Measurements of kinetic stability, blood-brain barrier permeability and cytotoxicity for two thulium based contrast agents

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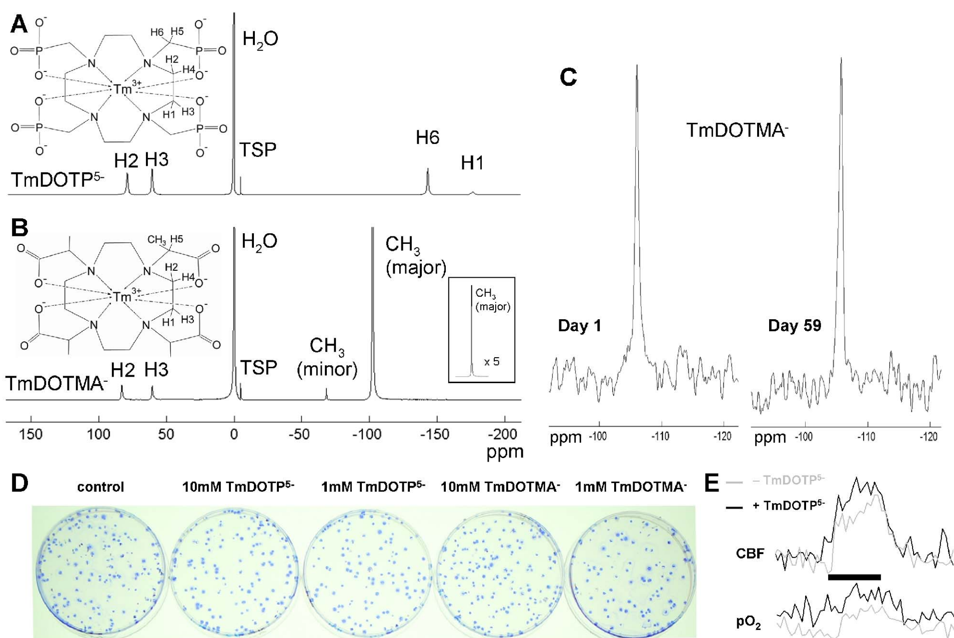
INTRODUCTION

Successful translation of smart contrast agents (SCAs) from the benchtop into biological use depends on several key intermediate steps. The measurement of *ex vivo* kinetic inertness, *in vivo* blood-brain barrier permeability and *in vivo* cytotoxicity are three important steps in assessing the feasibility of using any SCA for any ulterior biological studies. Over the past decade, a new non-invasive method for simultaneous measurements of temperature and pH was developed, based on the strong dependence on temperature and pH of the proton chemical shifts from the complex between the thulium ion and the macrocyclic chelate 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetra (methylene phosphonate) or $TmDOTP^{5-}$ **(Fig.1A) [1,2,3,4,5]**. Thanks to the high sensitivity of each resonance on temperature and pH, models can be developed **[2,3]** to determine both temperature and pH simultaneously and very accurately in the rat brain **[4]**. More recently, a new temperature probe was introduced, also relying on thulium as paramagnetic ion, but for which the macrocyclic chelate is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethyl-1,4,7,10-tetraacetate or $TmDOTMA^-$ **(Fig.1B) [6]**. The methyl ^1H chemical shift of $TmDOTMA^-$ is pH-independent **[6]**. In the present work, we measured *ex vivo* kinetic inertness, *in vivo* blood-brain barrier permeability and *in vivo* cytotoxicity of $TmDOTP^{5-}$ and $TmDOTMA^-$ agents.

MATERIALS AND METHODS

***In vivo* animal studies:** Sprague-Dawley rats were tracheotomized and artificially ventilated (70% N_2O, 30% O_2). During the animal preparation, isoflurane (1 to 2 %) was used for induction. An intraperitoneal line was inserted to administer α-chloralose (46 ± 4 mg/kg/hr) and an intravenous line was used to administer D-tubocurarine chloride (1 mg/kg/hr) or SCA (1-2 mmol/kg). An arterial line was used to monitor physiological parameters (blood pH, pO_2, pCO_2) throughout the experiment. The anesthetized rats were prepared with renal ligation as previously described **[7].** All ^1H NMR data **(Fig. 1A, 1B and 1C)** were acquired on an 11.7 T Bruker vertical-bore spectrometer (Billerica, MA). CBF was measured by a laser Doppler flowmetry probe (830 nm) and pO_2 was measured by an oxygen fluorescence probe (485/600 nm).
***In vivo* cytotoxicity:** CCL16 Chinese hamster lung cells **(Fig.1D)** were used to measure the *in vivo* cytotoxicity. The colony-forming ability of the cells in the cultures exposed to the SCAs was compared with that of cells from untreated cultures to calculate the relative surviving fraction. The cells were grown as monolayer cultures in Petri dishes containing cell culture medium (Dulbecco's Modified Eagle's Medium) supplemented with 15% fetal bovine serum. Following standard procedures, monolayers were prepared by plating cells suspended from exponentially growing stock cultures in flasks. Next, the growth medium was removed and replaced by either fresh growth medium, or medium containing SCA up to a maximum concentration of 10 mM. The cells were exposed for 6 hours to the SCAs, during which the cells were maintained in a humidified 95% air and 5% CO_2 environment at 37 °C. After the exposure, the medium on the cultures was removed, the cultures were washed twice with Hanks' balanced salt solution to remove debris and residual SCA, trypsinized, and counted with a Coulter counter. The cells were plated for colony formation in four dishes for each concentration and were allowed to grow in a humidified 95% air and 5% CO_2 environment at 37 °C for 7 days. The colonies were then fixed, stained, and counted. Colony counting was performed both visually and using the automated cell counting system MACE (Weiss Associates, Branford, CT). Based on the colony count, survival rates were calculated and expressed in terms of colony-forming ability of the exposed cells.



RESULTS AND DISCUSSION

Kinetic stabilities of the SCAs in blood extracts from the sagittal sinus were estimated by the intensity of their ^1H NMR resonances over time. For $TmDOTMA^-$, the results indicate that there is no change in the intensity of the methyl group resonance from day 1 to day 59 **(Fig.1C)**. Similar results were obtained for the $TmDOTP^{5-}$ agent, indicating that the complex between the macrocyclic chelates and Tm^{3+} is stable over long periods of time (months). It has been proposed **[6]** that $TmDOTP^{5-}$ does not cross the blood-brain barrier. Our observations suggest that $TmDOTP^{5-}$ does cross the blood-brain barrier quite efficiently, which allows quantifying brain temperature and pH **[7]**. Concomitant ^1H signals of $TmDOTP^{5-}$ in blood plasma and cerebral spinal fluid (CSF) confirm that the majority of the *in vivo* MR signals emanate from tissue **[7]**. SCAs most likely enter the extracellular space despite the fact that they are charged. A plausible delivery path into the extracellular space may be through the fenestrated vessels of the circumventricular organs. The *in vivo* cytotoxicity tests consistently showed survival rates, expressed in terms of colony-forming ability of the exposed cells, between 90 and 95% compared to the controls **(Fig.1D)**. Unfortunately, the half lethal dose (LD_{50}) values of $TmDOTP^{5-}$ or $TmDOTMA^-$ in the rat have not been measured specifically, but results in our laboratory **[7]** suggest that an infusion dose of 1-2 mmol/kg results in stable systemic physiology without affecting normal brain function **(Fig.1E)**. Localized changes in CBF and pO_2 during forepaw stimulation in α-chloralose anesthetized rats are nearly identical before and after SCAs infusion **(Fig.1E, gray and black lines, respectively)**. Moreover, LD_{50} values for similar lanthanide complexes such as $YbDOTMA^-$ **[8]** or $GdDOTA^-$ **[9]** are 10.5 mmol/kg and 11.4 mmol/kg, respectively, suggesting that the LD_{50} for the SCAs may be at least an order of magnitude higher than the doses used in our experiments. In summary, our results indicate that these two SCAs are kinetically stable, they cross the blood-brain barrier, they clearly exclude acute toxicity on Chinese hamster lung cells and they demonstrate that the brain's activity is unaffected by their presence in the extracellular space.

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