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Brief Article

Glucosylated Deferiprone and Its Brain Uptake: Implications for Developing Glucosylated Hydroxypyridinone Analogues Intended to Cross the Blood−Brain Barrier

Sourav Roy, Jane E. Preston, Robert C. Hider, and Yong Min Ma*

Pharmaceutical Science Division, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, United Kingdom

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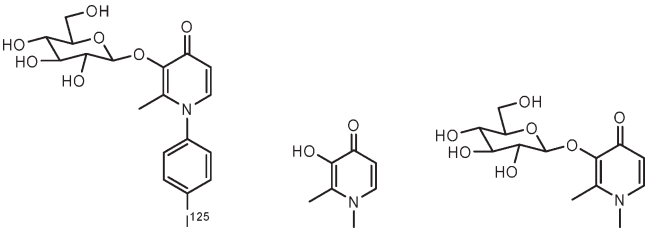
This report presents that Deferiprone, the only clinically used 3-hydroxypyridin-4-one (HPO), is able to penetrate the blood−brain barrier (BBB) in guinea pigs, whereas its glucosylated analogue is unable to do so. This finding is contrary to published information suggesting that the glucosylation of HPOs is a viable means of enhancing the brain uptake of this group of compounds.

Introduction

Deferiprone is an orally active molecule used clinically to treat iron overload associated with regularly transfused β thalassemia patients. Recently, Deferiprone has been shown to possess beneficial effects on patients suffering from Friedreich's ataxia (FA[a]).[1] FA is a genetically inherited mitochondrial disease characterized by decreased production of frataxin, a mitochondrial iron chaperone, which leads to mitochondrial iron accumulation and related toxicity. This is due to a mutant FXN gene that normally encodes for frataxin.[1,2] In Friedreich's ataxia, the reduced levels of frataxin leads to iron accumulation in mitochondria, resulting in oxidative stress-induced cellular damage, most importantly affecting sensory neurons, the myocardium, and endocrine glands.[1] Recent clinical trials on FA-affected patients clearly indicated the ability of Deferiprone (**2**, Figure 1) to cross the human BBB and to produce beneficial effects as an iron chelator. In principle, HPOs, which can achieve a higher brain uptake than Deferiprone, could be prospective lead molecules for the therapy of iron-dependent neurodegenerative disorders such as Alzheimer's disease (AD) and FA.[3]

In an attempt to achieve a novel means of improving the brain uptake of HPOs, a recent report claimed that *O*-glucosylation of 3-hydroxypyridin-4-ones, in general, is a viable means of enhancing BBB permeation of HPOs.[4] This conclusion was based on studies of a radiolabeled glucosylated HPO (**1**, Figure 1). This compound was reported to possess a similar brain uptake in rat to that of thiourea, a molecule which is known to possess a moderate ability for brain uptake. A 60 s in situ brain perfusion in rat was performed to determine the brain uptake of this radiolabeled glucosylated HPO (**1**, Figure 1).[4] As a result of this study, we decided to directly compare the brain uptake of Deferiprone and its glucosylated analogue (**3**, Figure 1).



1 2 3

Figure 1. Radiolabeled 1,4-dihydro-1-(4-[^{125}I]iodophenyl)-2-methyl-4-oxo-pyridin-3-yl) β-D-glucopyranoside (**1**), Deferiprone (**2**), and glucosylated Deferiprone (**3**).

Methods

A novel route of synthesis was developed to synthesize the *O*-glucosylated analogue of Deferiprone (Scheme 1). The glucosylation technique was adapted from Kroger.[5] Briefly, maltol (**4**, Scheme 1) was reacted with glucopyranosyl bromide (**5**, Scheme 1) in the presence of tetrabutylammonium bromide (TBAB) and 1 M NaOH at 35 °C for 3 h to obtain the 3-hydroxyl functionalized maltol (**6**, Scheme 1), which was converted into the corresponding pyridinone (**3**, Scheme 1) in good yield (82%) by reacting with methylamine at 70 °C with simultaneous cleavage of acetyl esters. The final product (**3**, Scheme 1) was crystallized from ethanol as pale-yellow crystals. The compound was identified and characterized using ^{13}C and ^{1}H NMR, electrospray, and high resolution mass spectroscopy. The purity of the compound was determined by HPLC method.

To compare the brain uptake of Deferiprone and **3** in guinea pigs, an in situ brain perfusion technique was used.[6,7] All animal procedures were in accordance with the Home Office, Animals (Scientific Procedures) Act 1986, UK. Briefly, anesthetized adult male guinea pigs were dissected to expose the common carotid arteries and the jugular veins. Each common carotid artery was cannulated with fine polyvinyl chloride tubing carrying a warm (37 °C) and well aerated (aerated with 95% O_2, 5% CO_2) perfusate containing 800 μM of either **2** or **3** in Ringer solution (NaCl 117 mM, KCl 4.7 mM, $MgSO_4$ 0.8 mM, $NaHCO_3$ 24.8 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, D-glucose 10 mM, bovine serum

*To whom correspondence should be addressed. Phone: +44 (0)207 848 4844. Fax: +44 (0)207 848 4800. E-mail: yongmin.ma@kcl.ac.uk.

[a]Abbreviations: HPO, 3-hydroxypyridin-4-one; BBB, blood−brain barrier; FA, Friedreich's ataxia; AD, Alzheimer's disease; TBAB, tetrabutylammonium bromide; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid.

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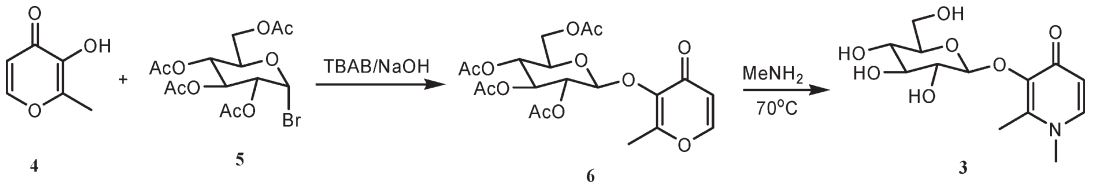
Scheme 1. Synthesis of Glucosylated Deferiprone (**3**)



albumin 39 g/L, Evans blue, 0.055 g/L), pumped at a flow rate of 6 mL/min. The perfusate also contained ^{3}H-mannitol as the vascular space marker. At the end of a 20 min perfusion, the animal was decapitated and the brain was isolated and dissected to remove the choroid plexuses and the cerebellum. It was identified from independent trial experiments that the time required to reach steady-state with the given experimental parameters, i.e., flow rate of 6 mL/min and HPO concentration of 800 μM, was 20 min. It was also understood from trial studies that these parameters were necessary to produce a reasonable concentration of Deferiprone in guinea pig brains that could be detected by the HPLC system used to determine the concentration of these HPOs in the brain and perfusate extracts. This lower threshold of HPO detection by the HPLC system was established at ~3−5 μM. Perfusate samples from the right and the left carotid artery cannulas were also collected at the end of each perfusion as sample perfusates.

The brain samples were homogenized with double the brain weight of physiological buffer (HEPES 100 mM, NaCl 141 mM, KCl 4 mM, $CaCl_2$ 2.8 mM, $MgSO_4$ ($3H_2O$) 1 mM, NaH_2PO_4 ($2H_2O$) 1 mM, and D-glucose 10 mM) and three times the brain weight of 30% dextran solution. Brain homogenate aliquots were taken for scintillation spectroscopy to determine the amount of brain-impermeable ^{3}H-mannitol present. The remaining brain homogenate was divided into two equal portions. One half was vortexed with trifluoroacetic acid (TFA) (9.1% final concentration) and centrifuged at 4 °C and 5400*g* for 30 min. The clear supernatant was collected as the whole brain extract. The other half of brain homogenate was centrifuged at 4 °C and 5400*g* for 15 min. The top layers of the brain tissue and the supernatant were collected, leaving behind the capillaries precipitated at the bottom of the eppendorf tubes. This brain and supernatant was then vortexed with TFA (9.1% final concentration) and further centrifuged at 4 °C and 5400*g* for 15 min. The clear supernatant was collected as the capillary depleted brain extract. Aliquots of the perfusate fluid were collected for scintillation spectroscopy to determine the amount of ^{3}H-mannitol present. Alongside this, the perfusate fluid was stripped of albumin by vortexing an aliquot with TFA (9.1% final concentration), followed by centrifuging the resultant mixture at 4 °C and 5400*g* for 20 min. The clear supernatant was collected as the perfusate extract.

A high performance liquid chromatography (HPLC) method was used to determine the concentration of the HPOs (**2** and **3**) in the brain and perfusate extracts using a standard method as developed by Liu et al.[8] Briefly, a reversed phase polymer column (PLRP-S 300 Å, 15 cm × 0.46 cm, internal diameter 8 μM) was used with a mobile phase produced by a buffer (1-heptanesulfonic acid sodium salt, 5 mM in HPLC water and pH adjusted to 2.0 using hydrochloric acid) and acetonitrile on the HPLC system. A linear gradient of 2−35% of acetonitrile over 20 min at a flow rate of 1 mL/min was followed by a 5 min post run period using 2% acetonitrile and 98% buffer.

The brain and perfusate extracts from experiments perfusing the glucosylated HPO (**3**, Figure 1) in guinea pigs required additional workup before being injected into the HPLC for determination of its concentration in these samples. These extracts were shaken in a water bath at 50 °C for 2 h to ensure 100% hydrolysis of the glucosylated HPO (**3**, Figure 1) to release the equivalent amount of Deferiprone. The amount of TFA present in the brain and perfusate extracts was sufficient to ensure complete hydrolysis of **3** in 2 h as was identified from prior independent trials. Thus, the concentration of **3** in these extracts was calculated as the equivalent detectable concentration of Deferiprone present following this typical workup. Each area under the curve produced by the brain and perfusate samples for both these compounds was recorded. The concentration of Deferiprone in these samples was then calculated from a standard Deferiprone calibration curve ranging from 5 to 800 μM.

Brain uptake or brain distribution of Deferiprone and 3 were calculated as K_p and determined using eq 1.[9]

$$K_p = (C_{br}/C_p) - (C_{brv}/C_{pv}) \quad (1)$$

Here, C_{br} and C_p are concentrations of the test compounds (**2** and **3**) in the brain and the perfusate, respectively. C_{brv} and C_{pv} are the concentrations of the vascular marker (^{3}H-mannitol) in the same. K_p is expressed in mL g^{-1}. A K_pW value was calculated using the values obtained from the whole brain fraction, and a K_pCD value was calculated using the values obtained from the capillary depleted fraction. K_pW indicates the brain distribution of the compounds in the brain as a whole, and K_pCD indicates the distribution of the compounds only in the brain parenchyma (devoid of the associated vasculature).

Results and Discussion

Table 1 summarizes the physicochemical constants and brain uptake values at steady-state for Deferiprone (**2**) and glucosylated Deferiprone (**3**, Figure 1). It was initially hypothesized that, compared to a moderately lipophilic compound like morphine (logP = 0.89),[10] which has a steady-state K_p of 0.54−0.74 mL/g (in rat),[11] the HPOs in this study which are relatively hydrophilic would be less efficient than morphine in crossing the BBB. The brain uptake value of morphine in rat is merely used as a qualitative reference to indicate the brain uptake of the experimental molecules through passive diffusion. This is in line with the current understanding that the central nervous system environment is well conserved across the species including man. This consideration is a useful tool in predicting the brain uptake of different molecules through passive diffusion.[12] However, the K_pW and K_pCD values of Deferiprone indicated that on average about 5% of the perfusate concentration of a BBB permeable HPO may be obtained in the brain using the in situ perfusion parameters used in this study. A K_pW of 0.05 ± 0.005 mL/g (n = 12) and K_pCD of 0.044 ± 0.006 mL/g (n = 12) was

Table 1. Physicochemical Constants and Brain Uptake Values of Deferiprone and Glucosylated Deferiprone (**3**)

compd	mol wt (Da)	clogP[a]	pK_{a1}[b]	pK_{a2}	K_pW (mL/g)	K_pCD (mL/g)
1	489	0.031	n/a	n/a	n/a	n/a
Deferiprone	139	−0.77	3.56	9.64	0.052 ± 0.0046	0.044 ± 0.0056
3	301	−2.65	2.88	n/a	< 0.001	< 0.001

[a] clogP was determined using Molinspiration (www. Molinspiration.com). [b] pK_a values were determined by spectroscopic titration.

determined for Deferiprone. In contrast, no brain uptake was observed for **3** ($n = 4$) using the method described. This might be expected in view of the extremely hydrophilic clogP value (Table 1) associated with this molecule. The observation that glucosylated deferiprone (**3**) completely fails to cross the BBB may indicate that the BBB hexose transporters[13] are not involved in the permeation of this class of compound. A thorough in situ perfusion study using a series of glucosylated analogues of Deferiprone may be employed to arrive at a definite conclusion regarding the mechanism of BBB permeamtion of these compounds.

Conclusions

The novel synthetic route used to synthesize **3** is a convenient and efficient way of preparing glucosylated HPOs with high yield. The in situ brain perfusion technique and the associated brain extraction and HPLC method developed for this study are sufficiently sensitive to detect the brain uptake of moderate to highly BBB permeant HPOs. This comparative study clearly indicates that glucosylation of a bidentate HPO, in general, cannot be relied upon as a general means of enhancing the brain uptake of this molecular class.

Experimental Section

General Procedure. Maltol (**4**) was purchased from Cultor Food Science. 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**5**) and TBAB were obtained from Sigma-Aldrich. Melting points were determined using an Electrothermal IA 9100 digital melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker NMR spectrometer. Chemical shifts (δ) are reported in ppm downfield from the internal standard tetramethylsilane (TMS). Mass spectra (ESI-MS and HRMS) were carried out by the Mass Spectrometry Facility, School of Biomedical and Health Science, King's College London. The purity of the compound **3** was greater than 95% as certified by HPLC method.

All chemicals used to prepare the plasma substitute Ringer solution and the physiological buffer were obtained from Sigma-Aldrich, UK. ^{3}H-Mannitol was obtained from PerkinElmer, UK. Adult male Dunkin−Hartley guinea pigs (350−500 g) were used in the experiments. Intraperitoneal injection of 50 mg/kg of ketamine (100 mg/mL, Pfizer, Sandwich, UK), 200 μg/kg of medetomidine hydrochloride (1 mg/mL, Pfizer, Sandwich, UK), and 25000 IU/kg of heparin (25000 IU/ml, CP Pharmaceuticals, Wrexham, UK) were used to anaesthetize the animals. Dissection was carried out on a warm water bed maintained at 37 °C with constantly circulating warm water using a heater and circulator (Tecam C-400). The perfusion was carried out started using a Watson Marlow 505S peristaltic pump (Watson Marlow Pumps Group, Cornwall, UK). During the course of the in situ perfusion the arterial pressure was monitored using a digital pressure transducer (Harvard Instruments, USA). After the perfusion, all brain portions were weighed using a Viacon microbalance (Acculab, Surrey, UK). Brain homogenate aliquots used to determine the amount of the radioisotope present in the brain were treated with tissue solubilizer (Soluene, Canberra-Packard, UK) and left for 24 h. A scintillation cocktail liquid (Perkin-Elmer, USA) was added to each of the scintillation vials, and samples were counted on a LKB Wallac 1219 Rackbeta liquid scintillation counter (Wallac, MD).

(2-Methyl-4-oxopyran-3-yl) 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside (6). Maltol (**4**) (4.84 g, 38.4 mmol), 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**5**) (5.26 g, 12.8 mmol), and tetrabutylammonium bromide (4.13 g, 12.8 mmol) were dissolved in dichloromethane (45 mL) and warmed to 35 °C. A solution of sodium hydroxide (1N, 45 mL) was added, and the mixture was stirred for 3 h at 35 °C. Ethyl acetate (300 mL) was added, and the organic phase was washed three times with sodium hydroxide (1N), twice with water, once with brine, dried, and finally concentrated. The residue was purified by column chromatography (EtOAc:hex = 1:1) to afford white crystals (43%); mp 145−146.5 °C [lit.[14] 147 °C]. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.63 (d, $J = 5.7$ Hz, 1H, maltyl CH-6), 6.34 (d, $J = 5.7$ Hz, 1H, maltyl CH-5), 5.35 (d, $J = 7.9$ Hz, 1H, H-1), 5.29 (t, $J = 9.5$ Hz, 1H, H-3), 5.19 (dd, $J = 7.9, 9.6$ Hz, 1H, H-2), 5.12 (t, $J = 9.6$ Hz, 1H, H-4), 4.20 (dd, $J = 4.5, 12.3$ Hz, 1H, H-6a), 4.13 (dd, $J = 2.6, 12.3$ Hz, 1H, H-6b), 3.68−3.64 (m, 1H, H-5), 2.31 (s, 3H, maltyl CH_3), 2.14 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 2.03 (s, 3H, CH_3), 2.02 (s, 3H, CH_3).

(1,4-Dihydro-1,2-dimethyl-4-oxo-pyridin-3-yl) β-D-glucopyranoside (3). To a solution of **6** (2.5 g) in 100 mL of mixed solvent (EtOH:H_2O = 1:1) was added 20 mL of 40% methylamine aqueous solution and the mixture was stirred at 70 °C overnight. After evaporation to remove the solvents, the residue was purified by crystallization (EtOH) to obtain pale-yellow crystals (82%); mp 241−243 °C (decomp). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.77 (d, $J = 7.4$ Hz, 1H, pyridyl CH-6), 7.28 (s, 1H, OH, D_2O exchangeable), 6.29 (d, $J = 7.4$ Hz, 1H, pyridyl CH-5), 5.01 (s, 1H, OH, D_2O exchangeable), 4.93 (d, $J = 3.4$ Hz, 1H, OH, D_2O exchangeable), 4.46 (t, $J = 5.6$ Hz, 1H, OH, D_2O exchangeable), 4.35 (d, $J = 7.6$ Hz, 1H, H-1), 3.67 (s, 3H, pyridyl NCH_3), 3.65−3.64 (m, 1H, H-6a), 3.48−3.45 (m, 1H, H-6b), 3.18−3.10 (m, 4H, H-2, H-3, H-4 and H-5), 2.43 (s, 3H, pyridyl CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.8 (pyridyl C-4), 144.8 (pyridyl C-3), 144.1 (pyridyl C-2), 141.2 (pyridyl C-6), 115.2 (pyridyl C-5), 106.7 (C-1), 77.4 (C-5), 77.0 (C-2), 73.9 (C-3), 69.4 (C-4), 61.0 (C-6), 41.4 (NCH_3), 13.2 (CH_3). ESI-MS: 302 $(M+1)^+$. HRMS: calcd for $C_{13}H_{20}NO_7$ $(M+1)^+$, 302.1240; found, 302.1230.

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