In Vitro Mitochondrial Labeling using Mito-Carboxy Proxyl (Mito-CP) Enhanced Magnetic Resonance Imaging

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Introduction

Nitroxide radicals, due to a single unpaired electron, exhibit T_1-contrast enhancement. Nitroxides have also been shown to exhibit T_1 contrast enhancement *in vivo* [1]. Recent evidence suggests that Mito-Carboxy Proxyl (Mito-CP), depicted in Figure 1, preferentially targets mitochondria [2]. It is thought that the Mito-CP is taken up by the mitochondria for two reasons [3,4]. First, lipophilic cations, such as the triphenylphosphonium cation, distribute their charge over a large surface area allowing them to easily penetrate the lipid bilayers. Second, uptake of lipophilic ions through the lipid bilayers is increased 10-fold for every 61.5 mV difference in the membrane potential. This would explain the uptake of Mito-CP across the plasma membrane (30-60 mV) and across the mitochondria membrane (150-180 mV). The purpose of this study was two fold: (1) to measure the relaxivity of Mito-CP in solution and (2) to verify that Mito-CP is taken up by mitochondria. A MR contrast agent specific to mitochondria would provide a marker of metabolic and/or mitotic activity. Pathologies, such as tumors, where metabolic activity is significantly increased would benefit from spatial identification *in vivo*.

Materials and Methods

All studies were preformed at 3.0T using a local 3.5 cm diameter in-house quadrature coil. *Relaxivities of CP and Mito-CP*. 2.0 mM CP and 2.0 mM Mito-CP were prepared as previously described [2]. Both solutions were diluted with Dulbecco's Phosphate Buffered Saline (DPBS) to the following concentrations: 1.0 μM, 10.0 μM, 100.0 μM, 1.0 mM, 10.0 mM, and 20.0 mM. Longitudinal relaxation times (T_1) for each solution and relaxivities (R_1) for CP and Mito-CP were calculated using a standard spin echo sequence (T_E=9 msec, T_R=15,000 msec) at various inversion times (T_I=100 msec incrementing by 100 msec to 4000 msec and 50, 150, 1050, 1150 msec).

In Vitro Mitochondria. Isolated rabbit mitochondria were separated into three eppendorf tubes each containing 40 μL of 15 mg/ml mitochondria. Six eppendorf tubes were prepared as described in Table 1. Note the succinate is needed to activate mitochondria's respiratory chain, necessary for normal function. All tubes were incubated at 37°C for 10 minutes. Immediately following incubation all tubes were centrifuged at 1,000 x g for 4 minutes at 4°C. The supernatant was then transferred into another eppendorf tube. The mitochondria in tubes 4-6 were resuspended with the addition of 50 μL of DPBS and allowed to naturally settle. All tubes were simultaneously imaged using a fast spin echo inversion recovery sequence (T_E=24.25 msec, T_R=15,000 msec, and an echo train of 16) at various inversion times for initial estimates of T_1 and a standard spin echo sequence (T_E=21 msec, T_R=15,000 msec) at various inversion times (T_I=63, 250, 500, 750, 1000, 1200, 1250, 1275, 1300, 1325, 1350, 1375, 1400, 1450, 1600, 1700, 1750, 1800, 1850, 1900, 2500, 3000, 3500, 4000 msec). T_1 maps were then generated from the spin echo inversion recovery data.

Results and Discussion

Depicted in Figure 2 are the longitudinal relaxation times of the various concentrations of the solutions and the fits used to estimate R_1 for CP and Mito-CP. CP and Mito-CP were found to have longitudinal relaxivities of 0.1546 ± 0.00567 $mM^{-1}sec^{-1}$ and 0.2322 ± 0.0151 $mM^{-1}sec^{-1}$, respectively. T_1 weighted images at an inversion time of 1900 msec are displayed in Figure 3. The overlaid numbers correspond to the labels from Table 1. The mitochondria in tubes 4-6 settled to the tip of the eppendorf tube. The mitochondria treated with the Mito-CP (#5) were found to have an enhanced T_1 due to the presence of the Mito-CP. The mitochondria treated with CP (#6) did not enhance considerably compared to mitochondria alone (#4) demonstrating the necessity of the triphenylphosphonium cation for uptake into mitochondria. Ignoring partial voluming, an estimated intramitochondrial Mito-CP concentration of 0.143 mM was calculated using the estimated longitudinal relaxivity and the longitudinal relaxation time from the untreated mitochondria. This would suggest at least a 140-fold increase in concentration within the mitochondria compared to the incubating solution. These results indicate that Mito-CP allows for MRI visualization of mitochondria. Further *in vitro* cell and *in vivo* animal studies will be pursued to validate Mito-CP as a viable *in vivo* contrast agent. This is the first demonstration of the potential for a new MR contrast agent, such as Mito-CP, to specifically target mitochondria.

Figure 1: Chemical structures of CP and Mito-CP

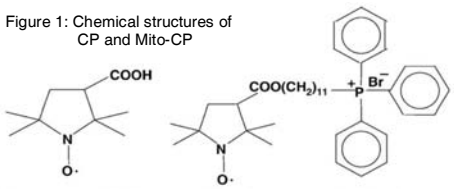


Table 1: Detail description of each eppendorf tube's contents.

Eppendorf Tube	Label	Contents
1	Buffer	20 uL 0.1 M succinate 180 uL DPBS
2	Buffer + 10 uM Mito-CP	20 uL 0.1 M succinate 180 uL DPBS 1.0 uL 2mM Mito-CP/EtOH
3	Buffer + 10 uM CP	20 uL 0.1 M succinate 180 uL DPBS 1.0 uL 2mM CP/EtOH
4	Mitochondria	40 uL 15 mg/mL mitochondria 20 uL 0.1 M succinate 140 uL DPBS
5	Mitochondria + 10 uM Mito-CP	40 uL 15 mg/mL mitochondria 20 uL 0.1 M succinate 139 uL DPBS 1.0 uL 2.0 mM Mito-CP/EtOH
6	Mitchondria+ 10 uM CP	40 uL 15 mg/mL mitochondria 20 uL 0.1 M succinate 139 uL DPBS 1.0 uL 2.0 mM CP/EtOH
4b	Supernatant #4	
5b	Supernatant #5	
6b	Supernatant #6	

Figure 2: Longitudinal relaxation times of various concentrations of CP and Mito-CP.

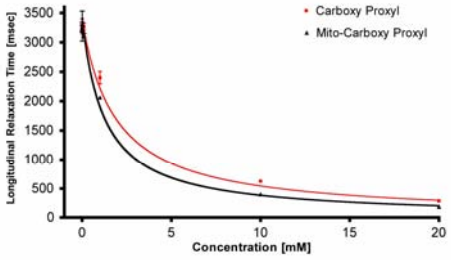
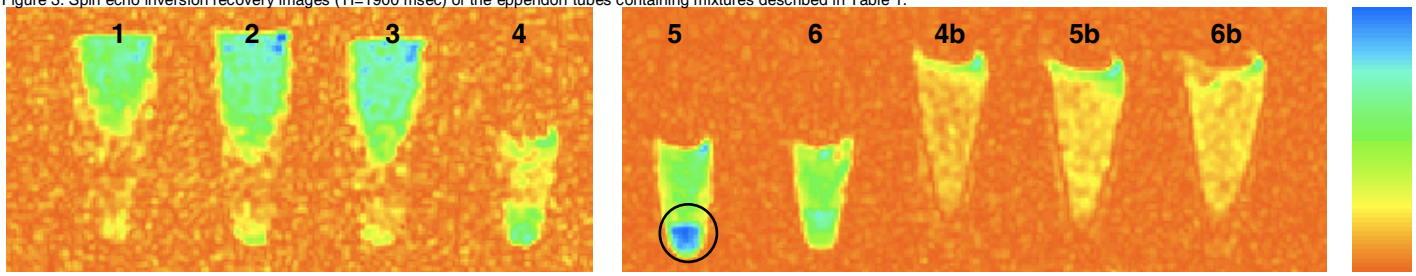


Figure 3: Spin echo inversion recovery images (TI=1900 msec) of the eppendorf tubes containing mixtures described in Table 1.



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