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● *Original Contribution*

THE UPTAKE OF Mn-DPDP BY HEPATOCYTES IS NOT MEDIATED BY THE FACILITATED TRANSPORT OF PYRIDOXINE

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Manganese-dipyridoxal diphosphate (Mn-DPDP) is a liver-selective contrast agent selectively taken up by the hepatocytes. Because of the analogy of structure with pyridoxine (vitamin B_6), it was previously suggested that this compound can be selectively taken up by the facilitated transport of vitamers B_6. To understand the uptake mechanism, an in vivo binding study was performed based on a competition between ^{54}Mn-DPDP and pyridoxine on the one hand, and Mn-DPDP and [^{3}H]pyridoxine on the other. We found that the [^{3}H]pyridoxine levels in the liver were not significantly different 5 min after intravenous administration of several doses of Mn-DPDP (5 nmol/kg to 50 μmol/kg): 5.0 ± 0.3% of the injected dose/g tissue. The content of ^{54}Mn (administered as ^{54}Mn-DPDP) in the liver was not affected by a saturation dose of pyridoxine (1 mmol/kg) and was found to be constant (±10% of the injected dose/g tissue) for 60 min. These experiments showed that the uptake of Mn-DPDP is not mediated by the transporter of pyridoxine.

Keywords: **Dipyridoxal diphosphate (Mn-DPDP); [^{3}]pyridoxine; Pyridoxine.**

INTRODUCTION

Manganese-dipyridoxal diphosphate (Mn-DPDP) (Fig. 1, top) is a manganese chelate derived from vitamin B_6 (pyridoxal 5′-phosphate) and is specifically taken up by the hepatocytes.[1] Chelation of Mn(II) is necessary to decrease the high acute toxicity of the free metal ions.[2] Initial evaluation of Mn-DPDP revealed it considerably decreased acute toxicity (LD_{50} in mice was 0.3 and 5.5 mmol/kg for $MnCl_2$ and Mn-DPDP, respectively) and was highly extracted by the liver.[3] Subsequently, Mn-DPDP has been used to assess different experimental liver and pancreas pathologies and was the first hepatobiliary magnetic resonance (MR) contrast agent to undergo clinical trials.[4–9]

Although this compound is in clinical trials, the uptake mechanism of Mn-DPDP still remains unknown. The analogy of structure of Mn-DPDP with pyridoxine suggested that the complex can be taken up by the transporter of pyridoxine.[10–12] Other analogs of pyridoxine, such as pyridoxal, pyridoxal-phosphate, and pyridoxic acid are indeed transported inside the hepatocytes using the same facilitated transport. To our knowledge, there is no conclusive proof that Mn-DPDP is able to recognize this receptor. This knowledge is essential for knowledge of whether Mn-DPDP could be considered a receptor imaging contrast agent. Because the expression of the receptors is often modified in the case of pathologies,[13] and because it is known that diseases such as cancer affect the metabolism of pyridoxine,[14,15] it could be very useful to characterize the mode of penetration of Mn-DPDP inside hepatocytes. To understand the uptake mechanism, an in vivo binding study was performed based on competition between ^{54}Mn-DPDP and pyridoxine on the one hand, and Mn-DPDP and [^{3}H]pyridoxine on the other. This in vivo competition is the most straightforward method, classically performed with receptor contrast agents used in positron emission tomography,[16,17] and

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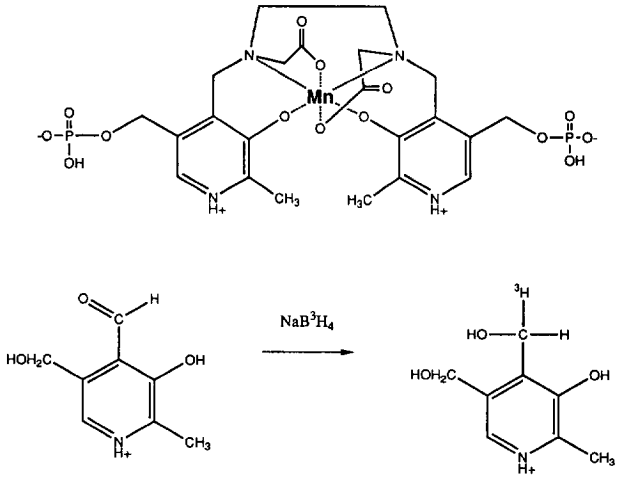

Fig. 1. Chemical structures. (Top) Structure of Mn-DPDP. (Bottom) Radiolabeling of pyridoxine using the reduction of pyridoxal by boro[^{3}H]hydride. Note the analogy of structures between Mn-DPDP and pyridoxine.

has been already performed with nuclear MR (NMR) contrast agents.[18]

MATERIALS AND METHODS

Chemistry

Preparation of ^{54}Mn-DPDP. The complex was prepared by transchelation: To 50 μl of Mn-DPDP (Byk-Gulden; 145 mol/ml) was added 10 μl of $^{54}MnCl_2$ (Dupont de Nemours; 175.77 mCi/mg). The volume was adjusted to 3 ml with water (solution A). The radiochemical purity was monitored by paper chromatography (Whatman no. 1; eluent:methanol/water, 7/3) and the radioactivity on the plate was analyzed with a Bioscan System 200 imaging scanner. After 30 min, the radiochemical purity was constant and > 95%.

Synthesis of [^{3}H]pyridoxine. The chemical synthesis is shown in Fig. 1, bottom. The method is adapted from Zuomin[10] and involves the reduction of pyridoxal by boro[^{3}H]hydride. Briefly, 4.8 μmol of pyridoxal hydrochloride (Aldrich) was dissolved in sodium hydrogen carbonate (1 M; final volume 4.5 ml) and the pH was adjusted to 8 with NaOH. Then, 75 mg of sodium boro[^{3}H]hydride (Dupont de Nemours; 8.31 Ci/mmol) was added. The reaction mixture was stirred for 1 h at room temperature, and the reaction was stopped by adding an excess of concentrated hydrogen chloride. The acidic excess was then neutralized using NaOH. The mixture was evaporated under reduced pressure. The remaining salts were removed by several cycles of dissolution in methanol filtration evaporation. The radiochemical purity was monitored by thin-layer chromatography (silicagel; eluent:acetone/dioxane/NH_3 25%:45/45/10). The radioactivity on the plate was analyzed with a Bioscan System 200 imaging scanner. On the other hand, the ^{1}H and ^{13}C NMR spectra of pyridoxine obtained by the same method, using nonradiolabeled borohydride, showed NMR spectra identical to the reference compound. For preparation of the solution for injection (solution B), a part of the residue was dissolved in a solution of 0.2 mg/ml cold pyridoxine hydrochloride (Aldrich) in NaCl 9% (final vol. act.: 75 $\times$ 10^6 cpm/ml).

In Vivo Studies

Male NMRI mice (average weight, 30 g) were used in all experiments. The injections were performed in a caudal vein. Three kinds of experiments were performed. We validated the dose necessary to inhibit the hepatocyte pyridoxine transporter by competition between traces of [^{3}H]pyridoxine and unlabeled pyridoxine. We also made a competition study between [^{3}H]pyridoxine and Mn-DPDP to demonstrate the potential recognition of the pyridoxine transporter by the Mn-DPDP complex. Finally, we performed a competition between ^{54}Mn-DPDP and an inhibiting dose of pyridoxine to demonstrate if this complex needs the pyridoxine transporter to be taken up by hepatocytes.

Inhibiting dose of pyridoxine. Two groups of 10 mice were injected in a tail vein with 1 ml/kg of solution B (2 μmol/kg of [^{3}H]pyridoxine): one group without previous treatment (control) and one group 5 min after injection of 1 mmol/kg of cold pyridoxine. At 5 min after the injection of [^{3}H]pyridoxine, the mice were anesthetized under diethylether and sacrificed by cervical dislocation, and the liver was sampled. The livers were carefully washed with NaCl 9% solution. After weighing, the samples were dissolved in 1 ml Soluene 350 (Camberra Packard; 1 night at room temperature), discolored by adding 1 ml hydrogen peroxide (small portions of 100 μl), and mixed with 10 ml of Hionic-Fluor (Camberra Packard). The radioactivity (β-emission) of the samples was counted using a Wallac 1410 liquid scintillation counter (Pharmacia) 24 h later to avoid chemiluminescence problems. The efficiency of the counting was > 25%.

Competition Mn-DPDP/[^{3}H]pyridoxine. The competition study was performed between [^{3}H]pyridoxine and several dosages of Mn-DPDP (0.005, 0.05, 0.5, 5, and 50 μmol/kg). Two populations of mice received 1 ml/kg of solution B (2 μmol/kg of [^{3}H]pyridoxine): the control group without previous treatment and the other group 5 min after injection of a solution of Mn-DPDP (10 mice/group). At 5 min after the injection of [^{3}H]pyridoxine, the mice were anesthetized under

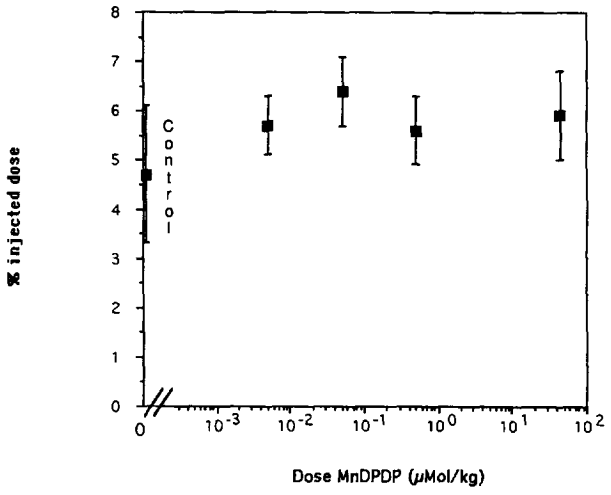

Fig. 2. Effect of the injection of different amounts of Mn-DPDP on the uptake of the [^{3}H]pyridoxine by the liver. Radioactivity incorporated into the liver (average ± standard deviation) 5 min after the injection of [^{3}H]pyridoxine. Note that previous injection of Mn-DPDP in different doses did not affect the uptake of [^{3}H]pyridoxine.

diethylether and sacrificed by cervical dislocation, and the liver was sampled. The radioactivity present in the liver was analyzed as described previously.

Competition ^{54}Mn-DPDP/pyridoxine. A biodistribution study was performed on two populations of mice who received 5 µmol/kg ^{54}Mn-DPDP (solution A, 2.5 µmol/ml): a control group (20 mice) without previous treatment and another group after injection of 1 µmol/kg pyridoxine. As described above, the mice were sacrificed 5, 15, 30, 45, and 60 min after injection. The livers were sampled. After weighing, the radioactivity (γ emission) of the samples was measured using a Packard 5230 scintillation spectrometer.

RESULTS

Inhibiting Dose of Pyridoxine

A delay time of 5 min between injection and sampling of the liver was chosen because a preliminary study demonstrated that the uptake of [^{3}H]pyridoxine by the liver is rapid and reaches a plateau 5 min after the injection of this compound (data not shown). The competition between [^{3}H]pyridoxine and cold pyridoxine (1 mmol/kg) showed that this uptake was saturable. A significant difference ($p < .01$, t-test) in the radioactivity found inside the liver was observed between the control group and the group that received an injection of 1 mmol/kg pyridoxine 5 min before the administration of the radiolabeled pyridoxine: 5.0 ± 0.3% of the injected dose/g tissue and 3.8 ± 0.2% injected dose/g tissue (mean ± standard error of the mean).

Competition Mn-DPDP/[^{3}H]pyridoxine

The results of the competition between Mn-DPDP and [^{3}H]pyridoxine are shown in Fig. 2. Mn-DPDP did not compete (no significant differences; $p > .01$, t-test) with the uptake of [^{3}H]pyridoxine by the liver, even though we used amounts at a dose as high as 10 times the clinical dose of Mn-DPDP (5 µmol/kg).

Competition ^{54}Mn-DPDP/pyridoxine

The results of the competition between ^{54}Mn-DPDP and pyridoxine are shown in Fig. 3. The uptake of ^{54}Mn-DPDP was not affected by saturation of the pyridoxine transporter.

DISCUSSION

The concept of MR receptor imaging is very attractive because it could potentially combine the advantages of the mapping of receptors (as classically obtained in nuclear medicine, particularly in positron emission tomography) and the high resolution of MR imaging. Up to now, only few compounds based on coated ultrasmall superparamagnetic iron oxide (USPIO)[19–21] or targeted nitroxides[18,22] have been checked for their ability to specifically bind to targeted receptors. Because USPIO particles are very effective with the relaxation times of the neighboring water protons, they are ideal for this purpose; indeed, the problem of saturation of the receptors cannot

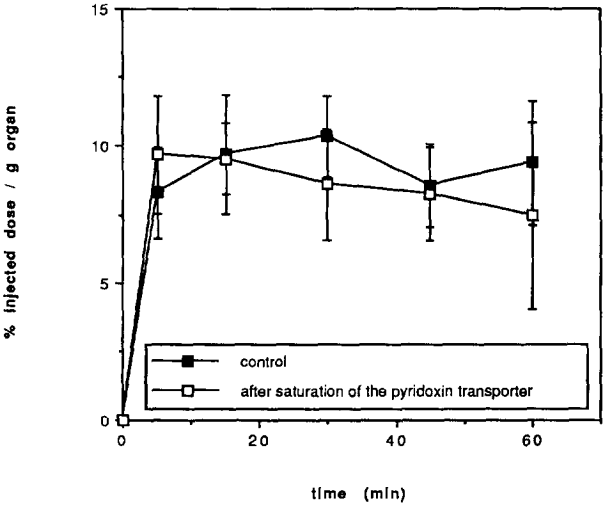

Fig. 3. Uptake kinetics of ^{54}Mn-DPDP (5 µmol/kg) by the liver without (control) or after saturation of the pyridoxine transporter by intravenous injection of 1 mmol/kg of pyridoxine. Radioactivity incorporated into the liver (average ± standard deviation) 5 min after the injection of ^{54}Mn-DPDP.

occur at the dose used to induce contrast on the MR image. However, their large size limits their applicability to receptor-mediated endocytosis such as asialoglycoprotein receptor in liver[19,20] or cholecystokinin receptor in pancreas,[21] and cannot be enlarged to the targeting of transporters or receptors of small molecules. Nitroxide-based compounds present high chemical versatility and can be functionalized by highly specific carriers.[18,22] However, because of the low relaxivity and consequent low efficiency of the NMR image, injection of large amounts of contrast agent is necessary and leads to non-specific contrast effects.[22] Among the paramagnetic metal-based compounds, several complexes were designed for the specific recognition of receptors.[23] However, no complex was definitely established to recognize specific receptors because of the lack of a binding study.

Mn-DPDP was a potential candidate as a specific receptor contrast agent of the pyridoxine transporter expressed at the sinusoidal pole of hepatocytes. As analogs of pyridoxine are also recognized by this transporter,[10,12] and because of the analogous structure of Mn-DPDP with vitamers B_6 (Fig. 1), it was suggested that the complex can be taken up by the pathway of pyridoxine. Our present study clearly shows that Mn-DPDP does not require the presence of the pyridoxine transporter to be taken up by the liver: No significant differences in the level of Mn-DPDP were observed in the liver with or without saturation of the pyridoxine transporter (Fig. 3). Moreover, the competition study between Mn-DPDP and [^{3}H]pyridoxine clearly demonstrates that Mn-DPDP does not recognize the pyridoxine transporter (Fig. 2), which means that the chemical modifications brought to the pyridoxine structure lead to a loss in ability of the paramagnetic complex to bind to the transporter. The actual mechanism in the uptake of the paramagnetic center by the liver still needs to be elucidated. We recently demonstrated the dissociation of the Mn-DPDP complex in biological media and the consequences on relaxivity.[24] Our results suggested that the paramagnetic moieties entering the hepatocytes are free Mn^{2+} on the one hand and intact complex on the other; the dechelation continues after entering the hepatocytes.[24] Consequently, the elimination pathways of Mn and DPDP are quite different: Mn is excreted more in the feces, while the ligand is essentially excreted in urine[25] after dephosphorylation and metal exchange.[26] Considering the pathway used for entering inside hepatocytes, the uptake of the free Mn^{2+} can be ascribed to the presence of a selective transporter for this cation.[27] The uptake of the complex is hypothetical: passive diffusion through the membrane or nonspecific uptake by an anion transporter.

The actual and practical consequence of these results is the unsuitability of using Mn-DPDP as a tracer of modifications in the expression of the pyridoxine transporter. This means that the clinical applications of Mn-DPDP should be found essentially in systematic studies of the usefulness of this contrast agent in pathologies (such as the correlation between contrast, tumor differentiation, and vascularization[28]), rather than in rational prediction of the basis of changes in the expression of receptors induced by a particular pathology.

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