Glucose Promoiety Enables Glucose Transporter Mediated Brain Uptake of Ketoprofen and Indomethacin Prodrugs in Rats

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The brain uptake of solutes is efficiently governed by the blood−brain barrier (BBB). The BBB expresses a number of carrier-mediated transport mechanisms, and new knowledge of these BBB transporters can be used in the rational targeted delivery of drug molecules for active transport. One attractive approach is to conjugate an endogenous transporter substrate to the active drug molecule to utilize the prodrug approach. In the present study, ketoprofen and indomethacin were conjugated with glucose and the brain uptake mechanism of the prodrugs was determined with the in situ rat brain perfusion technique. Two of the prodrugs were able to significantly inhibit the uptake of glucose transporter (GluT1)-mediated uptake of glucose, thereby demonstrating affinity to the transporter. Furthermore, the prodrugs were able to cross the BBB in a temperature-dependent manner, suggesting that the brain uptake of the prodrugs is carrier-mediated.

Introduction

The development of new pharmacologically active drugs for central nervous system (CNS[a]) disorders often fails because of poor brain uptake of these molecules.[1] The brain uptake of solutes is efficiently governed by the blood−brain barrier (BBB). The BBB segregates the CNS from the systemic circulation, and its main physiological functions include maintaining homeostasis at the brain parenchyma and protecting the brain from potentially harmful chemicals. The BBB is primarily formed from capillary endothelial cells, which differ from the other tissues.[2] The brain capillary endothelial cells are very closely joined together by tight intercellular junctions that efficiently restrict the paracellular diffusion of hydrophilic drugs.[3] In addition to being a selective structural diffusion barrier, the BBB constitutes an efficient functional barrier for solutes crossing the cell membrane. The high metabolic activity of brain capillary endothelial cells,[4] as well as effective efflux systems that actively remove solutes from brain tissue and return them back to the bloodstream,[2,5−7] create a great challenge for potential neurotherapeutics. Furthermore, the BBB expresses a number of specific carrier-mediated inward transport mechanisms that ensure an adequate nutrient supply for the brain,[8] and new knowledge of these endogenous BBB transporters can be used in the rational reformulation of drug molecules for active transport.[9] A small molecule may diffuse through 40 μm space in about 1 s, which indicates that after passage across the BBB, the drug is almost instantly distributed within the whole cerebral tissue.[1,10] These physiological facts indicate that the vascular route would be very promising in drug delivery to the brain if nutrient transporters present at the BBB could be targeted.

The glucose transporter (GluT1) is present both on the luminal and the abluminal membrane of the endothelial cells forming the BBB.[11] GluT1 transports glucose and other hexoses and has the highest transport capacity of the carrier-mediated transporters present at the BBB, being therefore an attractive transporter for prodrug delivery.[12] Several in vitro studies have been performed with different drug molecules in order to determine the ability of their glycosyl derivatives to bind to GluT1. In addition, systemically delivered glycosyl derivatives of 7-chlorokynurenic acid, L-dopa, and dopamine have been shown to have pharmacological activity in the CNS of rodents.[13−16] According to the GluT1 model published by Mueckler and Makepeace (2008),[17] the hydroxyl group of glucose situated at the carbon 6-position goes into a hydrophobic pocket in the transporter protein substrate binding site. In addition, the hydroxyl group at the carbon 6-position does not form a hydrogen bond with the transporter that would be crucial for the affinity. Fernandez et al.[18] synthesized several glycosyl derivatives of dopamine and tested the affinity of the prodrugs to GluT1 in human erythrocytes. Dopamine was linked to glucose with different linkers at the carbon 1-, 3-, and 6-positions of glucose. The results of glucose uptake inhibition showed that the glucose derivatives that were conjugated at position 6 had the best affinity for GluT1. Therefore, the hydroxyl group at the carbon 6-position is likely the most potential functional group to which to attach the drug molecule in order to maintain the affinity of the glucose conjugate for the GluT1 transporter. These previous studies have indeed demonstrated that glucose conjugates can bind to GluT1 and that the derivatives are centrally available, but none of these studies verified the ability of conjugates to cross the BBB via GluT1. Therefore, the overall aim of the present study was to show with two model compounds ketoprofen and indomethacin, that GluT1 can be utilized to carry nonsubstrate drugs into the brain by conjugating a drug molecule to D-glucose with bioreversible linkage. Four glucose prodrugs synthesized and studied are ketoprofen−glucose produg (**4**), indomethacin−glucose prodrug (**6**), indomethacin−glycolic acid−glucose prodrug (**10**) and indomethacin−lactic acid−glucose prodrug (**14**) (Figure 1).

Results and Discussion

Chemical and Enzymatic Stability of Prodrugs. The degradation of prodrugs **4** and **6** was studied in aqueous buffer solution of pH 7.4 at 37 °C. The degradation of **4** and **6** followed pseudo-first-order kinetics with the half-lives of 88.6 ± 0.0 and 73.4 ± 1.6 h (mean ± sd, $n = 2$), respectively (Table 1).

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[a] Abbreviations: CNS, central nervous system; BBB, blood−brain barrier; DMSO, dimethyl sulfoxide; GluT1, glucose transporter; IC_{50}, half maximal inhibitory concentration; PA, permeability-surface area; V_d, volume of distribution.

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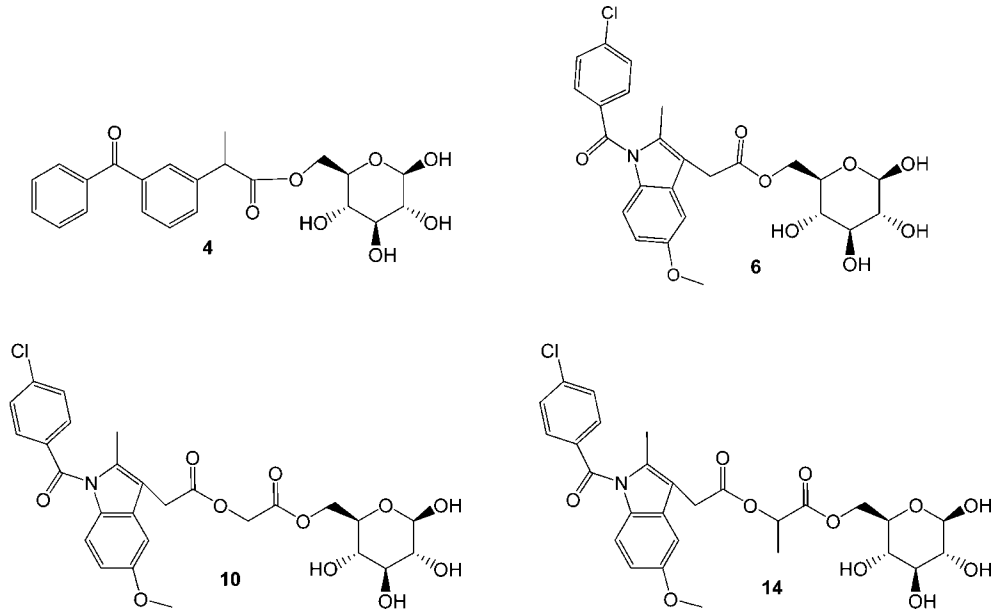

Figure 1. Chemical structures of ketoprofen prodrug **4** and indomethacin prodrugs **6**, **10**, and **14**.

Table 1. Molecular Weight, Hydrolysis Rates in Phosphate Buffer Solution, and Polar Surface Area of the Prodrugs[a]

prodrug	molecular weight (g/mol)	$t_{1/2}$ (h) 37 °C phosphate buffer (pH 7.4)	polar surface area (Å^2)
4	416	88.5 ± 0.0	133.52
6	519	40.5 ± 2.5	145.99
10	577	3.0 ± 0.2	172.29
14	591	9.5 ± 0.0	172.29

[a] Mean ± sd, $n = 2$.

Therefore, both **4** and **6** demonstrated sufficient chemical stability in aqueous solutions for further evaluation.

Compound **4** was susceptible to enzymatic hydrolysis, as it was quantitatively cleaved to ketoprofen and glucose in 20% rat brain homogenate and in 50% rat liver homogenate. The enzymatic hydrolysis followed pseudo-first-order kinetics, the half-lives being 43.5 ± 3.6 and 8.6 ± 0.6 min (mean ± sd, $n = 3$) in brain homogenate and liver homogenate, respectively. Therefore, **4** undergoes bioconversion to ketoprofen and glucose in the brain tissue. However, **4** is also highly susceptible to enzymatic hydrolysis in liver, which may compromise its effective brain drug delivery in vivo. Enzymatic hydrolysis studies of **6** in neither brain nor liver homogenate resulted in the formation of indomethacin, which makes the prodrug not suitable for brain delivery. Because indomethacin was not released from the prodrug, the hydrolysis products and the half-life of **6** were not identified. In an attempt to solve the problem, we synthesized two prodrugs (**10** and **14**) with glycolic acid and lactic acid linker group between glucose and indomethacin. The synthesis of these prodrugs is described in the Supporting Information. The degradation of prodrugs **10** and **14** was studied in aqueous buffer solution of pH 7.4 at 37 °C. The degradation of **10** and **14** followed pseudo-first-order kinetics with the half-lives of 3.0 ± 0.2 and 9.5 ± 0.0 h (mean ± sd, $n = 2$). Therefore, **10** and **14** are not stable enough to be tested with in situ rat brain perfusion technique. Although **6** did not release the parent drug in brain tissue, it is reasonable to determine the brain uptake of **6**. Because **6** has higher molecular weight than **4**, determining the uptake mechanism of both prodrugs may give more insight into the GluT1 ability to transport molecules across the BBB.

Determination of the Brain Uptake Mechanism for Prodrugs 4 and 6. In the present study, modified in situ rat brain perfusion technique was used for the determination of the brain uptake of the prodrugs.[19] In situ technique was chosen for the determination of the prodrug uptake because it provides insight into the accurate mechanism of the BBB penetration.[20] In situ rat brain perfusion technique is described in detail in our previous study.[21]

The in situ rat brain perfusion technique has not been previously used to determine the brain uptake of glycosyl conjugates. Therefore, the suitability of the technique for glycosyl conjugate uptake determination was studied by confirming the presence of functional GluT1-transporters in rat BBB with [^{14}C]D-glucose, which is an endogenous substrate for the GluT1.[11] The uptake of molecules across the BBB was quantified by determining the permeability−surface area (PA) product. The PA product of 0.2 μCi/mL [^{14}C]D-glucose was determined to be 0.0042 ± 0.0002 mL/s/g (mean ± sd, $n = 4$) (Figure 2). In addition, the determination of cerebrovascular GluT1 functional expression was carried out with competition assay by perfusing [^{14}C]D-glucose (0.2 μCi/mL) with 20 mM concentration of glucose. This coperfusion resulted in a brain uptake of 0.0011 ± 0.0003 mL/s/g (mean ± sd, $n = 3$) (73.8% inhibition) of [^{14}C]D-glucose, thereby demonstrating functional expression of cerebrovascular GluT1. The PA product was also determined using 5 °C perfusion medium which resulted in 76.2% inhibition of the PA product of [^{14}C]D-glucose to 0.001 ± 0.0003 mL/s/g (mean ± sd, $n = 3$). This further suggests that the uptake of [^{14}C]D-glucose in the in situ rat brain perfusion test method was carrier-mediated because the carrier-mediated uptake is reduced when the temperature is lowered.[21,22] However, the uptake of [^{14}C]D-glucose is not completely inhibited by low temperature, which suggests that some activity of GluT1 is still present at the BBB. There is also the possibility that a part of the uptake of [^{14}C]D-glucose is due to passive diffusion. However, 80 μM **6** is able to inhibit the [^{14}C]D-glucose uptake almost completely (96.7% inhibition), which indicates that there is no passive uptake of [^{14}C]D-glucose present at the BBB.

The ability of **4** and **6** to bind into GluT1 was studied by coperfusing increasing concentrations of the prodrugs with

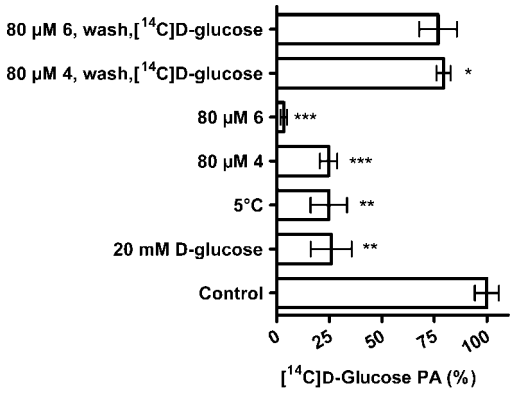

Figure 2. Mechanism of **4** and **6** rat brain uptake. The PA product of 0.2 μCi/mL [^{14}C]D-glucose in absence or presence of D-glucose, low temperature, **4** or **6**. The control PA 0.0042 ± 0.0002 mL/s/g (mean ± sd, $n = 4$) is decreased to 0.0011 ± 0.0003 (mean ± sd, $n = 3$) (73.8% inhibition) in the presence of 20 mM D-glucose and to 0.001 ± 0.0003 mL/s/g (mean ± sd, $n = 3$) (76.2% inhibition), when using 5 °C perfusion medium. The PA product of [^{14}C]D-glucose decreased to 0.00103 ± 0.00014 mL/s/g (75.5% inhibition) and 0.00014 ± 0.00005 mL/s/g (96.7% inhibition) (mean ± sd, $n = 3$) after perfusing the brain with **4** and **6**, respectively. After washing **4** from the brain capillaries, the PA product of 0.2 μCi/mL [^{14}C]D-glucose was 0.0033 ± 0.0001 mL/s/g (mean ± sd, $n = 3$). The PA product of 0.2 μCi/mL [^{14}C]D-glucose was 0.0032 ± 0.0003 mL/s/g (mean ± sd, $n = 3$) after **6** was washed from the brain capillaries. An asterisk denotes a statistically significant difference from the respective control (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, Brown Fortsythe, followed by Dunnett T3-test).

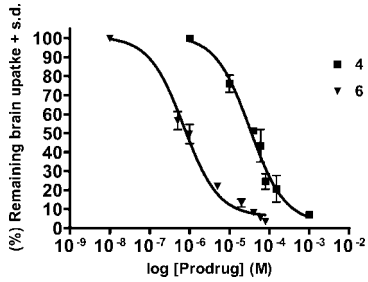

Figure 3. Inhibition of 0.2 μCi/mL [^{14}C]D-glucose uptake by **4** and **6**. IC_{50} values are 32.85 ± 8.17 μM and 0.71 ± 0.04 μM for **4** and **6**, respectively. Data are mean ± sd ($n = 2$). IC_{50} values are calculated with nonlinear regression analysis using GraphPad Prism 4.0 for Windows.

[^{14}C]D-glucose. The prodrugs were able to inhibit the uptake of [^{14}C]D-glucose in a concentration-dependent manner (Figure 3). Nonlinear regression analysis was used to determine the half maximal inhibitory concentration (IC_{50}) values of **4** and **6**. The IC_{50} values were 32.85 ± 8.17 μM for **4** and 0.71 ± 0.04 μM for **6**, which indicates that **6** has higher affinity for GluT1 compared to **4**.

To further study the binding kinetics of the prodrugs to GluT1, the PA product of [^{14}C]D-glucose was determined after perfusing rat brain first with the prodrugs at 80 μM for 30 s, followed by washing the prodrug from the brain capillaries with 30 s perfusion of prodrug-free perfusion medium and finally perfusing the rat brain with 0.2 μCi/mL [^{14}C]D-glucose for 30 s. This resulted in the PA products of [^{14}C]D-glucose 0.0033 ± 0.0001 mL/s/g and 0.0032 ± 0.0003 mL/s/g (mean ± sd, $n = 3$) for **4** and **6**, respectively, indicating that the binding of **4** and **6** to the GluT1 is reversible (Figure 2).

These results show that **6** has higher affinity for GluT1 than **4**, and both of the prodrugs have higher affinity for GluT1 than D-glucose. This higher inhibition caused by the prodrugs

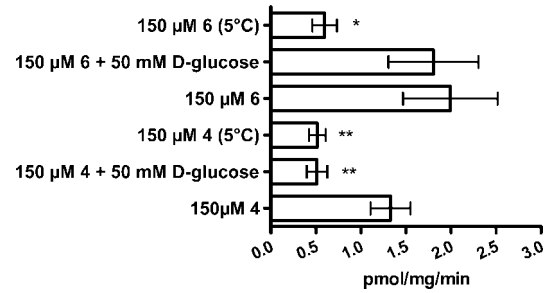

Figure 4. The brain uptake of **4** and **6**. The uptake of **4** with 150 μM concentration is 1.33 ± 0.18 pmol/mg/min and addition 50 mM D-glucose to the perfusion medium decreased significantly (61.4% inhibition) the brain uptake to 0.513 ± 0.009 pmol/mg/min (mean ± sd, $n = 3$). Perfusion medium at 5 °C decreased significantly the uptake of **4** to 0.515 ± 0.065 pmol/mg/min (mean ± sd, $n = 3$) (61.3% inhibition). The brain uptake of 150 μM **6** was 1.993 ± 0.429 pmol/mg/min (mean ± sd, $n = 3$), and after the addition of 50 mM D-glucose to the perfusion medium, the uptake was 1.806 ± 0.353 pmol/mg/min (mean ± sd, $n = 3$). The decrease of the perfusion medium temperature to 5 °C resulted in brain uptake of 0.599 ± 0.098 pmol/mg/min (mean ± sd, $n = 3$) (69.9% inhibition). An asterisk denotes a statistically significant difference from the respective control (**$P < 0.01$, *$P < 0.05$, one-way ANOVA, followed by Dunnett *t*-test). Columns 150 μM **4** and 150 μM **6** were used as controls in Dunnett *t*-test.

compared to D-glucose could be due to the higher molecular weight of the prodrugs. It is proposed that as the substrate binds to the GluT1 binding site, the transporter protein cavity occludes the bound substrate and then opens at the opposite side of the membrane where the bound substrate can dissociate.[23] The sterical hindrance caused by higher molecular weight of the prodrugs could slow the conformational change of the transporter, and as the transporters are occupied by the prodrugs, the transporters are not able to facilitate the uptake of [^{14}C]D-glucose. Therefore, the low IC_{50} values only suggest that the prodrugs are able to bind to GluT1 and the ability cross the BBB is not necessarily achieved.

Brain Uptake Determination of the Prodrugs. In addition to determining the binding of the prodrugs to GluT1, we also determined the brain uptake of the prodrugs across the BBB. The brain uptake of the prodrugs was determined with the in situ rat brain perfusion technique using 150 μM concentration at 60 s perfusion time (Figure 4). In the perfusion of 150 μM, both **4** and **6** indeed resulted in detectable amounts of both prodrug in brain tissue. The brain uptake of 150 μM **4** was 1.33 ± 0.18 pmol/mg/min (mean ± sd, $n = 3$), and an addition of 50 mM D-glucose to the perfusion medium decreased the brain uptake 61.4% to 0.513 ± 0.009 pmol/mg/min (mean ± sd, $n = 3$) (Figure 4). Finally, brain uptake of **4** was determined at 150 μM concentration in a 5 °C perfusion medium. Lower temperature decreased the uptake of **4** 61.3% to 0.515 ± 0.065 pmol/mg/min (mean ± sd, $n = 3$). The brain uptake of **4** was also determined with 450, 1000, 3500, and 15000 μM concentrations, but the uptake was not saturable within this concentration range (Figure 5). It is possible that because of poor affinity of **4** for GluT1 and high capacity of the transporter (K_m value of D-glucose uptake is 11 mM and V_{max} is 1420 nmol/min/g),[12] the uptake did not saturate, although the uptake of **4** was mainly carrier-mediated. Another explanation for the lack of uptake saturation could be that passive diffusion of **4** is significant enough to hide the saturation of Glut1-mediated uptake.

The brain uptake of 150 μM **6** was 1.993 ± 0.429 pmol/mg/min (mean ± sd, $n = 3$) (Figure 4) and after the addition of 50 mM D-glucose to the perfusion medium the uptake was 1.806 ± 0.353 pmol/mg/min (mean ± sd, $n = 3$). The

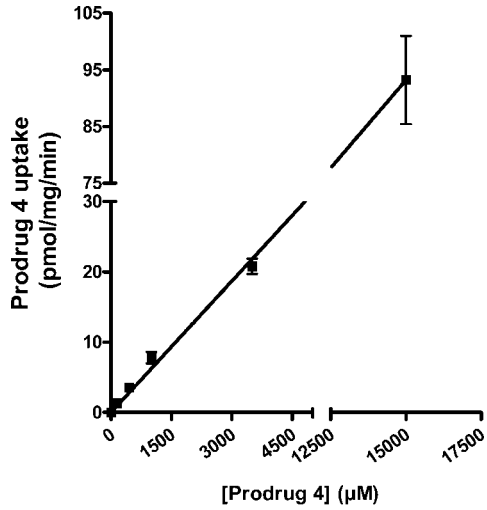

Figure 5. Relationship between concentration of the perfusion medium and brain uptake of prodrug **4**. The uptake of **4** was 1.33 ± 0.18, 3.55 ± 0.33, 7.78 ± 0.68, 20.77 ± 0.78, and 93.21 ± 5.51 pmol/mg/min (mean ± sd, $n = 3$) using 150, 450, 1000, 3500, and 15000 μM concentrations, respectively.

Table 2. Capillary Depletion Analysis after 60 s Perfusion of **4** and **6** with 450 μM Concentration, Followed by Washing the Prodrug from the Capillaries with 30 s Perfusion[a]

right cerebrum	V_d (mL/g)
4	
whole brain	0.0054 ± 0.0002
supernatant	0.0050 ± 0.0006
pellet	*b*
6	
whole brain	0.0155 ± 0.0044
supernatant	0.0135 ± 0.0052
pellet	*b*

[a] Mean ± sd, $n = 3$. [b] Below lower limit of detection.

decrease of the perfusion medium temperature to 5 °C resulted in brain uptake of 0.599 ± 0.098 pmol/mg/min (mean ± sd, $n = 3$) (69.9% inhibition). The uptake of **6** was slightly higher than the uptake of **4**, and the uptake was not decreased by addition of D-glucose in the perfusion medium, unlike the uptake of **4**. Because addition of D-glucose did not affect the uptake of **6**, the uptake of **6** is either due to passive diffusion or the affinity of **6** for GluT1 is much higher than the affinity of D-glucose.

The passive diffusion of the prodrugs across the BBB is probably limited because the polar surface areas of the prodrugs are over 90 Å^2 (Table 1).[24] In addition, the uptake of both prodrugs was significantly decreased by low temperature, which indicates that the uptake of the prodrugs is carrier-mediated. The low temperature could not inhibit the uptake of the prodrugs entirely, which may indicate that the brain uptake of the prodrugs is partly due to passive diffusion. However, the uptake of [^{14}C]D-glucose was not entirely inhibited by low temperature and leaving some transporter activity present at the BBB. Therefore, the fraction of passive diffusion of the whole brain uptake cannot be determined without noncompeting GluT1 inhibitor. To our knowledge, such an inhibitor suitable for in situ rat brain perfusion does not exist.

Capillary Depletion Analysis. Capillary depletion analysis of brain samples from perfused brain showed that the prodrugs are present in the supernatant fraction, which consists of brain parenchyma (Table 2). The concentration of the prodrugs in the endothelial cell enriched pellet fraction was below the lower limit of detection. However, because the uptake of the prodrugs determined from the whole brain is higher compared to brain parenchyma (supernatant), a fraction of the prodrugs is captured into the endothelial cells.

Conclusion

In this study, two glucose prodrugs **4** and **6** were synthesized and their ability to cross the BBB via GluT1 was determined with in situ rat brain perfusion technique. Both **4** and **6** demonstrated reversible concentration dependent inhibition of brain uptake of the radiotracer [^{14}C]D-glucose in the in situ rat brain perfusion model, indicating that the prodrugs bind to the GluT1. In addition, three factors strongly suggest that the brain uptake of **4** and **6** is GluT1-mediated. First, both prodrugs were able to cross the BBB and gain entry into the brain tissue. Second, the uptake of **4** and **6** was decreased significantly when 5 °C perfusion medium was used, indicating that the uptake is carrier-mediated. Furthermore, the high polar surface areas of **4** and **6** indicate that passive diffusion across the BBB is limited.

The uptake of **4** was decreased when 50 mM concentration of D-glucose was added in to the perfusion medium. In addition, the uptake of **4** did not saturate even in 15 mM concentration, which suggests that the affinity of **4** for Glut1 is low and the capacity of the transporter is high. It is also possible that passive diffusion of **4** is significant enough to hide the saturation of Glut1-mediated uptake. In the case of **6**, the uptake did not decrease when 50 mM D-glucose was added into the perfusion medium. However, the unspecific inhibition of GluT1 using lowered temperature suggests that the uptake of **6** is, at least, partly GluT1-mediated. The lack of inhibition of **6** brain uptake by 50 mM D-glucose indicates that the affinity of **6** for the transporter is much higher than the affinity of D-glucose or **4**. This hypothesis is further supported by the low IC_{50} value of **6**.

These results strongly suggest that a hydrophilic drug can be attached to the hydroxyl group of D-glucose at the carbon 6-position and still maintain the affinity of the D-glucose promoiety for the GluT1 transporter. However, glucose as a promoiety has several limitations. The structure of glucose limits the amount of drug molecules that can be linked with biodegradable bonds with it. Practically only drugs that bear a carboxyl acid group can be linked without a linker/spacer with glucose. In addition, the stability of ester prodrugs in systemic circulation might not be adequate for clinical use of ester prodrugs for drug brain targeting. Therefore this prodrug technology demands further development before being fully applicable to oral drug delivery. In summary, drug molecules as large as indomethacin can be conjugated with D-glucose and GluT1 is able to mediate the uptake of the conjugate across the BBB into the brain parenchyma.

Experimental Section

General Synthetic Methods. Commercial reagents were obtained from major chemical suppliers and used without further purification unless otherwise noted. ^{1}H (500.13 MHz) and ^{13}C (125.77 MHz) NMR spectra were recorded on a Bruker Avance 500 spectrometer in $CDCl_3$ and CD_3OD. Chemical shifts (δ) are reported in ppm relative to $CDCl_3$ and CD_3OD (7.26 and 3.31 for ^{1}H and 77.0 and 49.0 for ^{13}C), respectively. Electrospray ionization mass spectra (ESI-MS) were obtained on a LCQ ion trap mass spectrometer equipped with an electrospray ionization source (Finnigan MAT, San Jose, CA). Elemental analyses (CHN) were carried out with a Thermo Quest CE Instruments EA 1110 CHNS-O elemental analyzer. Flash chromatography was performed on J. T. Baker silica gel for chromatography (pore size 60 Å, particle size 50 μm). The synthesis of **2** and the prodrugs **4**, **6**, **10**, and **14** are described in Schemes 1−3, found in the Supporting Information.

All tested compounds possessed purity of at least 95% and was determined using combustion analysis method.

Synthesis of Ketoprofen−Glucose Produg (4). 3,4,5,6-Tetrakis(trimethylsilyloxy)tetrahydro-2*H*-pyran-2-yl)methyl 2-(3-benzoylphenyl) propanoate (**3**) (0.98 g, 1.39 mmol) was dissolved in dichloromethane (20 mL), solution was cooled to 0 °C, and TFA (6 mL) was added. After 4 h, $NaHCO_3$ (2.34 g, 27.8 mmol) was added to the solution and stirred 30 min, and the mixture was concentrated. Raw material was purified by flash chromatography eluting with dichloromethane, gradually increasing polarity to 85:15 dichloromethane:methanol to give product as a white solid. Yield 0.50 g (86%). ^{1}H NMR (Methanol-d_4) δ 1.51 (d, $J = 6.76$ Hz, 3H, $-CH_3$), 3.21−3.49 (m, 3H, C*H*), 3.67 (q, $J = 4.71$ Hz, 2H, C*H*), 3.91−4.49 (m, 3H, C*H* + CH_2), 5.02 (d, $J = 3.40$ Hz, 1H, −C*H*), 7.48−7.79 (m, 9H, Ar*H*). ^{13}C NMR (Methanol-d_4): δ 17.8 ($-CH_3$), 45.1 (−*C*H), 63.9 ($-CH_2$), 70.4 (−*C*H), 72.4 (−*C*H), 74.0 (−*C*H), 76.6 (−*C*H), 92.6 (−*C*H), 128.3 (2C, Ar*C*H), 128.5 (Ar*C*H), 128.6 (Ar*C*H), 128.8 (Ar*C*H), 129.8 (2H, Ar*C*H), 131.9 (Ar*C*H), 132.6 (Ar*C*H), 137.4 (Ar*C*), 137.7 (Ar*C*), 141.3 (Ar*C*), 174.4 (*C*O), 197.2 (*C*O). MS: *m*/*z* calcd for $C_{22}H_{24}O_8$ $[M]^+ = 416.2$; found 434.0 $[M + NH_4]^+$. Elemental anal. calcd for $C_{22}H_{24}O_8$ C: 63.45; H: 5.81; found $C_{22}H_{24}O_8$ C: 63.13; H: 5.91.

Synthesis of Indomethacin−Glucose Prodrug (6). Indomethacine (0.389 g, 1.09 mmol) and 1,2,3,4-tetra-*O*-trimethylsilyl-β-D-glucopyranose (0.500 g, 1.07 mmol) and DMAP (0.013 g, 0.107 mmol) were dissolved in 25 mL of dichloromethane at room temperature. DCC (0.253 g, 1.23 mmol) was added and the mixture was stirred for 5 h at room temperature. Formed urea was filtered, and filtrate was extracted with 5% acetic acid (20 mL), H_2O (20 mL), and dried with Na_2SO_4 and concentrated. Formed solid (**5**) was dissolved in acetonitrile (30 mL) and solution was cooled to 0 °C and TFA (0.75 mL) was added. After one hour, $NaHCO_3$ (1.79 g, 21.3 mmol) was added to the solution and mixture was concentrated. Raw material was purified by flash chromatography eluting with dichloromethane, gradually increasing polarity to 80:20 dichloromethane:methanol to give product as a white solid. Yield 0.30 g (71%).

^{1}H NMR (Methanol-d_4): δ 2.27 (s, 3H, $-CH_3$), 3.10−3.32 (m, 2H, C*H*), 3.50−3.64 (m, 2H), 3.68 (s, 2H, CH_2) 3.79 (s, 3H, $-CH_3$), 3.94−4.50 (m, 2H, C*H* + CH_2), 5.05 (d, $J = 3.00$ Hz, 1H, −C*H*), 6.63 (dd, $J = 2.13$ Hz, $J = 8.97$ Hz, 1H, Ar*H*), 6.82 (t, $J = 9.23$ Hz, 1H, Ar*H*), 6.88 (d, $J = 2.11$ Hz, 1H, Ar*H*), 7.51 (d, $J = 8.37$ Hz, 2H, Ar*H*), 7.63 (d, $J = 8.49$ Hz, 2H, Ar*H*).

^{13}C NMR (Methanol-d_4): δ 13.4 ($-CH_3$), 30.5 ($-CH_2$), 56.1 ($-CH_3$), 65.2 ($-CH_2$), 71.6 (−*C*H), 73.7 (−*C*H), 74.7 (−*C*H), 76.2 (−*C*H), 93.9 (−*C*H), 102.6 (Ar*C*H), 112.5 (Ar*C*H), 113.9 (*C*), 115.7 (Ar*C*H), 130.1 (2C, Ar*C*H), 131.8 (Ar*C*), 131.9 (Ar*C*), 132.2 (2C, Ar*C*H), 135.4 (*C*), 136.8 (Ar*C*), 140.0 (Ar*C*), 157.3 (Ar*C*), 169.8 (*C*O), 172.7 (*C*O).

MS: *m*/*z* calcd for $C_{25}H_{26}ClNO_9$ $[M]^+ = 519.1$; found 537.0 $[M + H_2O]^+$.

Elemental anal. calcd for $C_{25}H_{26}ClNO_9$ C: 57.75; H: 5.04; N: 2.69; found $C_{25}H_{26}ClNO_9 \cdot {}^3/_2H_2O$ C: 54.90; H: 5.34; N: 2.56.

HPLC Assay. The prodrug concentrations were analyzed by Agilent 1100 HPLC system (Agilent Technologies Inc., Waldbronn, Karlsruhe, Germany) that consisted of a binary pump G1312A, a vacuum degasser G1379A, an automated injector system autosampler Hewlett-Packard 1050, an UV-detector Hewlett-Packard 1050 variable wavelength detector, and an analyst software Agilent ChemStation for LC Systems Rev. A.10.02. The detector wavelength was set at 256 nm when **4** was analyzed and at 222 nm when indomethacin prodrugs were analyzed. A mixture of acetonitrile (50%) and a 0.02 M phosphate buffer solution of pH 6.0 (50%) at a flow rate of 1 mL/min was used as a mobile phase. Ketoprofen samples were analyzed using a mixture of acetonitrile (50%) and a 0.02 M phosphate buffer solution of pH 2.5 (50%). Reversed-phase HPLC was conducted with a Zorbax RP-18 column (150 mm × 4.6 mm, 5 μm, Agilent Technologies, Little Falls Wilmington, DE).

Chemical and Enzymatic Stability of the Prodrugs. The rate of chemical hydrolysis of the prodrugs were studied in aqueous phosphate buffer solution of pH 7.4 (0.16 M, ionic strength 0.5) at 37 °C. An appropriate amount of prodrug was dissolved in 10 mL of preheated buffer, and the solutions were placed in a thermostatically controlled water bath at 37 °C. At appropriate intervals, samples were taken and analyzed for remaining prodrug by HPLC. Pseudo-first-order half-time ($t_{1/2}$) for the hydrolysis of the prodrugs was calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug versus time.

The rate of enzymatic hydrolysis of the prodrugs were studied at 37 °C in rat brain homogenate, which was diluted to 20% (v/v) with isotonic 0.16 M phosphate buffer of pH 7.4 and in rat liver homogenate, which was diluted to 50% (v/v) with isotonic 0.16 M phosphate buffer of pH 7.4. The concentration of esterase enzymes in the homogenates was not determined. The solutions were kept in a water bath at 37 °C, and 0.2 mL of homogenate/buffer mixture was withdrawn and added to 0.2 mL of acetonitrile to precipitate protein from the sample. After immediate mixing and centrifugation, the supernatant was analyzed for remaining prodrug and released ketoprofen or indomethacin by the HPLC. Pseudo-first-order half-time ($t_{1/2}$) for the hydrolysis of the prodrugs was calculated from the slope of the linear portion of the plotted logarithm of remaining prodrug against time.

Polar Surface Area. Polar surface areas of the prodrugs were calculated using the method by Ertl et al.[25] implemented in Molecular Operating Environment.[26]

In Situ Rat Brain Perfusion Technique. Adult male Wistar rats (200−230 g) were supplied by the National Laboratory Animal Centre (Kuopio, Finland) for the rat brain perfusion studies. Rats were anesthetized with ketamine (90 mg/kg, ip) and xylazine (8 mg/mL, ip), and their right carotid artery system was exposed. The right external carotid artery was ligated, and the right common carotid artery was cannulated with PE-50 catheters filled with 100 IE/mL heparin. The in situ rat perfusion technique is described in more detail in our previous study.[21]

Determination of the Brain Uptake Mechanism for the Prodrugs. The ability of the prodrugs to bind into GluT1 was studied with the in situ rat brain perfusion technique. The 100% PA product of [^{14}C]D-glucose was determined after 30 s perfusion of 0.2 μCi/mL [^{14}C]D-glucose solution. In a competition study, [^{14}C]D-glucose (0.2 μCi/mL) was coperfused with increasing concentration of **4** or **6** for 30 s. The concentrations ranged from 0.1 to 1000 μM for **4** and 0.01 to 80 μM for **6**. To study whether the binding of **4** and **6** to GluT1 was reversible, the PA product of [^{14}C]D-glucose was determined after perfusing rat brain first with **4** or **6** at 80 μM for 30 s, followed by washing the prodrug from the brain capillaries with 30 s perfusion of prodrug-free perfusion medium, and finally perfusing the brain with 0.2 μCi/mL [^{14}C]D-glucose for 30 s.

Brain Uptake Studies of the Prodrugs. The prodrug brain uptake studies were performed with the in situ rat brain perfusion technique. The prodrugs were dissolved in dimethyl sulfoxide (DMSO) and then added to the perfusion medium, resulting in 1% (v/v) DMSO solution. After adjusting the pH to 7.4, the solution was filtered with 0.45 μm Millex-HV filters. The rat brains were perfused 60 s with 37 °C perfusion medium containing prodrug. After perfusion, the remaining prodrug was washed from the brain vasculature with cold prodrug-free perfusion medium (5 °C) for 30 s. The prodrug concentration of the perfusion medium was analyzed by HPLC after each perfusion to confirm that prodrug had stayed intact. The brain uptake was also determined by inhibiting carrier-mediated uptake unspecifically with low temperature. The brain capillaries were first washed for 30 s with cold prodrug-free perfusion medium (5 °C), followed by perfusion for 60 s with cold prodrug solution (5 °C), and washed again with cold prodrug-free perfusion medium (5 °C).

Capillary Depletion Analysis. Capillary depletion analysis was carried out as previously described by Triguero et al.[27] Brain samples (right brain hemisphere) were weighed and homogenized in glass homogenizer with 1.5 mL of physiological buffer. After homogenization, 2 mL of 26% dextran solution was added and the mixture was further homogenized. The homogenate was separated into two microcentrifuge tubes and centrifuged 15 min (5400*g*, 4 °C). The resulting supernatant consisting of the brain parenchyma and the pellet rich in

cerebral capillaries were separated and prepared for analysis with HPLC. Volume of distribution (V_d) values for the homogenate, supernatant fraction, and the capillary pellet were calculated.

Brain Sample Preparation. The quantification of **4** from the rat brain samples was performed by analyzing the total concentration of formed ketoprofen after its enzymatic release from **4** during 24 h of incubation in 37 °C. Ketoprofen was isolated from the rat brain samples by protein precipitation and solid phase chromatography. A complete brain hemisphere was homogenated with 2.5 mL of water to produce 3.0 mL of homogenate. The samples were acidified with 300 μL of 2 M hydrochloric acid and vortexed for 5 min. Then 4.0 mL of acetonitrile was added and the homogenates were vortexed for 2 min. The homogenates were centrifuged for 10 min (7500*g*, 20 °C), and the supernatants were collected. The supernatants were diluted with 6.0 mL of water and applied to the preconditioned and equilibrated C18 solid phase extraction cartridges (Discovery DSC-18; Supelco, Bellefonte, PA). The cartridges were first washed with 2.0 mL of 1% (v/v) acetic acid solution to maintain ketoprofen in the un-ionized form and then washed with 2.0 mL of water. The analytes were eluted with 6.0 mL of acetonitrile, and evaporated to dryness under a nitrogen stream at 40 °C. Prior to analysis, samples were reconstituted in 50% (v/v) acetonitrile in water and filtrated. External standards were used for the brain samples. The calibration curve of the brain method was linear over a range of 0.2−10 nmol/brain hemisphere, and samples containing high concentration of ketoprofen were diluted prior to analysis. The lower limit of quantification for spiked samples was 0.2 nmol of prodrug/brain hemisphere.

The quantification of **6** from the rat brain samples was performed by isolating **6** from the rat brain samples by protein precipitation and solid phase chromatography. A complete brain hemisphere was homogenated with 2.5 mL of water to produce 3.0 mL of homogenate. The samples were acidified with 300 μL of 2 M hydrochloric acid and vortexed for 5 min. Then 4.0 mL of acetonitrile was added and the homogenates were vortexed for 2 min. The homogenates were centrifuged 10 min (7500*g*, 20 °C) and the supernatants were collected. This procedure was repeated. Acetonitrile was evaporated from the supernatants under a nitrogen stream at 40 °C, after which the supernatants were diluted with 6.0 mL of water and applied to the preconditioned and equilibrated C18 solid phase extraction cartridges. The solid phase extraction was performed like for **4**. Prior to analysis, samples were reconstituted in 50% (v/v) acetonitrile in water and filtrated. External standards were used for the brain samples. The calibration curve of the brain method was linear over a range of 0.3−5.0 nmol/brain hemisphere. The lower limit of quantification for spiked samples was 0.3 nmol of prodrug/brain hemisphere.

Data Analyses. The results from the brain uptake experiments are presented as mean ± sd of at least three independent experiments. Statistical differences between groups were tested using Brown Fortsythe, followed by Dunnett T3-test (Figure 2) and one-way ANOVA, followed by two-tailed Dunnett *t*-test (Figure 4). The normality of the data was tested using Shapiro−Wilk test. The IC_{50} values (Figure 3) were determined by nonlinear regression analysis using GraphPad Prism 4.0 for Windows. All statistical analyses were performed using SPSS 14.0 for Windows.

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Supporting Information Available: General synthetic procedures for compounds **1**−**3** and **7**−**14**. This material is available free of charge via the Internet at http://pubs.acs.org.

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