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Synthesis of glucose-chlorambucil derivatives and their recognition by the human GLUT1 glucose transporter

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Abstract

A limitation of the use of chemotherapeutic agents against intracerebral tumors lies on their poor uptake into the central nervous system. An approach to enhance brain delivery is to design agents that are transported into the brain by one of the saturable nutrient carriers of the blood–brain barrier, the highly efficient brain and erythrocyte glucose transporter isoform GLUT1. Since the GLUT1 hexose transporter of the blood–brain barrier is also present on erythrocytes, new compounds designed to be transported by the GLUT1 transporter were studied on human erythrocytes, which represent unique, easily accessible human GLUT1 expressing cells. In this paper we describe the synthesis of four glucose-chlorambucil derivatives, namely methyl 6-*O*-4[bis(2-chloroethyl)amino]benzenebutanoyl-β-D-glucopyranoside (**3**), 6-*O*-4-[bis(2-chloroethyl)amino]benzenebutanoyl-D-glucopyranose (**6**), methyl 6-{4-[bis(2-chloroethyl)amino]benzenebutanoylamido}-6-deoxy-β-D-glucopyranoside (**9**) and 6-{4-[bis(2-chloroethyl)amino]benzenebutanoyl amido}-6-deoxy-D-glucopyranose (**10**), and the study of their interactions with the GLUT1 transporter of the human erythrocytes. All four compounds were able to inhibit [^{14}C]glucose uptake in a concentration-dependent manner. One of them, compound **6**, exhibited an approximately 160-fold higher inhibition of [^{14}C]glucose uptake by the GLUT1 transporter than glucose itself. Compound **6** was also able to inhibit [^{3}H]cytochalasin B binding to erythrocytes with approximately 1000-fold higher efficacy than does glucose. The inhibition of glucose uptake was entirely reversible, indicating that it was not due to alkylation of a nucleophilic group of the hexose transporter. The above results suggested specific interactions of compound **6** with the hexose transporter protein. Uptake studies of [^{14}C]compound **6** indicated, in addition, some non-specific interactions with intact and open erythrocyte membranes: only a small amount of the bound [^{14}C]compound **6** can be displaced by cytochalasin B. Collectively, these findings led us to conclude that the interactions of compound **6** with GLUT1 are presumably that of a non-transported inhibitor.

Keywords: GLUT1 transporter; Glucose; Chlorambucil; Blood–brain barrier; Erythrocyte

1. Introduction

The structure of cerebral vessels, which are constitutive of the blood–brain barrier, is composed of endothelial cells with tight junctions. This provides an efficient protection of the brain against exogenous hydrophilic substances (Pardridge, 1988). Moreover, while several lipophilic substances can readily cross the blood–brain barrier (Levin, 1980; Van Bree et al., 1988), other hydrophobic compounds such as vinblastine, vincristine or cyclosporin have a very low brain penetration (Begley et al., 1990; Greig et al., 1990; Pardridge, 1991; Safa and Tamai, 1990) due to the presence at the blood–brain barrier of the multidrug resistance P-glycoprotein (Cordon-Cardo et al., 1989) which pumps the drugs out of the blood–brain barrier endothelial capillary cells.

Because of the above mentioned limitations, the chemotherapy of cerebral tumors relies principally on highly hydrophobic alkylating agents such as busulfan, thiothepa, nitrosourea, or mustard derivatives (Malkin and Shapiro, 1988), which are not substrates of the P-glycoprotein. However, because of their lipophilicity, these drugs are highly myelotoxic and are associated with several side

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effects. New strategies for cancer chemotherapy are hence necessary, and particularly the development of more hydrophilic, thus less myelotoxic, antitumor agents.

Because of the blood–brain barrier, brain transport of essential hydrophilic nutrients and exit of metabolic wastes are mediated by a number of specific carriers. The large and uninterrupted energy demand of the brain is provided almost exclusively by glucose, which is transported through the blood–brain barrier by one of the facilitative glucose transporters, GLUT1. Thus, it was estimated that the glucose consumption of the brain, which represents about 2% of the whole body weight, amounts to 30% of the whole body glucose consumption, and the brain endothelium transports about ten times its weight of glucose per minute (Dick et al., 1984; La Manna and Harik, 1985). This is reflected by a comparison of the V_{max} of various blood–brain barrier facilitated uptake processes, which indicates a 15–3000-fold higher value for the hexose transporter than for other transporters, such as monocarboxylic acids, neutral amino acids, etc. (Pardridge, 1983). This high level of cerebral glucose uptake suggests GLUT1 to be a useful carrier to deliver glucose-conjugated drugs efficiently and selectively to the brain. Indeed, the GLUT1 transporter is a highly efficient facilitative glucose carrier protein, which is also responsible for rapid glucose capture by erythrocytes.

This opportunity of the presence of the same GLUT1 passive transporter on the blood–brain barrier and on erythrocytes represents a unique advantage, since easy access to human erythrocytes allows structure-activity studies directly on this well documented human transport system. In the present report, we describe the synthesis of several derivatives of glucose, in which the dialkylating antitumor compound chlorambucil was linked on the C-6 position (Fig. 1), and we have studied the interaction of these compounds with the GLUT1 transporter of the human erythrocytes.

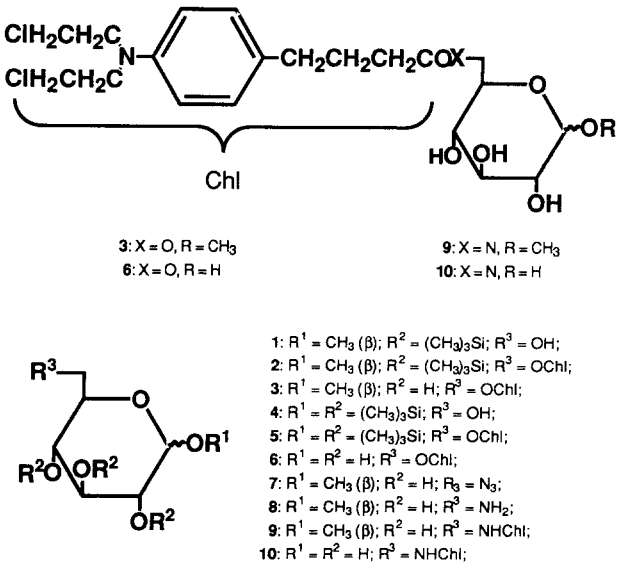

Fig. 1. Schematic formulas of synthesized compounds. Chl = chlorambucil residue.

2. Materials and methods

2.1. Materials

Chemicals: [1-^{14}C]D-glucose (50 mCi/mmol) and [4(n)-^{3}H]cytochalasin B (15 Ci/mmol) were purchased from Amersham (France). Other chemicals were from Sigma (France), Aldrich (France) or Prolabo (France).

2.2. Methods

2.2.1. General chemical methods

All reactions were conducted under nitrogen with exclusion of the light. Solutions were concentrated to dryness under reduced pressure below 30°C. Flash chromatography (Still et al., 1978) was carried out with Silica Gel 60 (230–400 mesh) and dichloromethane (*A*), 1:1 ethyl acetate/dichloromethane (*B*), ethyl acetate (*C*), 7:3 ethyl acetate/methanol (*D*), hexane (*E*), 8:2 hexane/ethyl acetate (*F*) and 1:1 hexane/ethyl acetate (*G*) as eluents. Thin layer chromatography was performed on Silica Gel 60 F_{254} (Merck, Darmstadt, Germany) and spots were visualized either by treatment with 20% sulfuric acid followed by heating, or by examination under UV light. ^{1}H-NMR spectra were recorded with tetramethylsilane as the internal standard, using a Brucker MSL 300 spectrometer. Microanalyses were performed by the Service Central d'Analyse du CNRS (Vernaison, France). Compounds were analyzed for C, H and N. Analytical results were within 0.5% of the theoretical values.

2.2.2. Synthesis of the ester linked chlorambucil derivatives

*2.2.2.1. Methyl 6-O-4-[bis(2-chloroethyl)amino] benzenebutanoyl-β-D-glucopyranoside (**3**), 6-O-4-[bis(2-chloroethyl)amino] benzenebutanoyl-D-glucopyranose (**6**) and ^{14}C-labelled **6** ([^{14}C]compound **6**).* To a solution of methyl 2,3,4,6-tetra-*O*-trimethylsilyl-β-D-glucopyranoside (4.65 g, 9.65 mmol) in acetone (19.3 ml) were added methanol (28.9 ml) and acetic acid (1.447 ml), and the solution was stirred for 7 h at room temperature. After the addition of solid $NaHCO_3$ (2.2 g), the mixture was concentrated, and the residue was purified by flash chromatography (continuous gradient from solvent *E* to solvent *F*) to yield crystalline **1** (2.42 g, 61%), m.p. 70°C, $[\alpha]_D^{20}$ − 37.5° (c 0.5, chloroform), R_f 0.256 (solvent *F*). Anal. ($C_{16}H_{38}O_6Si_3$) C, H. A stirred solution of **1** (205 mg, 0.5 mmol) in dry

dichloromethane (5 ml) was treated with chlorambucil (160 mg, 0.525 mmol), 4-dimethylaminopyridine (DMAP) (3.1 mg, 0.025 mmol) and 1,3-dichlorohexylcarbodiimide (DCC) (113 mg, 0.55 mmol). The mixture was kept for 1 h at room temperature and then filtered. The filtrate was diluted with dichloromethane and washed with dilute acetic acid and water, dried and concentrated. Flash chromatography (continuous gradient from solvent *E* to solvent *F*) of the residue gave **2** as a syrup (345 mg, 99%), $[\alpha]_D^{20}$ + 17° (c 0.22, chloroform), R_f 0.340 (solvent *F*). Anal. ($C_{30}H_{55}Cl_2NO_7Si_3$) C, H, N. Ester **2** (69 mg, 0.1 mmol) in nitromethane (600 μl) was treated with 90% trifluoroacetic acid (300 μl) for 25 min at room temperature and then concentrated. The residue was dissolved in ethyl acetate and concentrated. This procedure was repeated three times. The residue was stored in vacuo in an exsiccator over NaOH for 24 h, and then purified by flash chromatography (continuous gradient from solvent *A* to solvent *C*) to yield **3** as a white powder (42 mg, 88%), $[\alpha]_D^{20}$ − 9.3° (c 0.2, ethanol), R_f 0.132 (solvent *C*). Anal. ($C_{21}H_{31}Cl_2NO_7$) C, H, N. ^{1}H-NMR (DMSO-d_6 + D_2O): δ 6.82 (4 H; m; aromatic protons); 4.26 (1 H; m; $J_{5,6}$ 2.18 Hz; $J_{6a,6b}$ 11.78 Hz; H-6a); 4.06 (1 H; m; $J_{5,6b}$ 6.4 Hz; H-6b); 4.05 (1 H; d; $J_{1,2}$ 7.85 Hz; H-1); 3.67 and 3.51 (8 H; 2 s; chloroethyl); 3.32 (3 H; s; OMe); 3.33 (1 H; m; H-5) 3.14 (1 H; t; $J_{3,4}$ 8.8 Hz; H-3); 3.05 (1 H; t; $J_{4,5}$ 9.3 Hz; H-4); 2.93 (1 H; t; $J_{2,3}$ 8.8 Hz; H-2); 2.45, 2.27 and 1.74 (6 H; 3 t; 3 CH_2).

Compound **6** was prepared according to the procedure described in the synthesis of **3** with only minor modification. Briefly, penta-*O*-trimethylsilyl-D-glucopyranose (5.4 g, 10 mmol) in acetone (20 ml), anhydrous methanol (30 ml) and acetic acid (1.5 ml), yielded crystalline **4** (3.33 g, 71%), m.p. 59–60°C, $[\alpha]_D^{20}$ + 83° (c 0.2, chloroform), R_f 0.215 (9:1 hexane/ethyl acetate). Anal. ($C_{18}H_{44}O_6Si_4$) C, H. Compound **4** (140 mg, 0.3 mmol) in dry dichloromethane (1.2 ml) treated with chlorambucil (96 mg, 0.315 mmol) DMAP (1.83 mg, 0.015 mmol) and DCC (68 mg, 0.33 mmol) gave **5**, isolated as an oil (200 mg, 88%), $[\alpha]_D^{20}$ + 57° (c 0.2, chloroform), R_f 0.218 (9:1 hexane/ethyl acetate). Anal. ($C_{32}H_{61}Cl_2NO_7Si_4$) C, H, N. A solution of **5** (169 mg, 0.223 mmol) in acetone (1.12 ml) and methanol (1.12 ml) was treated with 0.001 M HCl (2.24 ml) for 25 min at 60°C, then neutralized with Amberlite IR-45 (OH^-) resin, filtered and concentrated. Flash chromatography (continuous gradient from solvent *C* to 9:1 ethyl acetate/methanol) of the residue gave amorphous **6** (90 mg, 87%), $[\alpha]_D^{20}$ − 22° (c 0.2, ethanol), R_f 0.360 (9:1 ethyl acetate/methanol). Anal. ($C_{20}H_{29}Cl_2NO_7$) C, H, N. ^{1}H-NMR (DMSO-d_6 + D_2O): δ 6.84 (4 H; m; aromatic protons); 4.88 (1 H; d; $J_{1,2}$ 3.6 Hz; H-1α); 4.28 (1 H; d; $J_{1,2}$ 7.69 Hz; H-1β); 3.11 (1 H; dd; $J_{2,3}$ 9.65 Hz; H-2α).

From 2.04 mg (0.011 mmol, 0.98 mCi/mmol, 10.8 mCi) of [1-^{14}C]glucose 2.8 mg (0.006 mmol, 5.9 mCi, 53% overall yield) of ^{14}C-**6** compound were obtained.

2.2.3. Synthesis of the amide linked chlorambucil derivatives

*2.2.3.1. Methyl 6-[4-[bis(2-chloroethyl)amino]benzenebutanoylamido]-6-deoxy-β-D-glucopyranoside (**9**) and 6-[4-[bis(2-chloroethyl)amino]benzene-butanoylamido]-6-deoxy-D-glucopyranose (**10**).* To a mixture of anhydrous methyl β-D-glucopyranoside (606 mg, 3.12 mmol), triphenylphosphine (835 mg, 3.18 mmol) and dry lithium azide (765 mg, 15.6 mmol) in anhydrous dimethylformamide (15 ml) was added carbon tetrabromide (1.055 g, 3.18 mmol), and the solution was stirred for 20 h at room temperature and at 55°C for 2.5 h. After the addition of methanol the mixture was concentrated, the residue was partitioned between dichloromethane (20 ml) and water (25 ml). The organic layer was extracted with two additional portions of water (10 ml) and the combined aqueous solutions were concentrated. Flash chromatography (continuous gradient from solvent *C* to solvent *D*) of the residue gave amorphous **7** (440 mg, 64%), $[\alpha]_D^{20}$ − 102° (c 0.2, ethanol), R_f 0.527 (8:2 ethyl acetate/methanol). Anal. ($C_7H_{13}N_3O_5$) C, H, N. A mixture of azide **7** (219 mg, 1 mmol) and 10% palladium on carbon (75 mg) in ethanol (10 ml) was stirred under hydrogen at room temperature for 1.5 h. The catalyst was removed and the ethanol evaporated giving amorphous **8** (180 mg, 93%) which was immediately used for the next step without further purification, R_f 0.036 (8:2 ethyl acetate/methanol). A stirred solution of **8** (90 mg, 0.465 mmol) in ethanol (5 ml) was treated with chlorambucil *p*-nitrophenyl ester (188 mg, 0.44 mmol) and triethylamine (87 μl, 0.625 mmol). The mixture was kept for 4 h at room temperature and then concentrated. Flash chromatography (continuous gradient from solvent *C* to 8:2 ethyl acetate/methanol) of the residue gave crystalline **9** (177 mg, 79%), m.p. 111–111.5°C, $[\alpha]_D^{20}$ − 12.8° (c 0.2, ethanol), R_f 0.256 (8:2 ethyl acetate/methanol). Anal. ($C_{21}H_{32}Cl_2N_2O_6$) C, H, N. A solution of **9** (89 mg, 0.185 mmol) in 0.035 M HCl (0.9 ml) was heated for 25 min at 100°C, then neutralized with Amberlite IR-45 (OH^-) resin, filtered and concentrated. Flash chromatography (continuous gradient from solvent *C* to solvent *D*) of the residue gave amorphous **10** (70 mg, 81%), $[\alpha]_D^{20}$ − 7.7° (c 0.15, ethanol), R_f 0.325 (8:2 ethyl acetate/methanol). Anal. ($C_{20}H_{30}Cl_2N_2O_6$) C, H, N. ^{1}H-NMR ($CDCl_3$): δ 6.84 (4 H; m; aromatic protons); 6.27 (1 H; t; $J_{NH,6a}$ 4.83 Hz; $J_{NH,6b}$ 7.52 Hz; NH-6); 4.23 (1 H; d; $J_{1,2}$ 7.72 Hz; H-1); 3.66 (8 H; m; chloroethyl); 3.53 (3 H; s; OMe); 2.54, 2.23 and 1.90 (6 H; 3 t; butanoyl CH_2).

2.3. Biological methods

2.3.1. Preparation of intact erythrocytes and membranes

Fresh erythrocytes drawn from healthy blood donors, collected on citrate and remaining after removal of platelets and leukocytes, were washed at room temperature in ten volumes of isotonic phosphate buffered saline (PBS), cen-

trifuged (15 min, 2500 × g), resuspended for 20 min at 37°C with fresh PBS, and centrifuged and washed three more times in the same way at room temperature. The erythrocytes thus obtained were resuspended in PBS to a hematocrit of 30% (controlled by microhematocrit centrifugation) and kept at 4°C.

We prepared open erythrocyte membranes according to a slightly modified method of Steck and Kant (1974), briefly by hemolysing erythrocytes prepared as described above with 40 volumes of ice-cold sodium phosphate solution at pH 8, and centrifugation (10 min, 22000 × g), followed by resuspension and 20 min incubation at 37°C in PBS, and three subsequent similar washings and centrifugations in PBS. The prepared membranes were kept at 4°C in PBS.

2.3.2. Erythrocyte incubation with synthesized compounds or other inhibitors

Compounds were solubilized in PBS or dimethyl sulfoxide (DMSO) (usually 240 mM stock solution). Final DMSO concentration did not exceed 4% to prevent hemolysis of erythrocytes. Glucose uptake was determined at room temperature on 40 μl of erythrocyte-PBS suspension. Glucose uptake was started by the addition of 10 μl of [1-^{14}C]D-glucose (final concentration 1 mM; 0.33 μCi/ml, final hematocrit 20%). Preliminary experiments showed that glucose uptake was linear with time up to 8 s, in agreement with previously published results (Wheeler and Whelan, 1988), and was proportional to the erythrocyte concentration up to a final hematocrit of 25%. In further experiments, glucose uptake was stopped after 8 s by adding 750 μl of an ice-cold blocking solution, modified from Jarvis (1988), containing phloretin (0.1 mM), $HgCl_2$ (2 μM) and cytochalasin B (16 μM) in an isotonic aqueous solution of NaCl (140 mM) and KI (2 mM). The resulting suspension was transferred to an Eppendorf microtube containing 200 μl of dibutyl phthalate and immediately centrifuged (1 min, 2500 × g). The cell pellet was thus rapidly separated from the reacting solution by the dibutyl phthalate layer. The upper solution was removed by aspiration, the tube was gently rinsed with ice-cold saline, and the dibutyl phthalate layer was then discarded. The pellet was treated with 1 ml of aqueous 6% trichloroacetic acid, centrifuged (1 min, 2500 × g), and the deproteinized supernatant was counted by liquid scintillation.

2.3.3. Binding of [^{3}H]cytochalasin B

Binding of [^{3}H]cytochalasin B was studied at room temperature. To 1 ml of erythrocytes (hematocrit 2%) were added various concentrations of [^{3}H]cytochalasin B, in the presence or the absence of an excess of cytochalasin B. After 1 h of incubation at room temperature, the solution was centrifuged (1 min, 2500 × g), the pellet was deproteinized by aqueous 6% trichloroacetic acid, centrifuged, and the supernatant was counted by liquid scintillation. A concentration of [^{3}H]cytochalasin B ranging from 0.1 to 2.55 μM was used for the saturation isotherm, which was performed twice on different batches of human erythrocytes.

2.3.4. Miscellaneous

Protein content was evaluated by the BCA method (Bradford, 1976), using bovine serum albumin as a standard.

To derive the number of [^{3}H]cytochalasin B binding sites per cell from the B_{max} value of 2.9 ± 0.2 pmol/mg protein, we determined that one liter of our erythrocyte stock solution at 45% hematocrit contained 5×10^{12} cells and 233 g of proteins. This led to a value of 8.1×10^4 binding sites per cell.

3. Results

3.1. Chemistry

Methyl 2,3,4,-tri-*O*-trimethylsilyl-β-D-glucopyranoside (**1**) was prepared from methyl 2,3,4,6-tetra-*O*-trimethylsilyl-β-D-glucopyranoside as previously described (Hurst and McInnes, 1965). Esterification of **1** with chlorambucil in the presence of DCC and DMAP (Hassner and Alexanian, 1978) gave the chlorambucil derivative **2**. Removal of the silyl groups from **2** with trifluoroacetic acid yielded the desired methyl β-D-glucoside derivative **3** carrying an ester-linked chlorambucil molecule on the C-6 position.

The corresponding glucose derivative with a free anomeric hydroxyl (**6**) could not be obtained by acid-catalyzed hydrolysis of the glycosidic bond without hydrolysis of the ester group at C-6. Compound **6** was more conveniently prepared from 1,2,3,4-tetra-*O*-trimethylsilyl-D-glucopyranose (**4**), using the same reaction sequence employed for the synthesis of **3**.

For the synthesis of glucose-chlorambucil derivatives linked by an amide bond, the key compound, methyl 6-azido-6-deoxy-β-D-glucopyranoside (**7**) was obtained from methyl β-D-glucopyranoside by the adaptation of a one-pot procedure described for the preparation of 5′-azido-nucleosides (Hassner and Alexanian, 1978). Regioselective azidation of methyl β-D-glucopyranoside by treatment with the reagent triphenylphosphine-carbon tetrabromide-lithium azide yielded a crystalline product with properties consistent with the 6-azide structure. The i.r. spectrum of **7** showed a strong absorption band at 2100 cm^{-1} indicative of an organic azide, which was located at the 6-position, since in the ^{1}H-NMR spectrum of its acetylated derivative, H-2 (δ 4.99), H-3 (δ 5.22) and H-4 (δ 4.97) were the most deshielded sugar protons. Catalytic (Pd/C) hydrogenation of azide **7** afforded the amine **8** which was immediately treated with the *p*-nitrophenyl ester of chlorambucil to give the amide **9**. Acid hydrolysis of **9** afforded **10** as mixture of α- and β-anomers.

3.2. Inhibition of glucose uptake by glucose, various glucose analogs and the synthesized glucose-chlorambucil derivatives

Glucose uptake by human erythrocytes was determined in the absence or the presence of preincubated GLUT1 inhibitor, 10 μM cytochalasin B. Following this standard procedure, the values of V_{max} and K_m for the specific GLUT1 mediated glucose uptake by human erythrocytes were respectively 40.5 ± 2.9 mmol · l_e^{-1} · min^{-1} and 9 ± 0.9 mM (l_e = erythrocyte volume in liter, quadruplicate determinations; data not shown). This result is in good agreement with previously published data (Speizer et al., 1985; Lowe and Walmsley, 1986).

Uptake at 8 s was then studied in the presence of various concentrations of the different glucose derivatives. Inhibition curves displayed a sigmoidal shape, as illustrated in the typical experiment of Fig. 2. In Table 1 are indicated the mean values of the IC_{50} inhibition constants deduced from several experiments performed with different human erythrocyte preparations from various blood donors. Values found for glucose and 3-*O*-methyl glucose agree with the observed K_m value of glucose uptake and are in the same order of magnitude as those previously reported (Helgerson and Carruthers, 1989). The four studied chlorambucil derivatives also inhibited [^{14}C]D-glucose uptake. However, this occurred for compounds **3**, **9** and **10** at a millimolar concentration, at which chlorambucil alone displayed an inhibitory effect, about 35% inhibition at 4 mM concentration, suggesting a non-specific membrane effect. Surprisingly, one of the derivatives prepared, compound **6**, presented a high inhibitory effect on glucose uptake by erythrocytes. This compound, which thus displayed the most interesting characteristics of recognition by the human GLUT1 transporter, was selected for further studies.

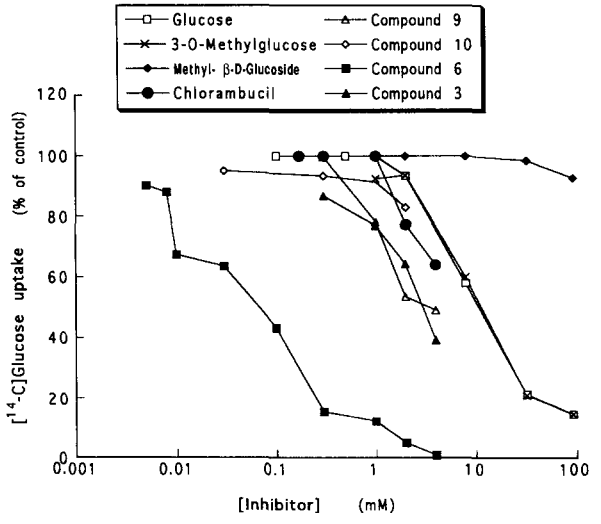

Fig. 2. Inhibition of [1-^{14}C]D-glucose uptake by glucose, various glucose derivatives, and chlorambucil. Uptake of [1-^{14}C]D-glucose (1 mM) by human erythrocytes was determined at room temperature for an 8 s incubation time in the presence of the indicated concentration of non-labeled compound. Glucose uptake represents specific GLUT1-mediated uptake deduced after subtraction of non-specific association evaluated in the presence of 10 μM cytochalasin B. All [1-^{14}C]D-glucose uptake values were determined in quadruplicate. Relative standard deviations were always lower than 4% of the values. For clarity, they are not shown here.

3.3. Reversibility of inhibition of glucose uptake by compound 6

Human erythrocytes were incubated for 20 min at 37°C with either compound **6**, or chlorambucil. The inhibitors were then removed from the erythrocytes by three consecutive resuspensions in 10 volumes of PBS and centrifugation/incubation steps. Glucose uptake determination gave similar results to the control in both cases (96.0 ± 7.0% and 96.9 ± 9.1% of control [^{14}C]D-glucose for compound **6** and chlorambucil, respectively; control value was 195.44 ± 44.5 pmol/mg protein after an 8 s uptake; data not shown). This indicated that GLUT1 inhibition by compound **6** was completely reversible, and that it was not due to chemical alkylation of the GLUT1 transporter.

3.4. Binding of [^{3}H]cytochalasin B to erythrocytes and inhibition by glucose and derivatives

The number of cytochalasin B binding sites to the GLUT1 transporter on human erythrocytes was determined with labeled [^{3}H]cytochalasin B, deducing the non-specific absorption observed with an excess of non-labeled cytochalasin B. From a Scatchard analysis of the saturation isotherm, we found K_d = 0.27 ± 0.03 μM, B_{max} = 2.9 ± 0.2 pmol/mg protein (data not shown). From the estimation of the number of erythrocytes in the sample (see Section 2.2), a number of 8.1×10^4 [^{3}H]cytochalasin B binding sites per cell were deduced, in good agreement with published data (Feugeas et al., 1991; Helgerson and Carruthers, 1987).

In the presence of glucose, of compound **6**, or of chlorambucil, the results were similar to those observed on glucose uptake: while chlorambucil had no or little effect, compound **6** inhibited [^{3}H]cytochalasin B binding with a slope characteristic of competitive inhibition, identical to that observed with glucose (Fig. 3).

The above results suggested that compound **6** was competing with glucose or cytochalasin B for the same site on the GLUT1 transporter. It was thus important to determine if compound **6** was either a substrate or an inhibitor of the GLUT1 transporter. For this purpose, we prepared a radiolabeled form of compound 6 as described in Section 2.2, and the radiolabeled [^{14}C]compound **6** was used in further experiments.

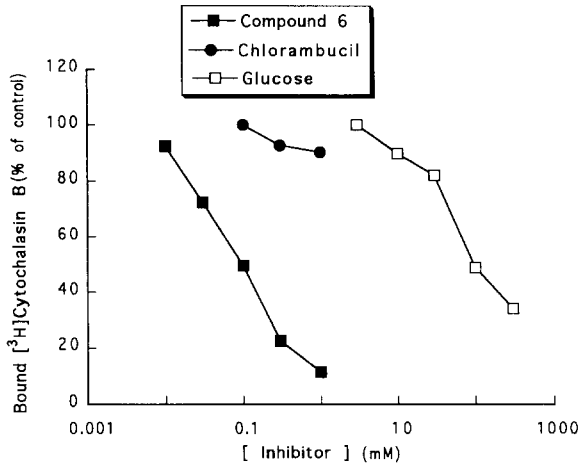

Fig. 3. Inhibition of [^{3}H]cytochalasin B binding to human erythrocytes by glucose, compound **6**, and chlorambucil. The binding of [^{3}H]cytochalasin B (0.1 μM concentration, 20 min of incubation at room temperature) to human erythrocytes was determined at room temperature after 20 min of incubation, in the presence of various concentrations of glucose, chlorambucil and compound **6**. The concentration of the lipophilic chlorambucil, which was dissolved in stock concentrations in DMSO, could not exceed 3 mM to avoid hemolysis. Error bars, which indicate S.D., are visible only when they are larger than the size of the symbols. This experiment was repeated twice, leading to similar values.

3.5. Studies of association of [^{14}C]compound 6 to erythrocytes and open erythrocyte membranes

Erythrocytes were incubated with various concentrations of radiolabeled [^{14}C]compound **6**, for uptake times of 5 s to 1 h. Identical values of radiolabeled [^{14}C]compound **6** were found associated with erythrocytes in the presence or the absence of saturating concentrations of the GLUT1 inhibitor cytochalasin B (10 μM) (Table 2) or of a 50 mM saturating concentration of substrate glucose (data not shown). Moreover, the association of [^{14}C]compound **6** to erythrocytes was significantly less dependent on the temperature than glucose uptake (Table 2).

This suggested that no net uptake of [^{14}C]compound **6** through the GLUT1 transporter occurred, and that the observed binding of [^{14}C]compound **6** to erythrocytes resulted mainly from non-specific association of this hydrophobic compound to the erythrocyte plasma membrane. A confirmation came from the fact that the amount of [^{14}C]compound **6** associated with open erythrocyte membranes was not dependent upon the presence or absence of 10 μM cytochalasin B, in contrast to the binding of radiolabeled cytochalasin B which was displaced by a saturating 10 μM concentration of cold cytochalasin B (data not shown).

Table 2
Temperature dependence of [1-^{14}C]D-glucose uptake, and of [1-^{14}C]compound **6** binding by human erythrocytes

Incubation time (s)	Cytochalasin B (μM)	[^{14}C]D-Glucose (pmol/mg protein)		[^{14}C]Compound **6** (pmol/mg protein)	
		15°C	22°C	15°C	22°C
8	0	13.8 ± 1.3	206.0 ± 44.7		
8	10	1.7 ± 0.1	10.6 ± 2.4		
60	0			219 ± 22	261 ± 22
60	10			176 ± 11	257 ± 22
180	0			172 ± 24	305 ± 27
180	10			198 ± 17	276 ± 29

Uptake of [1-^{14}C]D-glucose or of [1-^{14}C]compound **6** was determined as described in Section 2.2, at 15°C and 22°C, and at various incubation times: 8 s for glucose, and 60 or 180 s for compound **6**. Concentration of [1-^{14}C]D-glucose was 1 mM, and of compound **6** was 7.2 μM.

4. Discussion

Various strategies for increasing drug delivery to the brain have been considered. A widely used strategy appears to be the prodrug approach, in which the increased lipophilic nature of a prodrug may facilitate passive diffusion across the blood–brain barrier with subsequent conversion to the parent compound by simple chemical hydrolysis, by brain enzymatic biotransformation, or by the dihydropyridine-pyridinium salt type of redox system (Thomson et al., 1992; Bodor et al., 1981). Another approach explored the use of endogenous transporter proteins to target drugs to the brain. For example, several antineoplastic amino acid drugs such as melphalan or DL-2-amino-

Table 1
Inhibition of [^{14}C]D-glucose uptake into human erythrocytes by glucose derivatives

Compound	Modified position of D-glucose	*n*	IC_{50} ± S.D. (mM)
D-Glucose	–	1	10
Methyl β-D-glucopyranoside	O-1 (methyl)	2	≫ 100
Compound **3**	O-1 (methyl), O-6 (Chl ester) [a]	2	3.0 ± 0.3
Compound **9**	O-1 (methyl), O-6 (Chl amide)	2	3.0 ± 0.9
3-*O*-Methyl-D-glucose	O-3 (methyl)	2	16.0 ± 6.0
Compound **6**	O-6 (Chl ester)	4	0.065 ± 0.015
Compound **10**	O-6 (Chl amide)	2	≫ 4 [b]

IC_{50} values were deduced from the results of Fig. 4. Concentration of [^{14}C]D-glucose was 1 μM. *n* = number of separate experiments performed on different preparations of human erythrocytes from various blood donors. Within each separate experiment, each glucose uptake value was deduced from quadruplicate determinations. [a] Chl = chlorambucil residue (see Fig. 1). [b] Chlorambucil derivatives were dissolved in DMSO in 240 mM stock solutions, and DMSO-induced hemolysis prevented the use of inhibitor concentration higher than 4 mM.

7-bis[(2-chloroethyl)amino]-1,2,3,4-tetrahydro-2-naphthoic acid (Greig et al., 1987; Takada et al., 1991, 1992) have been shown to be taken up into the brain by the large neutral amino acid transporter. We have chosen the glucose transporter to mediate drug penetration into the brain for several reasons. First, because of the high efficiency of GLUT1 at the blood–brain barrier. Second, because an accelerated rate of glucose transport associated to the expression of GLUT1 is one of the most characteristic biochemical events of neoplastic cells and transformed phenotypes (Hatanaka, 1974; Yamamoto et al., 1990; Nishioka et al., 1992; Brown and Wahl, 1993; Ahn et al., 1993). This raises the possibility of specific drug targeting to the tumor, in addition to increased brain penetration.

Our results show that the newly synthesized glucose-chlorambucil derivatives, compounds **3**, **6**, **9** and **10**, were able to inhibit the transport of glucose by GLUT1. The inhibition of [^{14}C]glucose uptake was dependent upon the inhibitor's concentration and displayed an inhibition curve similar to that of the inhibition by glucose itself. However, in the concentration range where inhibition by **3**, **9** and **10** was observed, chlorambucil also exerted an inhibitory activity. In contrast, compound **6** displayed its activity at a 160-fold lower concentration. The inhibition was reversible, showing that it was not due to alkylation of some nucleophiles on the GLUT1 protein. Compound **6** was also able to inhibit [^{3}H]cytochalasin B binding to erythrocytes with a much higher efficiency than does D-glucose. The inhibition was dependent on the concentration of **6** and displayed an inhibition curve similar to that of the inhibition by glucose. Taken together, all the present data suggest that compound **6** specifically interacts with the glucose transporter protein. However, uptake studies with the [^{14}C]compound **6** indicated also non-specific interactions with the intact erythrocyte membrane, in addition to the specific effects: only little of the bound [^{14}C]compound **6** can be displaced by cytochalasin B. The same results were obtained with open erythrocyte membranes. Thus, we can conclude that the interaction of compound **6** with the GLUT1 is rather that of a non-transported inhibitor.

The above results show that the nature of interactions was dependent upon the structure of the sugar residue. For binding with high affinity, the presence of free anomeric hydroxyl group was necessary, the only difference between **3** and **6** being the methyl substitution at OH-1. This result is in agreement with the findings of Barnett et al. (1973, 1975). In contrast, during completion of this work, Polt et al. (1994) reported the blood–brain barrier penetration of several intraperitoneally administered enkephalin β-D-glucosides with bulky polypeptide substituents at the C-1. The authors suggested that the glucose transporter GLUT1 was responsible for the transport. However, this was not demonstrated, and further work by the same group indicated that GLUT1 was not responsible of the brain uptake of the glycosylated enkephalin analogs (Williams et al., 1995).

Investigations of the structural requirements of glucose and its derivatives for binding to the carrier of human erythrocytes (Barnett et al., 1973, 1975) suggested the existence of a large hydrophobic cleft adjacent to the C-4 and C-6 positions of bound glucose. Analogs with hydrophobic alkyl substituents at the C-6 position were found to have high affinity for the transporter. Although binding well to the exofacial site, the C-6 substituted analogs were not transported into the cell because of strong hydrophobic interactions between the sugar and some aromatic amino acids (probably Trp-388 or Tyr-293) at or near the exofacial binding site. In contrast, a glucose analog with a bulky hydrophobic substituent at the C-6, 6-deoxy-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4yl)-aminoglucose (NBDG), was demonstrated to be transported into human erythrocytes by the sugar transporter pathway, although at a very slow rate (Speizer et al., 1985). Our results with compound **6** suggest it to be rather an inhibitor (as were the 6-*O*-alkyl hexose derivatives) and not a transported ligand (as was NBDG).

The question arises, which is the reason for the observed affinity difference between compounds **6** and **10**, the only structural difference being the replacement of the ester linkage by an amide linkage at C-6. One of the possible explanations is given by molecular modeling studies. Chemical structure and energy calculations of the α- and β-anomers of compounds **6** and **10** demonstrated structural differences between the minimal energy forms of the ester and the amide. While in the ester the chlorambucil moiety occupies a space above and behind the sugar ring, leaving the sugar ring easily accessible to the transporter, and the chlorambucil moiety possibly interacting with the hydrophobic pocket, conversely, in the amide the chlorambucil moiety occupies a space at the opposite site, below the sugar ring. Subtle changes in the environment of C-6 can cause substantially different behavior with respect to the sugar transporter, as was observed for the 6-*O*-alkyl hexoses and NBDG. Further studies are necessary to determine the structural requirements which could best accommodate a relatively bulky substituent at C-6 with transport activity.

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