make it difficult to conduct repeat sampling and/or real time measurement of the circulating blood. Therefore in most cases of pharmacokinetic studies of nitroxyl radicals in mice, EPR measurements were carried out in mouse-tail instead of blood (Komarov et al., 1994). These results include pharmacokinetic information not only from the blood but also from tissue in the sampled region. In addition, it is not possible to determine the concentration of spin probes from *in vivo* EPR studies since the *in vivo* EPR signal intensity is not quantitative in most cases.

In our laboratory, the EPR related oxymetry, redox status estimation, and related functional imaging techniques are being investigated using mouse tumor models (Yamada et al., 2002; Matsumoto et al., 2003). Nitroxyl radicals are used in some of these studies to non-invasively

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assess tissue redox status. Additionally, nitroxyl radicals are used as *in vivo* radioprotectors. Therefore, the pharmacokinetic data of nitroxyl radicals in mouse blood will be useful to assess tumor redox status.

In this study, we evaluated the acute toxicity of nitroxyl spin probe after i.v. injection. We also describe a novel procedure to measure the time course of spin probe concentration in the blood of a mouse using X-band EPR spectroscopy. Using this technique, the pharmacokinetics of several nitroxyl spin probes were estimated and the pharmacokinetics results were also simulated. Suitable properties for *in vivo* redox probes and the radiation protection agents are discussed.

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Materials and Methods

Chemicals. TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl), TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), carboxy-TEMPO (4-carboxy-2,2,6,6-tetramethylpiperidine-N-oxyl), carbamoyl-PROXYL (3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl), carboxy-PROXYL (3-carboxy-2,2,5,5-tetramethylpyrrolidine-N-oxyl) were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO). TEMPONE (4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl) and amino-TEMPO (4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl) were purchased from Alexis Biochemicals (Milwaukee, WI). CAT-1 (4-trimethylammonium-2,2,6,6-tetramethylpiperidine-N-oxyl iodide) was purchased from Molecular Probes Inc. (Eugene, OR). TEMPO, TEMPONE, amino-TEMPO and CAT-1 were prepared as 50 mM. Other spin probes were prepared as 150 mM solutions. All solutions were adjusted to be pH 7 with addition of 1.0 N NaOH or HCl, then to be isotonic with addition of NaCl if required.

Animal. Female C3H mice were supplied by the Frederick Cancer Research Center, Animal Production (Frederick, MD). Animals, received at six weeks of age, were housed five per cage in climate controlled circadian rhythm-adjusted rooms and were allowed food and water *ad libitum*. Experiments are carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the National Cancer Institute Animal Care and Use Committee. Pharmacological experiments were performed within two weeks of their arrival at the facility.

Acute Toxicity by Intravenous Injection. Mice were anesthetized by isoflurane (1.5% in medical air, 700 mL/min). Several doses (1.50, 1.00, 0.75, 0.50, and 0.25 $\mu\text{mol/g}$ b.w.) were tried and acute toxicities were tested.

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Measurement of Time Course of the Spin Probe Concentrations in Blood. Each mouse was anesthetized by isoflurane (1.5% in medical air, 700 mL/min). The tail vein was cannulated to inject spin probe solution. The jugular vein was cannulated by PE-10 tubing. The jugular cannulation was through the X-band EPR cavity and the end of the cannulation tube was connected to a syringe. The schematics of the experimental arrangement to monitor blood levels of the spin probes continuously using EPR spectroscopy are shown in Figure 1. The cannulation line and cylinder were filled with heparinized saline. Spin probe solutions were injected via the cannula in the tail vein. Two different doses were tested for time course study of each probe except for TEMPO. The dose of TEMPO was 0.25 $\mu\text{mol/g}$ b.w. based on the maximum tolerated dose (MTD). Similarly, the higher dose was decided as 0.50 $\mu\text{mol/g}$ b.w. for amino-TEMPO and CAT-1, 1.00 $\mu\text{mol/g}$ b.w. for TEMPONE, and 1.50 $\mu\text{mol/g}$ b.w. for other probes. The lower dose was a half of higher dose. Immediately after the injection of the spin probe, blood was pumped up to the X-band EPR cavity (Varian E line, Palo Alto, CA) from the jugular vein cannulation and the EPR signal was measured. The blood was pushed back into the jugular vein after the measurement. The X-band EPR measurements were repeated over 20 min (30 min for carbamoyl-PROXYL, carboxy-PROXYL, and CAT-1) after injection of a spin probe solution. After each measurement, 10 ~ 30 μL of heparinized saline was additionally flushed. The EPR conditions were as follows; microwave frequency was 9.4 GHz, microwave power was 10 mW, magnetic field modulation frequency was 100 kHz, magnetic field modulation amplitude was 1.0 Gauss, time constant was 0.064 sec. The spin probe solutions were diluted by PBS at several concentrations and measured with same system to obtain the standard curves.

Data Analysis of the Time Course of the Spin Probe Concentrations in Blood. The estimation of the decay constant is affected by the selection of a time window, which may be

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subjective. To eliminate such empirical factors, we processed the data as follows. 1) Experimental end point was decided based on 0.05%, 0.5%, and 1.2% of predicted initial blood concentration C_0 for TEMPO derivatives, carbamoyl-PROXYL, and carboxy-PROXYL. The prediction of initial blood concentration was calculated by the dose and the initial distribution volume. A blood volume of 1.95 mL for a 25 g mouse (Sato, 1986) was used. The plasma volume was 1.07 mL for a 25 g mouse (55% of blood volume) (Rygaard and Povlsen, 1974; Kutscher and Schmalbach, 1975). The initial distribution volume was, in this time, the double of plasma volume. 2) The slow phase was estimated by the concentration region between the final concentration (at end point) and 5-fold of the final concentration and slow component k_β was thus calculated. 3) The fast phase was estimated by the concentration region between 10-fold of the final concentration and 80% of predicted initial blood concentration. 4) After deducting the slow phase components from the fast phase, the fast component k_α was calculated.

Simulation of Nitroxyl Decay Curve in Mouse Blood. A simulation of decay of spin probe in blood was carried out according with the following equations:

$$T_t = T_{t-1} \times \text{EXP}[-(k_D + k_U + k_B) \times \Delta t] + B_{t-1} \times [1 - \text{EXP}(-k_A \times \Delta t)] + T_i / 0.25 \times \Delta t \quad (1)$$

$$B_t = T_{t-1} \times [1 - \text{EXP}(-k_B \times \Delta t)] + B_{t-1} \times \text{EXP}[-(k_A + k_F) \times \Delta t] \quad (2)$$

$$\begin{aligned} N_t = N_{t-1} \times \text{EXP}[(k_D + k_U + k_B + k_R) \times \Delta t] \\ + B_{t-1} \times [1 - \text{EXP}(-k_A \times \Delta t)] \times N_{t-1} / T_{t-1} \\ + (T_{t-1} - N_{t-1}) \times [1 - \text{EXP}(-k_O) \times \Delta t] \\ + N_i / 0.25 \times \Delta t \quad (3) \end{aligned}$$

T_t : Concentration of total probe (nitroxyl radical + hydroxylamine) in blood (μM)

T_i : Expected initial concentration of total probe in blood (μM), ($T_i = D / V_0$)

B_t : Concentration of total probe in bile (μM)

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N_t : Concentration of nitroxyl radical in blood (μM)

N_i : Expected initial concentration of nitroxyl radical in blood (μM)

t : Data time point

Δt : Increment of data time point (min)

k_D : Distribution rate (min^{-1})

$$(T_{t-1} \geq D / V : k_D = k_{D0} \text{ or } T_{t-1} < D / V : k_D = k_{D0} \times \text{EXP}[-(D / V / T_{t-1} - 1)^{1/2}])$$

k_{D0} : Initial distribution rate (min^{-1})

k_U : Rate of urinary excretion (min^{-1})

$$(T_{t-1} \geq 2700 : k_U = 0.035 \times \text{EXP}[(2700 / T_{t-1} - 1)^6] \text{ or } T_{t-1} < 2700 : k_U = 0.035)$$

k_B : Rate of excretion into bile (min^{-1})

k_A : Rate of re-absorption from intestine (min^{-1})

k_F : Rate of excretion into feces (min^{-1})

k_R : Rate of reduction (min^{-1})

k_O : Rate of re-oxidation (min^{-1})

D : Dose (μmol)

V_0 : Initial distribution volume (g or mL)

V_f : Final distributable volume (g or mL)

$$(P \neq 0 : V_f = W \times \ln(50) / \ln(P) \text{ or } P = 0 : V_f = V_p)$$

V_p : Plasma volume (mL)

W : Animal body weight (g)

P : O/W partition coefficient.

The values used for the simulation was shown in Table 1. For initial 15 sec (0.25 min), T_t was assumed to increase linearly by injection, i.e. equation (1) and (3) has additional term (+ $T_i / 0.25$

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$\times \Delta t$). T_i is expected initial blood concentration of total probe which is given as D / V_0 . V_0 depends on the O/W partition coefficient, P . Expected initial blood concentration of nitroxyl radical N_i is same value as T_i when radical form was administered. N_i was set as 0 when hydroxylamine form was administered. T_t , B_t and N_t at time $t = 0$ are $T_0 = B_0 = N_0 = 0$.

Results

Acute Toxicity by Intravenous Injection. The results are shown in Table 2. To reduce the number of mice that would be required to determine precise MTD values, MTD values were approximated based on extrapolations between lethal and non-lethal doses. The MTD of TEMPOL should be between 2.00 and 1.50 $\mu\text{mol/g}$ b.w. which is in agreement with the previous report employing i.p. injection (MTD = 275 mg/kg b.w. = 1.6 $\mu\text{mol/g}$ b.w.) (Hahn et al., 1998). The MTD of TEMPO was below 0.25 $\mu\text{mol/g}$ b.w., which was approximately 1/6 that of TEMPOL. The MTD of TEMPONE should be between 1.00 and 1.50 $\mu\text{mol/g}$ b.w. which is lower than TEMPOL. A previous report (Hahn et al., 1998) also determined the MTD of TEMPONE by i.p. injection to be 225 mg/kg b.w. = 1.32 $\mu\text{mol/g}$ b.w., which was in the same range in the present study. The MTD of amino-TEMPO should be between 0.50 and 0.75 $\mu\text{mol/g}$ b.w., which is lower than TEMPOL and TEMPONE. However, a previous report (Hahn et al., 1998) showed the MTD of amino-TEMPO by i.p. injection to be 250 mg/kg b.w. = 1.46 $\mu\text{mol/g}$ b.w. The intraperitoneal injection route used in that study may have reduced the toxicity of amino-TEMPO. Octanol/water partition coefficients for the piperidine nitroxyl radicals have been reported in the following order; TEMPO \gg TEMPOL $>$ TEMPONE $>$ amino-TEMPO $>$ carboxy-TEMPO (Takechi et al., 1997). Hydrophilic amino-TEMPO injected i.p. may be retained in ascites for relatively long period rather than being transported into blood circulation through the cell membrane and might be prevented to reach target tissues/organs of toxicity. The MTD of CAT-1 should be between 0.50 and 0.75 $\mu\text{mol/g}$ b.w., which is in the same range as amino-TEMPO. Therefore, acute toxicity in mice by i.v. injection followed the order: TEMPO $>$ CAT-1 \approx amino-TEMPO $>$ TEMPONE $>$ TEMPOL. No acute deaths of mice were observed for other spin probes at the doses used in this study.

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Time Course of the Spin Probe Concentrations in Blood. Figure 2 shows a logarithmic plot of decay curves of several spin probes in mouse blood measured by EPR. Decay curves showed biphasic profiles except for TEMPO and CAT-1. Since the lipophilic TEMPO molecule adsorbed and remained in the PE-10 tube, quantification in the blood was inaccurate below 0.03 mM. Though data points could not be obtained for sufficient time periods because of high background to reliably characterize it, the decay curve of TEMPO (Fig. 2C) appeared to be biphasic as shown by simulation (Fig. 3C). In addition, TEMPO showed relatively low initial blood concentrations than the expected value from dose administered. CAT-1, which is an extra-cellular probe showed a tri-phasic behavior, consisting of a short first phase, followed by slow second phase, and then a final linear decrease.

A similar tri-phasic decay, as observed for CAT-1, was shown for another membrane impermeable molecule, tri[8-carboxy-2,2,6,6-tetrakis(2-hydroxymethyl)benzo[1,2-*d*:4,5-*d'*]bis(1,3)dithio-4-yl]methyl radical (Oxo63) which is a triarylmethyl radical used as EPR oxymetry probe (submitted for publication). The slow second phase was due to the saturation of urinary excretion. Oxo63 exhibited saturated excretion at a relatively high dose (1.50 $\mu\text{mol/g}$ b.w. bolus i.v. injection), while CAT-1 exhibited evidence of saturation at 0.25 $\mu\text{mol/g}$ b.w. bolus i.v. injection. Oxo63 has a diuretic effect, while no diuretic effect for any nitroxyl spin probes has been reported to our knowledge. The third phase of Oxo63 started when the plasma concentration fell below 2.7 mM, while the third phases of CAT-1 started below 0.15 mM as shown in Fig 2H. Total CAT-1 concentration, i.e. the total of reduced and oxidized forms, should be higher than the concentration indicated in Fig. 2H, since CAT-1 undergoes reduction in the mouse.

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The decay constants of various spin probes were calculated from those plots based on a 2-compartment model analysis except for TEMPO and CAT-1 as shown in Table 3. No marked differences were obtained between the high dose and the low dose except for TEMPOL and carboxy-TEMPO. In this experiment, the k_{β} values followed the order: TEMPONE > amino-TEMPO > TEMPOL > carboxy-TEMPO > carboxy-PROXYL > carbamoyl-PROXYL. This finding is in agreement with previous reports (Komarov et al., 1994; Takechi et al., 1997), except that several results reported for the order of TEMPO and amino-TEMPO was dependent on the experimental design and/or analysis. However, the k_{α} showed a different order. For low doses, k_{α} of the probes followed the order: amino-TEMPO > TEMPONE > TEMPOL > carboxy-TEMPO \approx carbamoyl-PROXYL > carboxy-TEMPO. For high doses, the k_{α} of TEMPONE and TEMPOL were similar.

Simulation of Nitroxyl Decay Curve in Mouse Blood. The urinary excretion rate k_U was determined to be 0.035 min^{-1} based on the pharmacokinetics data of the paramagnetic spin probe Oxo63. The pharmacokinetics of Oxo63 should be influenced by distribution, excretion, and less *in vivo* reduction. Therefore, k_U was obtained from the very late time region (30 ~ 60 min after injection) of the Oxo63 decay curve. The k_U should be a significantly smaller value i.e. being saturated when the $T_{1\rho}$ was higher than 2.7 mM, which is the value based on the pharmacokinetics of Oxo63. The distribution rate k_D might be larger than 0.5 min^{-1} , while it was difficult to estimate because it probably changes with time. For nitroxyl spin probe, the distribution rate k_D may be dependent on the distribution volume i.e., O/W partition coefficient and/or membrane permeability. The k_D may be faster for probes with low membrane permeability and their distribution may occur by penetration through cell interstitial spaces. In contrast, high membrane permeable probes should have a low k_D because of the distribution

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through cell membrane. The k_D gradually slows down when the T_{t-1} goes below D/V_f . The final distribution volume V_f should be estimated considering membrane permeability. V_0 is the initial distribution volume, which consists of the plasma volume and volumes associated with tissue cell interstices, cell membranes, and intracellular regions. Therefore, the V_0 is also dependent on membrane permeability. Lipophilicity may increase V_0 , while extremely high lipophilicity may tend to decrease V_0 because lipophilic probes may be within membranes. The k_B should also be dependent based on the membrane permeability. The re-absorption rate k_A was set to equal k_B and the fecal excretion rate k_F was set at 1/10 of the k_B . The re-oxidation rate k_O may depend on the k_R and also on the membrane permeability. Finally, reduction rate k_R was chosen depending on the shape of decay profile. The values used in simulation were selected considering the order of the redox potential and the partition coefficient shown in previous reports (Takechi et al., 1997; Krishna et al., 1992). The values used for simulations are shown in Table 1.

Simulated decay curves are shown in Fig. 3. Simulated decay profiles showed similar patterns as obtained in animal experiments. The desired values k_R followed the order of; TEMPO > TEMPOL > amino-TEMPO > TEMPONE >> carboxy-TEMPO > CAT-1 > carboxy-PROXYL >> carbamoyl-PROXYL. This order is dependent on the oxidation/reduction potentials within a class of probes, i.e. TEMPO or PROXYL derivatives.

Fig. 4 shows the simulation of nitroxyl radical concentration after the administration of 1.5 $\mu\text{mol/g}$ b.w. hydroxylamine for TEMPOL and carbamoyl-PROXYL. This simulation was carried out using the same equation by setting the expected initial concentration of radical form $N_i = 0$. The administration of the hydroxylamine form of carbamoyl-PROXYL may not give enough concentration of radical form (Fig. 4A). However, the administration of TEMPOL-H,

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hydroxylamine form of TEMPOL, can reach same radical concentration as TEMPOL are administered 3 min after administration (Fig. 4B).

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Discussion

The cytotoxicity of nitroxyl spin probes were reported in the order: TEMPO > TEMPONE > amino-TEMPO > TEMPOL (Kroll et al., 1999). TEMPONE was, however, less toxic than amino-TEMPO in mice. In a living mouse, TEMPONE may be metabolized to the less toxic TEMPOL at a relatively fast rate (Kroll and Borchert, 1999). After injection of TEMPONE, a small portion of TEMPOL signal appeared and gradually increased. TEMPONE almost completely disappeared within 10 min, after which time, only the TEMPOL signal was observed after 10 min. When potassium ferricyanide was added to collected blood 25 min after injection of TEMPONE, recovered signal was only TEMPOL (data not shown).

Generally, doses of commonly used pharmaceuticals are administered at concentrations less than 10 nmol/g in humans. The dose of spin probes used in this experiment was between 10 - 100 fold higher than this value. Saturation of excretion processes could occur at such a high dose. From the pharmacokinetic results of Oxo63, the urinary excretion rate was not so high (around 0.035 min^{-1}) (submitted for publication). When the blood concentration of the probe was higher than a certain extent, urinary excretion may be saturated. The time period of the saturated excretion may be shortened with decreasing dose, below which the decay curves of all probes become biphasic. The saturation period can also be shortened by membrane permeability of the probe i.e. increasing distribution volume and fecal excretion through bile. However, total concentrations of membrane permeable probes were kept in blood and/or tissues for relatively long time periods as shown in a previous report (Hahn et al., 1998) due to entero-hepatic circulation.

The decay curve profiles can be influenced by several rate factors, such as distribution, excretion, reduction and re-oxidation rates. The distribution rate should stabilize and should

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decrease when the probes distribute in the body. The urinary excretion could be saturated when the probe concentration in the blood is high. The urinary excretion rate will recover when the blood concentration of total probe decreases. The fecal excretion can also be expected for membrane permeable probes. The fecal excretion is comprised of three processes, i.e. excretion into bile, re-absorption from bile, and actual excretion into feces. The distribution rate and total excretion rate may change depending on blood and/or tissue concentration of the probe. Therefore, distribution and excretion may not exhibit first order decay kinetics, and may make the biphasic decay indistinguishable under conditions when saturation of excretion has not occurred. This process should influence the total probe kinetics. In addition to this, the different types of reduction rates should be considered, but mainly a reversible one-electron reduction and an irreversible reduction. Moreover, re-oxidation of hydroxylamine to nitroxyl radical should be considered. When the reduction rate predominates, the excretion phase can be neglected. However, in most of cases, the oxidation phase will make the profile pseudo-biphasic, because reduction and oxidation will reach an equilibrium, after which the decay rate depends primarily on the excretion rate. When the reduction rate was slow enough and the blood concentration of the probe was high enough, the saturation of total probe excretion was though obvious on the decay curve of reduced form as a triphasic profile with or without a slight shoulder (Fig. 2H).

Based on the factors discussed above, a simulation of the decay of nitroxyl radicals in blood was proposed as described in the methods. Although this simulation was not comprehensive, some observations related to the mechanism of *in vivo* nitroxyl decay can be noted. Several simulations of the decay kinetics showed that, a combination of distribution and excretion is responsible for the basic biphasic decay under non-saturated conditions of excretion. When the redox contribution to the signal loss was neglected, the slope of the fast phases was dependent

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mainly on the distribution volume and bile excretion rate, i.e. membrane permeability. The change in reduction and re-oxidation rates, i.e. redox status, could influence the slope of the fast phase and the time taken to transform from the fast phase to the slow phase, and then cancel each other, after which the decay curve will depend primarily on distribution and/or excretion. Therefore, the slow phase, especially at the later time regions after the administration of the probe, depends only on excretion rate. To estimate *in vivo* redox information from the decay constant of nitroxyl radicals in blood circulation, probes with fast reduction rates, such as the TEMPO, are sensitive because the reduction rates could predominate in the fast phase.

Most reported studies use nitroxyl radicals as redox probes where the EPR signal decay in a particular tissue and/or organ or the part of the body is followed. Different mechanisms for *in vivo* nitroxyl decay rate described in a previous reports (Kocherginsky and Swartz, 1995) may due to the ambiguity of measured volume and variation of treated phase. For the particular tissue and/or organ of interest, an additional compartment, which includes uptake phase and reduction phase, should be considered. Then, the decay curve will exhibit a peak followed by a biphasic profile with relatively slower initial fast phase or linear profile lacking fast phase. The reduction rate presumably predominates in EPR measurements of whole body or particular tissue and/or organ in the absence of gradients (Komarov et al., 1994; Kamatari et al., 2002). Therefore, redox status estimation using nitroxyls as probes will be more effective when implemented in combination with EPR imaging techniques.

One of the factors determining the suitability of nitroxyl radicals as redox probes is that the molecules should be able to go into cell. However, while estimating the decay rate as an index of redox status, any traces of saturated excretion on the decay curve may be confounding. Optimally, the fast phase and the time period needed to shift to the slow phase should be as long

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as possible. For *in vivo* EPR imaging, the administered dose should be as high as possible. The saturation of excretion might be negligible for membrane permeable probes, even at relatively high doses used. According to this hypothesis, TEMPOL and amino-TEMPO should be better redox probes among the compounds tested. While TEMPOL and amino-TEMPO may be more sensitive for redox status, carbamoyl-PROXYL is better for EPR imaging. Although the simulation of decay of carbamoyl-PROXYL showed almost no difference between total probe concentration and radical concentration, it is expected that the reduction rate will more responsive to the decay rate in tissues and/or organs.

Re-oxidation of the hydroxylamine to the corresponding nitroxyl radical is a feasible approach for radiation protection *in vivo*, since radiation protection is shown only for the radical form not for the hydroxylamine *in vitro* (Mitchell et al., 1991; Sasaki et al., 1998). Administration of the hydroxylamine to living mice yields radioprotection because it is re-oxidized to nitroxyl radical (Hahn et al., 2000). Re-oxidization may occur more efficiently in normal tissues compared to the hypoxic conditions expected in the tumor. Therefore, the nitroxyl radical shows radiation protection only for normal cells. To obtain selective protection, it is important to keep constant concentration of the hydroxylamine form in blood and/or tissues rather than radical form. Therefore, administration in the hydroxylamine form should be better, because of lower toxicity (Hahn et al., 2000; Zhdanov and Komarov, 1990). Of the probes studied in this investigation, optimal choices may be carbamoyl-PROXYL and TEMPOL. However, treatment with radical form of carbamoyl-PROXYL may result in a higher concentration of the radical, which protects not only normal tissues but also cancer and/or tumor tissues. Therefore, TEMPOL and/or TEMPOL-H may be most suitable for *in vivo* radiation protection.

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Using a novel X-band EPR technique, the pharmacokinetics of several nitroxyl spin probes were estimated and the suitability of these agents to probe *in vivo* redox status and as radiation protectors were described. EPR signal decay mechanisms of spin probes in the blood of living mice are analyzed by experiments and simulations. The one or two-compartment model analysis is not sufficient in such a case, since the decay mechanisms are complicated. Since *in vivo* reduction rates predicted by the simulation reflect the oxidation/reduction potential of the spin probe, comparison of *in vivo* decay rates between different spin probes is difficult by one or two-compartment model analysis. Considering simulation results, combination of distribution and excretion makes basic biphasic decay under non-saturated urinary excretion conditions. Redox information could be reflected in the initial phase. The slow phase, especially at later period, depends only on excretion rate, because the reduction and the oxidation are at equilibrium. Based on sensitivity, MTD, and membrane permeability conditions, TEMPOL or amino-TEMPO are better as redox probes in blood, while carbamoyl-PROXYL may be more suitable spin probe to investigate tissue redox status by means of EPR imaging. To expect the selective protection against radiation for normal tissues rather than cancer/tumor, re-oxidation of the probe in normal tissue is preferable. The hydroxylamine form of TEMPOL is probably a best radioprotector for *in vivo* use rather than TEMPOL.

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Legends for figures

Fig. 1. The schematics of the experimental arrangement to monitor blood levels of the nitroxyl spin probes continuously using X-band EPR spectroscopy.

Fig. 2. Decay curves of several nitroxyl spin probe in mouse blood obtained by the repeated measurements using X-band EPR. (A) carbamoyl-PROXYL, (B) carboxy-PROXYL, (C) TEMPO, (D) TEMPOL, (E) TEMPONE, (F) amino-TEMPO, (G) carboxy-TEMPO, and (H) CAT-1. Black marks indicate the higher doses and gray marks indicate lower doses.

Fig. 3. Simulation of decay curves of several nitroxyl spin probes in mouse blood. (A) carbamoyl-PROXYL, (B) carboxy-PROXYL, (C) TEMPO, (D) TEMPOL, (E) TEMPONE, (F) amino-TEMPO, (G) carboxy-TEMPO, and (H) CAT-1. Gray diamonds indicate simulated decay curves of total spin probe (hydroxyl amine + nitroxyl radical). Closed circles indicate simulated decay curves of nitroxyl radical form. Open circles indicate experimental results of higher doses in Fig. 2.

Fig. 4. Simulation of yield nitroxyl radical after administration of hydroxylamine. (A) carbamoyl-PROXYL and (B) TEMPOL. Gray diamonds indicate simulated decay curves of total spin probe (hydroxyl amine + nitroxyl radical). Closed circles indicate the simulation of yielding nitroxyl radical.

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Tabel 1. Values used for the simulation.

Probes	Dose [$\mu\text{mol/g}$]	V_0 [g]	k_{D0} [min^{-1}]	k_B [min^{-1}]	k_R [min^{-1}]	k_O [min^{-1}]
carbamoyl-P ^a	1.50	$V_p \times 3.0$	0.8	0.035	0.030	$k_R / 50$
carboxy-P ^b	1.50	$V_p \times 1.0$	6.0	0	0.055	0
TEMPO	0.25	$V_p \times 1.8$	0.5	0.035	8.0	$k_R / 50$
TEMPOL	1.50	$V_p \times 1.8$	0.5	0.035	4.0	$k_R / 50$
TEMPONE	1.00	$V_p \times 10.0$	0.4	0.035	0.75	$k_R / 50$
amino-T ^c	0.50	$V_p \times 4.0$	0.6	0.035	2.0	$k_R / 50$
carboxy-T ^d	1.50	$V_p \times 2.0$	0.8	0.01	0.3	$k_R / 50$
CAT-1	0.50	$V_p \times 1.0$	6.0	0	0.13	0

Plasma volume (V_p) = Blood volume (V_b) \times 0.55. ^a carbamoyl-PROXYL. ^b carboxy-PROXYL.

^c amino-TEMPO. ^d carboxy-TEMPO.

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Tabel 2. Acute toxicity of several spin probes.

Probes	Dose [$\mu\text{mol/g}$]	Result
TEMPO	0.50	A mouse died within 3 min after injection.
	0.25	Mice (4/4) survived until sacrificed (20 min after injection).
TEMPOL	2.00	A mouse died within 3 min after injection.
	1.50	Mice (4/4) survived until sacrificed (20 min after injection).
TEMPONE	1.50	Mice (2/2) died within 16 min after injection.
	1.00	Mice (4/4) survived until sacrificed (30 min after injection).
amino-TEMPO	1.00	Mice (2/2) died within 3 min after injection.
	0.75	Mice (2/2) died within 3 min after injection.
	0.50	Mice (4/4) survived until sacrificed (20 min after injection).
CAT-1	1.00	Mice (2/2) died immediately after injection.
	0.75	Mice (2/2) died immediately after injection.
	0.50	Mice (4/4) survived until sacrificed (30 min after injection).

Administrations were bolus i.v. injection. Numbers in parenthesis were number of mice affected/used.

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Tabel 3. Dose of several spin probes to a mouse.

Probes	High dose (sub-MTD or 1.50 $\mu\text{mol/g}$)			Low dose (Half of high dose)		
	n	k_{α} [min^{-1}]	k_{β} [min^{-1}]	n	k_{α} [min^{-1}]	k_{β} [min^{-1}]
carbamoyl-P ^a	4	0.691 ± 0.057	0.067 ± 0.009	4	0.791 ± 0.119	0.061 ± 0.002
carboxy-P ^b	4	0.391 ± 0.043	0.080 ± 0.002	4	0.484 ± 0.104	0.076 ± 0.007
TEMPOL	4	1.511 ± 0.347	0.238 ± 0.070	4	2.383 ± 0.147 *	0.195 ± 0.054
TEMPONE	4	1.584 ± 0.154	0.406 ± 0.064	4	1.752 ± 0.098	0.427 ± 0.043
amino-T ^c	4	2.878 ± 0.582	0.375 ± 0.016	5	3.066 ± 1.066	0.288 ± 0.053
carboxy-T ^d	4	0.696 ± 0.027	0.153 ± 0.031	4	0.809 ± 0.052 *	0.146 ± 0.018

Values are indicated as mean \pm SD. Decay constant of later phase k_{β} was calculated for the concentration region between final concentration (at end time point) and 5-fold of final concentration. The end time point was at 6.5 min for amino-TEMPO, at 8 min for TEMPONE, at 10 min for TEMPOL, at 20 min carboxy-TEMPO and at 30 min for PROXYL group. Decay constant of initial phase k_{α} was calculated for the concentration region between 10-fold of final concentration and 50 % of predicted initial blood concentration after deduct 2nd phase components. The prediction of initial blood concentration was calculated by blood volume as 7.78 mL/100 g b.w. and total dose. Significance was estimated by Student's or Welch's t-test. * indicates significance between high and low doses at $p < 0.05$. ^a carbamoyl-PROXYL. ^b carboxy-PROXYL. ^c amino-TEMPO. ^d carboxy-TEMPO.







