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Synthesis and biological testing of aminoxyls designed for long-term retention by living cells

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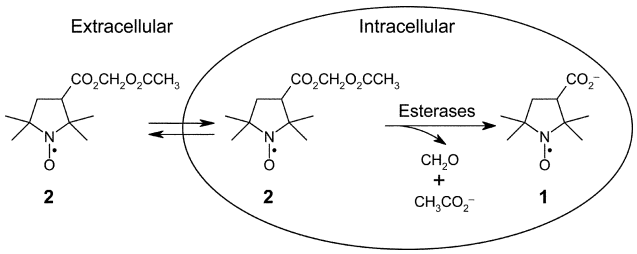
Owing to recent advances in electron paramagnetic resonance (EPR) imaging methodologies, it is now potentially possible to track and image, in real time *in vivo*, cells that had been tagged with aminoxyl spin probes. We had previously reported that living cells can accumulate 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**1**] to high (millimolar) intracellular concentrations through passive incubation with the corresponding acetoxymethyl (AM) ester [**2**]. In the present study, we show that under physiological conditions aminoxyl [**1**] is rapidly extruded by cells through an organic anion transport mechanism, resulting in an intracellular exponential lifetime ($t_{1/e}$ or τ) of just 9.84 min at 37 °C. Through successive rational structural modifications, we arrived at (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N*,*N*-diacetic acid [**10**], which can still be accumulated by cells to high intracellular concentrations, but which, with an intracellular exponential lifetime of $\tau = 114$ min, is well retained by cells for long periods of time, where one expects 14% retention even after 5 h. These results suggest that it should be feasible to use EPR imaging to perform *in vivo* tracking of populations of cells that have accumulated high intracellular levels of aminoxyls.

Introduction

The movement of specific populations of cells through the body occurs in normal physiology as well as pathophysiology. Examples include the "homing" of lymphocytes to specific tissues and organs, and the spread of metastatic cancer cells from the original malignant lesion to distant sites. The ability to track and visualize such cell movements within the body would be useful clinically and scientifically. With the development of low-frequency electron paramagnetic resonance (EPR) spectroscopy and imaging[1] and the ability to detect paramagnetic species *in situ*, *in vivo* and in real time,[2] tracking and imaging of cells within a living animal is now a real possibility. Before such studies can be undertaken, however, we must develop spin probes that can be loaded into cells at sufficiently high concentrations as to enable detection of the cells by low-frequency EPR spectroscopy. This is not a trivial task. The most common spin probes are the aminoxyls, which, until very recently, have not been successfully loaded into living cells at high concentrations. Moreover, aminoxyls, depending on their structure, can be susceptible to destruction by bioreduction.[3] Therefore, a successful spin probe that can be used to track living cells must 1) be readily loaded into cells at high concentration and 2) be retained intracellularly over long periods of time. We have recently demonstrated esterase-assisted accumulation of aminoxyl [**1**] to millimolar concentrations in living lymphocytes after incubation with the corresponding acetoxymethyl (AM) ester [**2**][4] (Scheme 1). In this paper, we report the synthesis and biological testing of new spin probes that meet the criteria given above.



Scheme 1 Esterase-assisted loading of aminoxyl [**1**] into a living cell.

Results and discussion

Once loaded into cells, a spin probe must be well retained by the cells at physiological temperature in order to be useful for cell tracking studies *in vivo*. As a starting point, we examined the cellular retention of aminoxyl [**1**] (Scheme 1). We loaded Jurkat lymphocytes with aminoxyl [**1**] by incubation with the AM ester [**2**] at 23 °C, essentially as described previously[4] (see Experimental section for details). We found that at 37 °C the amount of aminoxyl [**1**] retained intracellularly declined rapidly with time (circles in Fig. 1). The time course of decline was well-fitted by a single exponential, with a time constant (or lifetime) of $\tau = 9.84 \pm 0.06$ min, or equivalently the half-life was $t_{1/2} = 0.693 \times \tau = 6.82 \pm 0.04$ min. The rule of thumb that an exponential process is essentially (97%) complete after 5 half-lives implies that after 30–35 min very little aminoxyl [**1**] would be retained by the cells, as verified by the data at 40 and 80 min. We also estimated the concentration of aminoxyl [**1**] loaded into the lymphocytes. In three independent experiments, immediately after incubation with AM ester [**2**], the average intracellular concentration of aminoxyl [**1**] was found to be 2.27 ± 0.12 mM (see Experimental section for details). This confirmed what we have demonstrated previously[4], namely that through incubation with the AM ester [**2**] cells can accumulate aminoxyl [**1**] to high concentrations intracellularly.

To elucidate what process might underlie the rapid loss of aminoxyl [**1**] from the cells at 37 °C we performed a parallel experiment wherein lymphocytes loaded with aminoxyl [**1**] were incubated for 80 min at 23 °C rather than at 37 °C. Intracellular retention of aminoxyl [**1**] was markedly improved at 23 °C, with

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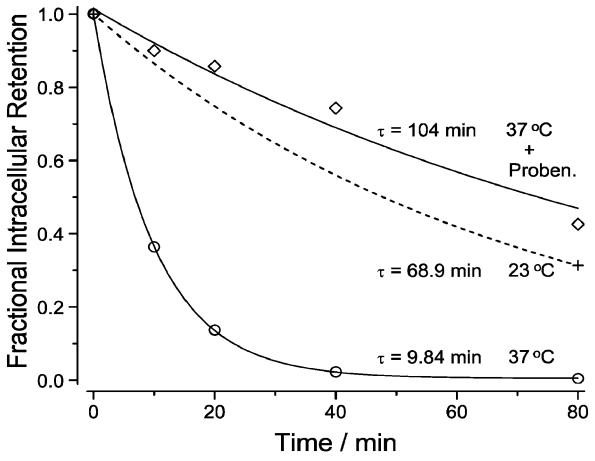
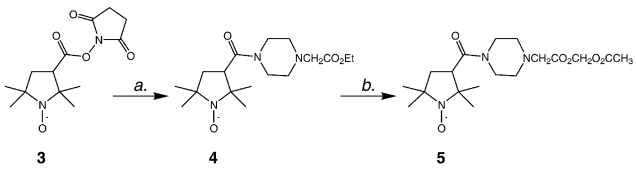


Fig. 1 Intracellular retention of aminoxyl [**1**] under different conditions: Cells lose aminoxyl [**1**] rapidly at 37 °C, but the rate of loss is greatly reduced at a lower temperature and in the presence of probenecid, an inhibitor of organic anion transport. Jurkat lymphocytes were allowed to accumulate aminoxyl [**1**] by incubation with the acetoxymethyl ester [**2**] as described in the Experimental section. Replicate suspensions of loaded lymphocytes in Hanks' Balanced Salt Solution (HBSS) were maintained at 37 °C for varying durations of time. Thereafter, the lymphocytes were separated from the HBSS by centrifugation. The amount of aminoxyl [**1**] in the cells and in the supernatant HBSS were assayed by EPR to permit calculation of the fraction of label retained intracellularly (see Experimental section for details). Circles (○) are data obtained at 37 °C in HBSS; diamonds (◇) are data obtained at 37 °C in HBSS containing 10 mM probenecid. The solid curves are least-squares fits to a single exponential of the form $y = y_0 + Ae^{-t/\tau}$, where τ is the exponential time constant, or lifetime, and is related to the half-life by $t_{1/2} = 0.693\tau$. Plus signs (+) represent data collected at 23 °C in HBSS and the dashed line is a single-exponential fit of the data. EPR spectrometer settings are detailed in the Experimental section.

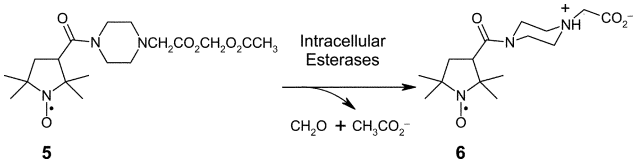
31% retention at 80 min as compared with 0% at 37 °C (+ in Fig. 1). This implies $\tau \approx 69$ min ($t_{1/2} \approx 48$ min) at 23 °C for a single-exponential time course (dashed line in Fig. 1). That is, loss of spin label was ~7-fold slower for a 14 °C decrease from 37 °C to 23 °C. Such a strong temperature dependence suggests that the loss of aminoxyl [**1**] could be mediated by a biological transport process.[5] Numerous studies suggest that cellular transporters of organic anions are ubiquitously expressed in the body. Organic anion transport has been observed in diverse cell types including neuronal cell lines,[6] pancreatic islet cells,[7] fibroblasts,[8] cardiac myocytes,[9] cells of the kidney tubules,[10] as well as cells of the immune system.[11] Probenecid and sulfinpyrazone are effective pharmacological inhibitors of such transport processes that extrude organic anions from cells.[11b,12] We tested the effect of probenecid and found that inclusion of 10 mM probenecid in the Jurkat cell suspension greatly improved the ability of the lymphocytes to retain aminoxyl [**1**] (diamonds in Fig. 1). Nonlinear least-squares fit of the data yielded an exponential time constant of $\tau = 104 \pm 12$ min ($t_{1/2} = 72 \pm 9$ min). Thus, the anion transport inhibitor slowed the loss of aminoxyl [**1**] at 37 °C by more than 10-fold. This result suggests that loss of the spin probe from Jurkat lymphocytes is mediated by organic anion transporters that actively extrude the anionic aminoxyl [**1**].

An organic anion extrusion mechanism suggests a potential approach to its circumvention. Cells retain the natural amino acids L-glutamate and L-aspartate, both of which bear a net negative charge at physiological pH but also contain a positively-charged group owing to the presence of the α-amino substituent. We investigated whether introducing such "mixed-charge" character into a xenobiotic molecule such as an aminoxyl could improve cellular retention. To test this we synthesized aminoxyl [**5**] from the known aminoxyl [**3**] (Scheme 2). Cleavage of [**5**] by cellular esterases is expected to release the mixed-charge aminoxyl [**6**] (Scheme 3).



3 4 5

Scheme 2 *Reagents and conditions*: (a) 1-(ethoxycarbonylmethyl)-piperazine, CH_2Cl_2; (b) *i*. KOH–CH_3OH, *ii*. $BrCH_2O_2CCH_3$, $(CH_3)_2SO$.



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Scheme 3 Esterase-catalyzed conversion of AM ester [**5**] into mixed-charge aminoxyl [**6**].

Replicate suspensions of lymphocytes loaded with aminoxyl [**6**] by incubation with the AM ester [**5**] were maintained at 37 °C for different periods of time. Thereafter, the amounts of aminoxyl [**6**] retained by the cells and extruded into the extracellular solution were assayed by EPR spectroscopy. As shown in Fig. 2, aminoxyl [**6**] (+) was much better retained than aminoxyl [**1**] (○). Least-squares analysis of the data yielded an exponential time constant of $\tau = 30.0 \pm 1.2$ min ($t_{1/2} = 20.8 \pm 0.8$ min) at 37 °C for aminoxyl [**6**]; a 3-fold improvement over aminoxyl [**1**]. By reference to a standard calibration curve, the average initial intracellular concentration of aminoxyl [**6**] was estimated to be 1.41 mM. Thus, introduction of mixed-charge character enhanced intracellular retention significantly without adversely affecting the ability of cells to accumulate the spin probe.

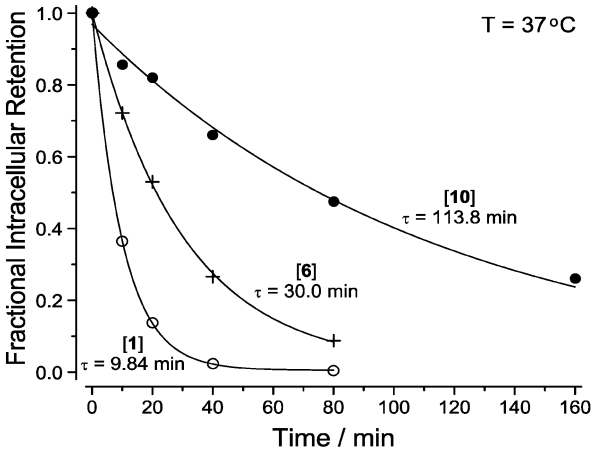
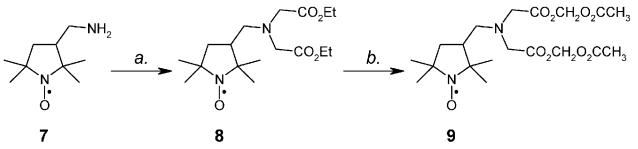


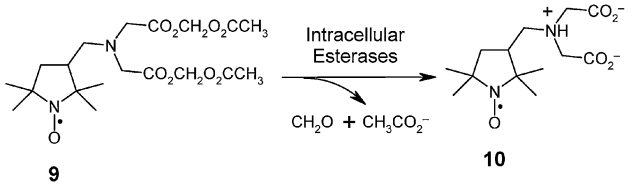
Fig. 2 Comparison of retention of aminoxyls [**1**], [**6**] and [**10**] by Jurkat lymphocytes at 37 °C. Suspensions of Jurkat lymphocytes loaded with aminoxyls were treated as in Fig. 1. Data for aminoxyl [**1**] (open circles, ○) are reproduced from Fig. 1 for ease of visual comparison. Data for aminoxyls [**6**] and [**10**] are represented by plus signs (+) and filled circles (●), respectively. Solid curves are least-squares fits to the single-exponential function, $y = y_0 + Ae^{-t/\tau}$, where τ is the exponential time constant, or lifetime, and is related to the half-life by $t_{1/2} = 0.693\tau$. EPR spectrometer settings are detailed in the Experimental section.

We next investigated whether a mixed-charge polyionic aminoxyl with increased net ionic charge would show further improved retention by cells. We synthesized aminoxyl [**9**] from the known aminoxyl [**7**] (Scheme 4). Esterase cleavage of [**9**] is expected to generate the mixed-charge aminoxyl [**10**] in the intracellular environment (Scheme 5).

When we examined lymphocytes that had been loaded by incubation with the AM ester [**9**], we found that intracellular retention of aminoxyl [**10**] was remarkably long-lived (●, Fig. 2). Nonlinear least-squares analysis yielded an exponential time constant of $\tau = 113.8 \pm 6.9$ min ($t_{1/2} = 78.9 \pm 4.8$ min) at 37 °C



Scheme 4 *Reagents and conditions*: (a) EtO_2CCH_2Br, K_2CO_3, $(CH_3)_2SO$–CH_3CN; (b) *i*. KOH–CH_3OH, *ii*. $BrCH_2O_2CCH_3$, $(CH_3)_2SO$.



Scheme 5 Esterase-catalyzed conversion of AM ester [**9**] into mixed-charge aminoxyl [**10**].

for aminoxyl [**10**]. This represents a close to 4-fold improvement over aminoxyl [**6**] and nearly 12-fold improvement over aminoxyl [**1**]. These results indicate that at physiological temperature cells can retain significant amounts of spin label even after 5 hours. We also examined how effectively incubation with the AM ester loaded aminoxyl [**10**] into cells. In two replicate measurements, we determined that the initial average concentration of [**10**] in loaded Jurkat lymphocytes was high at 2.1 ± 0.7 mM.

In summary, by introducing rational structural modifications, we have successfully made aminoxyl spin probes that simultaneously exhibit two desirable characteristics: 1) they can be accumulated to high intracellular concentrations through passive incubation of cells with the acetoxymethyl ester and 2) once loaded into cells, they are retained intracellularly for long periods of time under physiological conditions.

Experimental

Reagents

All chemical reagents and solvents were obtained from commercial vendors and used without further purification. Media and biochemicals for cell culture were from Biosource International (Rockville, MD). Probenecid was from Sigma-Aldrich (St. Louis, MO). IR spectra were recorded on a FT-IR spectrometer (Perkin-Elmer, Norwalk, CT) in $CHCl_3$. Melting points were obtained on a Thomas Hoover capillary melting point apparatus and were corrected.

[4-(2,2,5,5-Tetramethylpyrrolidin-1-oxyl-3-carbonyl)piperazin-1-yl]acetic acid acetoxymethyl ester [5]

To a CH_2Cl_2 (10 cm^3) solution of 3-[[(2,5-dioxo-1-pyrrolidinyl)-oxy]carbonyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**3**] (prepared as described in the literature[13]) (0.79 g, 2.79 mmol) was added 1-(ethoxycarbonylmethyl)piperazine (0.48 g, 2.79 mmol; Lancaster Synthesis Inc., Windham, NH). The reaction mixture was stirred overnight and evaporated to dryness, resulting in an oil which was then taken up in ice cold dilute HCl (5%) and extracted with $CHCl_3$ (three times). The remaining aqueous solution was made basic with Na_2CO_3 and extracted with $CHCl_3$ (three times). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residual oil was purified by flash column chromatography using silica gel 60 (230–400 mesh, $CHCl_3$–acetone, 9 : 1). The product solidified upon addition of hexane. Recrystallization from boiling hexane with dropwise addition of ethyl ether afforded [4-(2,2,5,5-tetramethyl-pyrrolidin-1-oxyl-3-carbonyl)-piperazin-1-yl]acetic acid ethyl ester [**4**] (0.71 g; 75%) as a yellow solid; mp 108–109 °C; (found: C, 60.02; H, 8.98; N, 12.25. Calc. for $C_{17}H_{30}N_3O_4$: C, 60.00; H, 8.82; N, 12.35); $\nu_{max}(CHCl_3)/cm^{-1}$: 1739 and 1640 (CO).

To a CH_3OH (10 cm^3) solution of [4-(2,2,5,5-tetramethyl-pyrrolidin-1-oxyl-3-carbonyl)-piperazin-1-yl]acetic acid ethyl ester [**4**] (0.5 g, 1.47 mmol) was added KOH (82 mg, 1.47 mmol). This reaction mixture was heated to ~50 °C for 3 h; disappearance of starting material was monitored by TLC (silica gel plates, $CHCl_3$–acetone, 1 : 1). After evaporating the solvent, the residue was taken up in H_2O (10 cm^3) and extracted with CH_2Cl_2 (three times). The aqueous solution was evaporated to dryness, and then DMSO (1 cm^3) and CH_2Cl_2 (10 cm^3) were introduced. This mixture was stirred at room temperature for 5 min, at which point bromomethyl acetate (0.22 g, 0.14 cm^3, 1.47 mmol; Aldrich, Milwaukee, WI) was added. The reaction was stirred at room temperature for 2 h. An ice–water mixture was then added and the organic solution was removed. The aqueous solution was extracted with CH_2Cl_2 (three times). The organic solution was dried over anhydrous Na_2SO_4, and evaporated to dryness. Any remaining DMSO was removed under a high vacuum. The residual oil was purified by flash column chromatography on silica gel 60 (230–400 mesh, $CHCl_3$–acetone, 9 : 1). The product solidified upon addition of hexane. Recrystallization from hexane–benzene yielded [4-(2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-carbonyl)piperazin-1-yl]acetic acid acetoxymethyl ester [**5**] (0.37 g; 65%) as a yellow solid; mp 71–72 °C; (found: C, 56.32; H, 7.99; N, 10.85. Calc. for $C_{18}H_{30}N_3O_6$: C, 56.25; H, 7.81; N, 10.94); $\nu_{max}(CHCl_3)/cm^{-1}$: 1765 and 1641 (CO).

(2,2,5,5-Tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N*,*N*-diacetic acid diethyl ester [8]

To a solution of 3-methylamino-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [**7**] (prepared as described in the literature[14]), (1.4 g, 8.1 mmol) in DMSO (2 cm^3) and CH_3CN (2 cm^3) was added anhydrous K_2CO_3 (3.39 g, 24.5 mmol). After stirring for 5 min, ethyl bromoacetate (2.73 g, 1.81 cm^3, 16.2 mmol) was added and the reaction mixture was stirred at room temperature for 3 h, at which point H_2O was added. The solution was washed with CH_2Cl_2 (three times). The organic solution was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residual oil was purified by flash column chromatography on silica gel 60 (230–400 mesh, $CHCl_3$–acetone, 48 : 2) to yield (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N*,*N*-diacetic acid diethyl ester [**8**] (1.9 g, 70%) as a thick orange oil; *m*/*z* (FAB) 344.2320 ($M^+ + H$. $C_{17}H_{32}N_2O_5$ requires 344.2311); $\nu_{max}(CHCl_3)/cm^{-1}$: 1733 (CO).

(2,2,5,5-Tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N*,*N*-diacetic acid diacetoxymethyl ester [9]

To a CH_3OH (10 cm^3) solution of (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N*,*N*-diacetic acid diethyl ester [**8**] (0.6 g, 1.7 mmol) was added KOH (200 mg, 3.6 mmol). The mixture was heated to ~50 °C for 3 h; disappearance of starting material was monitored by TLC (silica gel plates, ether). After evaporating the solvent, the residue was taken up in H_2O (10 cm^3) and extracted with CH_2Cl_2 (three times). The aqueous solution was evaporated to dryness then DMSO (1 cm^3) and CH_3CN (2 cm^3) were added. This mixture was stirred at room temperature for 5 min, at which point bromomethyl acetate (0.55 g, 0.35 cm^3, 3.6 mmol; Aldrich, Milwaukee, WI) was added. The reaction was stirred at room temperature for 3 h. Water (10 cm^3) and then CH_2Cl_2 (20 cm^3) were added and the organic solution was removed. The aqueous solution was further extracted with additional CH_2Cl_2 (three times). The organic solution was dried over anhydrous Na_2SO_4 and evaporated to dryness. Any remaining DMSO was removed under a high vacuum. The residual oil was purified by flash chromatography on silica gel 60 (230–400 mesh, $CHCl_3$–ethyl acetate, 45 : 5) to yield (2,2,5,5-tetramethylpyrrolidin-1-oxyl-3-ylmethyl)amine-*N*,*N*-diacetic acid diacetoxymethyl ester [**9**] (0.44 g, 60%) as a thick orange oil; *m*/*z* (FAB) 432.2095

(M^+ + H. $C_{19}H_{32}N_2O_9$ requires 432.2108); $\nu_{max}(CHCl_3)/cm^{-1}$: 1759 (CO).

EPR spectroscopy

For EPR spectroscopy, each sample was added to a quartz flat cell, which was introduced into the cavity of the spectrometer (model E-109, Varian Associates, Palo Alto, CA). The quartz cell was open to the atmosphere to allow free equilibration with air. EPR spectra were recorded at room temperature with the following instrument settings: microwave power, 20 mW; field set, 3340 G; sweep width, 100 G; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; response time, 0.5 s; sweep, 12.5 G min^{-1}. Receiver gain ranged from 1×10^3 to 1×10^4 for the lymphocyte experiments and 2×10^2 to 1×10^4 when constructing calibration curves. The hyperfine splitting constant of the aminoxyls is $A_N = 14.9$ G.

Loading of lymphocytes with aminoxyls

Jurkat lymphocytes (gift of Dr Alfredo Garzino Demo) were suspended in bicarbonate buffered RPMI 1640 medium, supplemented with 100 unit cm^{-3} penicillin and 100 μg cm^{-3} streptomycin, that also contained 40 μM of the acetoxymethyl (AM) ester of an aminoxyl and 0.0015% w/v Pluronic F-127 surfactant (BASF Corp., Washington, NJ). The cell suspension (2.2×10^6 cells cm^{-3}) was incubated at 23 °C for 70 min whilst being gently agitated by a rocker. After incubation, the cell suspension was centrifuged for 3.5 min at 1000 rpm, and the cell pellet was resuspended and centrifuged two more times in RPMI 1640 medium that contained 10% v/v fetal bovine serum but no AM ester. The final cell pellet was resuspended in Hanks' Balanced Salt Solution (HBSS) at a density of 3.6×10^6 cells cm^{-3}.

Assessing extrusion and retention of aminoxyls by lymphocytes

Replicate 5 cm^3 suspensions of lymphocytes in HBSS were transferred into 15 cm^3 polypropylene centrifuge tubes and incubated for varying periods of time at 37 °C in a stirred water bath, or at 23 °C on a mechanical rocker. In one series of experiments at 37 °C, the cells were incubated in the presence of 10 mM probenecid, an inhibitor of organic anion transport. At the end of the desired incubation period, the temperature of each sample was rapidly decreased to 10–15 °C with the aid of an ice–water bath. After incubation, each suspension was centrifuged for 3.5 min at 1000 rpm, and the supernatant HBSS was separated from the cell pellet, which was then resuspended in 400 μL of fresh HBSS and pulse-sonicated for 60 s at 20 °C to ensure complete cell lysis and release of aminoxyls into the HBSS. Recording EPR spectra of the resuspended cell pellet and the supernatant solution allowed quantitation of the amounts of intracellular and extracellular aminoxyl, respectively. The ratio of the intracellular aminoxyl to the total amount recovered in each sample yielded the fraction of the spin probe retained intracellularly ("Fractional Intracellular Retention" in figures).

Estimation of intracellular concentration of aminoxyls in lymphocytes incubated with the AM esters of aminoxyls

A Jurkat lymphocyte suspension at the same cell density as all samples used in the experiments (4.5×10^7 cells cm^{-3} HBSS) that had not been incubated with AM ester was pulse-sonicated (60 s, 20 °C) to ensure cell lysis. The lysate was clarified by centrifugation at 15 300 rpm for 6 min to sediment cellular debris. Standard solutions of the Na^+ salt of an aminoxyl in the clear cell lysate were made by serial dilution to bracket the concentration range of 10–1000 μM. An EPR spectrum was acquired for each standard solution; the height of the middle field peak was measured and plotted against the aminoxyl concentration to construct a standard calibration curve. From the EPR spectrum of the clear lysate of the cells that had been incubated with AM ester, the middle field peak height was measured and compared with the calibration curve, which allowed the aminoxyl concentration in the lysate to be determined. Knowing that the original suspension contained 1.8×10^7 cells cm^{-3} in 400 μL HBSS, and that 7.65×10^{-13} L is the average Jurkat cell volume,[15] the average intracellular aminoxyl concentration in intact cells could be estimated by straightforward volumetric calculation.

Data analysis

All data analysis, graphing and nonlinear least-squares curve fitting were performed through Origin software (OriginLab Corp, Northampton, MA). Reported values are mean ± standard error; standard errors of curve-fitting parameters are obtained from the nonlinear least-squares error matrix after χ^2 minimization.

Acknowledgements

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