Development of high-affinity ligands and photoaffinity labels for the D-fructose transporter GLUT5

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The GLUT5 transporter catalyses the specific uptake of D-fructose and can accept this hexose in its furanose and pyranose ring forms. The transporter does not accept fructose epimers and has very limited tolerance of bulky groups substituted at the 2-, 3-, 4- and 5-OH positions [Tatibouët, Yang, Morin and Holman (2000) Bioorg. Med. Chem. **8**, 1825–1833]. To further explore whether bulky groups can be tolerated at the primary OH positions, a D-fructose analogue with an allylamine group substitution to replace the 1-OH group was synthesized and was found to be quite well tolerated (K_i = 27.1 mM). However, this analogue occurs in multiple ring forms. By contrast, 2,5-anhydro-D-mannitol is a symmetrical molecule that occurs only in a furanose ring form in which C-1 and C-6 are equivalent. We have therefore synthesized new 2,5-anhydro-D-mannitol analogues (substituted at the equivalent of the 6-OH of D-fructose) and from studies in Chinese hamster ovary cells expressing GLUT5 cells report that (i) the allylamine derivative of 2,5-anhydro-D-mannitol is well tolerated (K_i = 2.66 mM); (ii) introduction of a di-nitrophenyl-substituted secondary amine group enhances affinity (K_i = 0.56 mM); (iii) introduction of amide-linked biotinylated photolabel moieties is possible without loss of affinity relative to 2,5-anhydro-D-mannitol but a small secondary amine spacer between the biotinylated photolabelling moiety and the fructofuranose ring increases affinity (fructose photolabel 2; K_i = 1.16 mM); (iv) introduction of a hydrophilic tartarate spacer between biotin and the diazirine photoreactive groups can be accomplished without reduction in affinity and (v) photoactivation of biotinylated fructose photolabels leads to specific biotin tagging of GLUT5. These data suggest that substitution of a secondary amine group (-NH) to replace the C-6 (or C-1) -OH of 2,5-anhydro-D-mannitol results in compounds of high affinity; the affinity is enhanced over 10-fold compared with D-fructose.

Key words: biotinylated ligand, fructose, membrane transport.

INTRODUCTION

Uptake of hexoses into mammalian tissues is mediated by a series of membrane transporters of the GLUT family [1]. Until fairly recently it was thought that there were just five proteins in this family (GLUTs 1–5) but since the cloning of the human genome it has become apparent that there are, in addition, eight other members of this family [2,3]. Based on sequence comparisons the family has been subdivided into three classes [2]. The first class has been most fully characterized and comprises GLUTs 1–4 and these are primarily glucose transporters with distinct tissue distributions. The second class is exemplified by GLUT5 and two new members, GLUTs 9 and 11. Based on the similarity to GLUT5 it is likely that GLUTs 9 and 11 are fructose transporters but the extent to which they may accept other hexoses has not been established. Likewise, the newly discovered GLUTs 6, 8, 10 and 12 constitute a third class. Class 2 and class 3 transporters have sequence identities with class 1 transporters that range from 29 to 42 %. The distinguishing feature of the class 3 transporters is that the extracellular glycosylation domain is between transmembrane segments 8 and 9 rather than transmembrane segments 1 and 2 as in class 1. The substrate specificity of this group has not been fully explored but it is known that GLUT8 [4,5] and GLUT10 [6] can transport D-glucose. Given that the widened GLUT family now includes D-fructose transporters, we considered that exploration of the specificity and tissue distribution of these proteins could be enhanced by the development of ligands and photoaffinity labels based on the fructofuranose ring. We now describe new analogues of this type and have characterized their interaction with the readily available GLUT5 isoform which has been expressed in Chinese hamster ovary (CHO) cells [7].

The GLUT5 transporter occurs mainly in intestinal tissue [8–13], kidney [14] and spermatozoa [8] but substantial levels are also present in adipose and muscle tissue [15–17]. The tissue expression levels of the GLUT5 transporter are altered in development and are responsive to alterations in D-fructose in the diet [18–20]. Furthermore, levels of the GLUT5 transporter in intestine are increased in experimental diabetes and type 2 diabetes [11,21–23]. Although changes of total cellular expression levels of GLUT5 are probably indicative of changes in the levels of this transporter at the plasma membrane, this cannot always be assumed and alterations in the subcellular distribution of GLUT5 during sorting can occur. For example, in polarized Caco2 cells, GLUT5 is mainly targeted to the apical membrane but is separated from membrane protein destined for trafficking to basolateral membranes at an intracellular sorting step [24,25].

Our approach to the investigation of cell-surface levels of the mammalian GLUTs 1–4 (the class 1 proteins) has been to use cell-membrane-impermeant photoaffinity probes. These molecules have been synthesized around bis-mannose [26–28] and bis-[29,30] and mono-glucose [31,32] analogues in which we have incorporated diazirine photolabelling moieties and, in some cases, biotin tags. We have previously found that the use of the

Abbreviations used: Boc, t-butoxycarbonyl; CHO, Chinese hamster ovary; DMF, dimethylformamide; FP, fructose photolabel; HRFABMS, high-resolution fast-atom-bombardment MS; KRP, Krebs–Ringer phosphate; TFA, trifluoroacetic acid.

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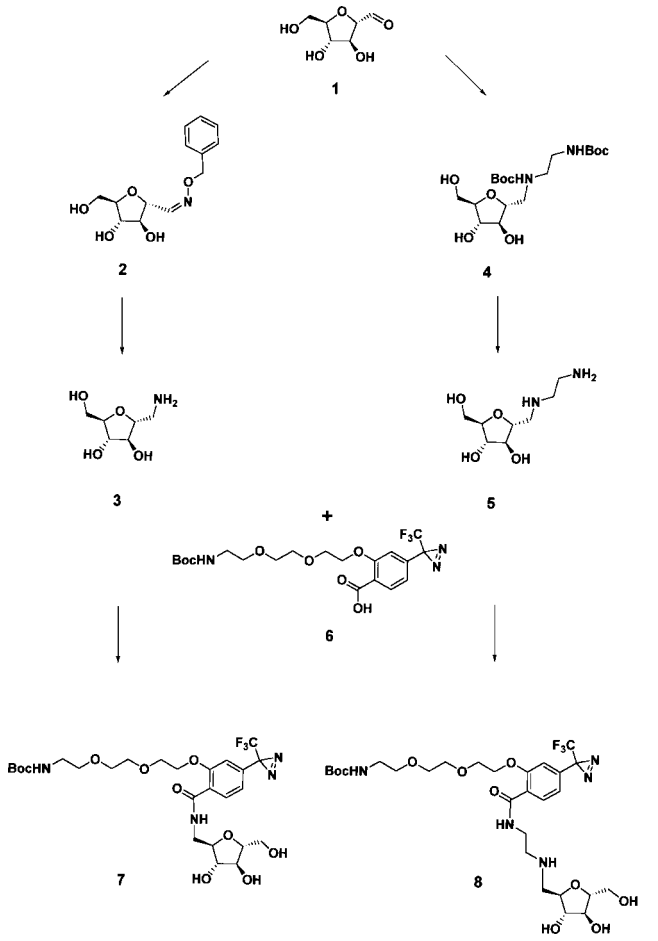
diazirine group introduces favourable photolabelling properties compared with the more commonly used azide and benzophenone photolabels [33,34]. The biotin tag has been used to separate plasma membrane from the internal reservoir pools of the transporters, following a streptavidin–agarose precipitation step [28]. In order to investigate the proportions of GLUT5 and other D-fructose transporters that are present at the surface of target cells, we sought to apply similar chemistries to a range of D-fructose analogues. This necessitated an investigation of the positions around the D-fructose structure that could accommodate bulky groups and, as described here, factors that could be used to increase the affinity of D-fructose analogues for its transporter.

We have previously reported that GLUT5 can accept D-fructose in its furanose and pyranose ring forms [35]. This transporter does not accept D-fructose epimers [35] or 5-thio-D-fructose in which the furanose ring oxygen is replaced by sulphur [36]. GLUT5 has very limited tolerance of bulky allyl groups substituted at 2-, 3-, 4- and 5-OH positions but 6-*O*-allyl-D-fructose is quite well tolerated [35]. D-Fructose in its furanose form is quite a symmetrical molecule while 2,5-anhydro-D-mannitol is structurally equivalent to 2-deoxy-D-fructofuranose. In addition, 2,5-anhydro-D-mannitol is a completely symmetrical molecule in which 1-OH and 6-OH are sterically indistinguishable. We have therefore used 2,5-anhydro-D-mannitol as a parent compound to explore the feasibility of developing high-affinity GLUT5 photolabels based on the fructofuranose ring.



Scheme 1 Synthesis of amine derivatives and photolabel precursor compounds from 2,5-anhydro-D-mannose

Two series of compounds have been synthesized that differ in composition around the link between the aryl-diazirine and the 2,5-anhydro-D-mannose moieties. The photolabel precursor compound **7** was composed of an aryl-diazirine moiety which was amide linked while compound **8** was composed of the same aryl-diazirine that was linked to an ethylenediamine spacer. This spacer allowed retention of hydrogen bonding from the secondary NH group. The synthetic routes to the photolabel precursors, and via compounds **2–6**, are described in the Experimental section.

EXPERIMENTAL

General chemical methods

Compounds were analysed by TLC and detected using UV, 2% *p*-dimethylaminocinnamaldehyde in methanol with 1% HCl for staining of biotin-containing compounds and 10% ammonium molybdate in 10% H_2SO_4 for staining of polyols. Preparative silica gel chromatography (Keiselgel 60; 230–400 mesh) was used to purify the products. The elution solvents were either ethyl acetate/methanol mixtures (for the more hydrophobic compounds) or ethyl acetate/methanol/water mixtures (for the more hydrophilic compounds). High-resolution mass spectra data were recorded on a Micromass Autospec.

Synthesis of amine derivatives of D-fructose and 2,5-anhydro-D-mannitol

The benzyloxime compound **2** (Scheme 1) was obtained in quantitative yield by treatment of 2,5-anhydro-D-mannose [37] in methanol with benzylhydroxylamine hydrochloride and triethylamine as a base. The product was purified by preparative silica gel chromatography. The mass, high-resolution fast-atom-bombardment MS (HRFABMS) m/z $(M+H)^+$, calculated for $C_{13}H_{18}NO_5$ was 268.1185; the mass found was 268.1181. The benzyloxime was catalytically hydrogenated using Raney nickel as a catalyst to give a quantitative yield of 1-amino-2,5-anhydro-D-mannitol (Scheme 1, compound **3**) with the same characteristics as described in [37].

The ethylenediamine derivative of 2,5-anhydro-D-mannose (Scheme 1, compound **5**) was prepared by reductive amination. A general method for synthesis of amine derivatives of 2,5-anhydro-mannose was followed [37]. 2,5-Anhydro-D-mannose in methanol was treated with 2 mol/mol of ethylenediamine and the resulting imine was reduced by sodium cyanoborohydride. The diamine groups in the crude product were protected by conversion to the t-butoxycarbonyl (Boc) derivative (Scheme 1, compound **4**), which was easier to purify than the diamine itself. This was obtained in 80% yield. The mass, HRFABMS m/z $(M+H)^+$, calculated for $C_{18}H_{35}N_2O_8$ was 407.2393; the mass found was 407.2394.

The allylamine derivative of D-fructose (Figure 1A) was synthesized from the isopropylidene-protected compound, 2,3,4,5-di-isopropylidene-D-fructopyranose [38]. This material was converted to the methane sulphonate ester using trifluoromethanesulphonate anhydride. This product was purified by silica gel chromatography and then the triflate group was displaced by allylamine in tetrahydrofuran with heating to 90 °C for 30 min. The product, 1-allylamine-1-deoxy-2,3,4,5-di-isopropylamine-D-fructopyranose, was purified by silica gel chromatography with a 50.5% yield. ^{1}H-NMR and ^{13}C-NMR confirmed the structure. The microanalysis calculated for $C_{15}H_{25}NO_5$ was C, 60.18%, H, 8.84% and N, 4.68%; we found C, 60.0%, H, 8.45% and N, 4.42%. The protecting isopropylidene groups were then removed using 4:1 trifluoroacetic acid (TFA)/water at room temperature

for 18 h. The TFA was removed by evaporation and the product (1-allylamino-D-fructose) was purified by silica gel chromatography. The microanalysis calculated for $C_9H_{17}NO_5 . CF_3COOH$ was C, 39.64 %, H, 5.44 % and N, 4.2 %; we found C, 39.9 %, H, 5.51 % and N, 3.94 %.

1-Allylamino-2,5-anhydro-D-mannitol (Figure 1A) was synthesized from 2,5-anhydro-D-mannose and a 1 molar equivalent of allylamine in methanol by reductive amination using cyanoborohydride. The product was purified by ion-exchange and silica gel chromatography with a 36 % yield. The mass, HRFABMS m/z $(M+H)^+$, calculated for $C_9H_{17}NO_4$ was 204.1236; the mass found was 204.1229.

Synthesis of dinitrophenyl derivatives of 2,5-anhydro-D-mannitol

1-Amino-2,5-anhydro-D-mannitol was treated with 1.5 mol/mol of dinitrofluorobenzene in dimethylformamide (DMF) with *N*-methylmorpholine as a base to give the dinitrophenyl amine derivative, compound **1** in Figure 2(A). The product was purified by silica gel chromatography and obtained in a 34 % yield. The mass, HRFABMS m/z $(M+H)^+$, calculated for $C_{12}H_{16}N_3O_8$ was 330.0937; the mass found was 330.0929. Treatment of 1-amino-2,5-anhydro-D-mannitol with 0.5 mol/mol of difluorodinitrobenzene under the same conditions gave the cross-linked bis-anhydromannitol product (compound **3**, Figure 2A) in a yield of 51 %. The mass, HRFABMS m/z $(M+H)^+$, calculated for $C_{18}H_{27}N_4O_{12}$ was 491.1625; the mass found was 491.1633. Treatment of the same amine with a 2-fold excess molar ratio of difluorodinitrobenzene gave a monofluoro-dinitrophenyl derivative but the fluoro group was displaced by -OH during the work under basic conditions to give compound **2** (Figure 2A). Following silica gel purification this compound was obtained in a 20 % yield. The mass, HRFABMS m/z $(M+H)^+$, calculated for $C_{12}H_{16}N_3O_9$ was 346.0887; the mass found was 346.0899.

Synthesis of fructofuranose photolabels

Fructose photolabels (FPs 1–4; Figure 3A) were prepared from the appropriate amine precursors. 1-Amino-2,5-anhydro-D-mannitol was coupled to the succinimide ester of compound **6** (Scheme 1), which was synthesized as described in [29]. The reaction was carried out in DMF with *N*-methylmorpholine as a base and the product was purified by silica gel chromatography to give compound **7** at a yield of 60 %. The mass, HRFABMS m/z $(M+H)^+$, calculated for $C_{26}H_{38}F_3N_4O_{10}$ was 623.2540; the mass found was 623.2557. The ethylenediamine derivative **5** (see Scheme 1) obtained by treatment of compound **4** with TFA was similarly coupled to compound **6** to give compound **8** in a yield of 40 %. The mass, HRFABMS m/z $(M+H)^+$, calculated for $C_{28}H_{43}F_3N_5O_{10}$ was 666.2962; the mass found was 666.2979.

The Boc group protecting the amino function in compound **7** was removed by treatment with TFA/water (4:1). Following removal of the TFA, the resulting amine was coupled to either biotinyl-aminohexanoyl-succinimide or biotinyl-ethylenediamino-tartaroyl-succinimide [31] to give the biotinylated photolabels FP1 and FP3, respectively. The reactions were carried out in DMF using *N*-methylmorpholine as a base. The products were purified by silica gel chromatography and were obtained with yields of 50–60 %. Identification of the products was by UV and biotin staining. The mass, HRFABMS m/z $(M+H)^+$, calculated for FP1, $C_{37}H_{55}F_3N_7O_{11}S$, was 862.3632; the mass found was 862.3632. The mass, HRFABMS m/z $(M+H)^+$, calculated for FP3 ($C_{37}H_{54}F_3N_8O_{14}S$) was 923.3432; the mass found was 923.3454. Similarly, the Boc group in compound **8** was removed by TFA treatment and the resulting amine was coupled to either biotinyl-hexanoyl-succinimide or biotinyl-ethylenediamino-tartaroyl-succinimide to give FP2 and FP4, respectively, in 50–60 % yields. No molecular ion for FP2 was found using fast-atom-bombardment and electrospray MS techniques. The compound was therefore converted to a Boc derivative and this gave a molecular ion. The mass, HRFABMS m/z $(M+H)^+$, calculated for the FP2-Boc compound $C_{44}H_{68}F_3N_8O_{13}S$ was 1005.4596; the mass found was 1005.4579. The mass, HRFABMS m/z $(M+H)^+$, calculated for FP4 ($C_{39}H_{59}F_3N_9O_{14}S$) was 966.3854; the mass found was 966.3864.

D-Fructose transport

CHO cells expressing GLUT5 (CHO-GLUT5 cells) [7] were grown to confluence in 19 mm culture dishes. Growth medium was removed by washing three times in Krebs–Ringer phosphate (KRP) buffer (130 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 10 mM Na_2HPO_4, pH 7.4). Cells were then maintained in this buffer at 37 °C for 10 min. Uptake was initiated by adding D-[U-^{14}C]fructose to give a final concentration of 1 mM and 0.3 μCi in 0.25 ml. After 10 min at 37 °C, 1 ml of ice-cold buffer containing 0.3 mM phloretin was added to terminate the reaction. The dishes were washed rapidly three times and then cell radioactivity was extracted into 0.5 ml of 0.1 M NaOH and detected by scintillation counting. The uninhibited uptake rate constant (V_o) was compared with the uptake in the presence of competitive inhibitors (V). K_i values were calculated from the equation $V_o/V = 1 + I/K_i$, where I is inhibitor concentration. It assumes that the concentration of the substrate is low in relation to its K_m for transport.

Photolabelling of GLUT5

CHO-GLUT5 cells were grown to confluence in 35 mm culture dishes and FPs 1–4 were added in KRP buffer to give the concentrations indicated in the Figure legends. Cells were irradiated at a peak wavelength of 300 nm for 1 min in a Rayonet RPR-100 photoreactor. Following irradiation, cells were washed three times in KRP buffer and scraped into 1 ml Hepes, EDTA, sucrose buffer (20 mM Hepes, 1 mM EDTA and 255 mM sucrose, pH 7.2) and homogenized in this buffer. The homogenized samples were then centrifuged at 100000 ***g*** for 30 min to obtain a total membrane fraction. This fraction was solubilized in 2 % Thesit detergent and then streptavidin–agarose precipitated as described in [28,32]. Proteins eluted from the streptavidin beads were resolved by SDS/PAGE (10 % gels), transferred to nitrocellulose and blotted with anti-GLUT5-C-terminal peptide antiserum [7] used in a 1:2000 dilution. Immunoreactive GLUT5 was detected with peroxidase-coupled secondary antibody and ECL reagents. Chemiluminescence was analysed using an Optichem detector with associated software (Ultraviolet Products).

RESULTS AND DISCUSSION

We have synthesized 1-allylamine derivatives of D-fructose and 2,5-anhydro-D-mannitol as potential ligands for GLUT5 to explore the spatial restrictions around the binding site that is adjacent to 6-OH of D-fructose. The structural relationship between these compounds and the extents to which substitutions at 1-OH of 2,5-anhydro-D-mannitol can occupy space in the binding site in GLUT5 that normally accepts the 6-OH of D-fructose are illustrated in Figure 1(A).

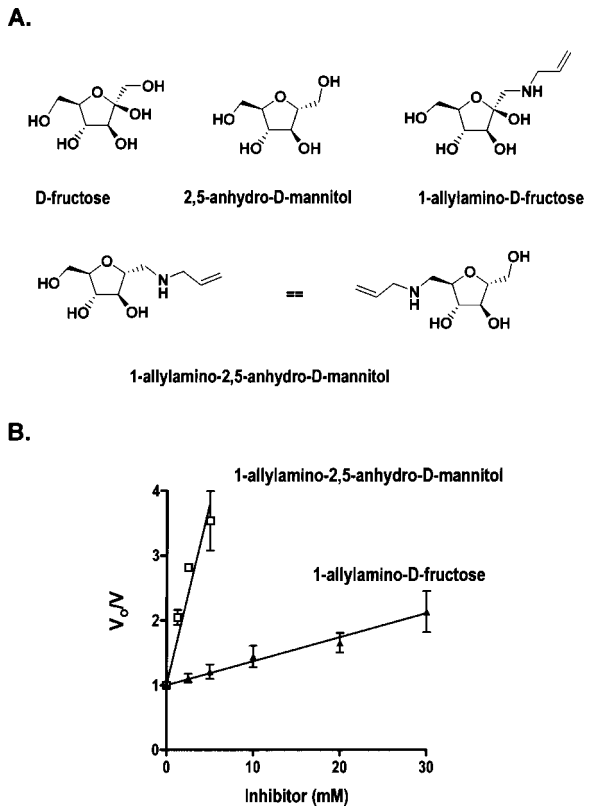


Figure 1 Comparison of the inhibition of D-fructose transport by allylamine derivatives on D-fructose and 2,5-anhydro-D-mannitol

(**A**) The α-furanose ring form of D-fructose is compared with 2,5-anhydro-D-mannitol. D-Fructose is present in solution in multiple ring forms while 2,5-anhydo-D-mannitol (because of the absence of 2-OH of D-fructose) can only adopt the indicated structure. Unlike D-fructose, 2,5-anhydro-D-mannitol is a totally symmetric molecule so that its 1-allylamine derivative can present the substituted group to the GLUT5 binding site in a position that is normally occupied by the 6-OH of D-fructose. These properties of 2,5-anhydro-D-mannitol allow it to interact strongly as an inhibitor of D-fructose transport into CHO-GLUT5 cells. (**B**) The uptake rate constants for D-fructose in the presence (V) and absence (V_0) of the inhibitors were used to calculate the K_i value according to the equation given in the Experimental section and are the means $\pm$ S.E.M. from three separate experiments. K_i values were: 1-allylamino-D-fructose, 27.1 $\pm$ 4.0 mM; 1-allylamino-2,5-anhydro-D-mannitol, 2.66 $\pm$ 0.03 mM. Some additional inhibitor concentrations were tested and used to calculate the K_i but these data are not shown for clarity.

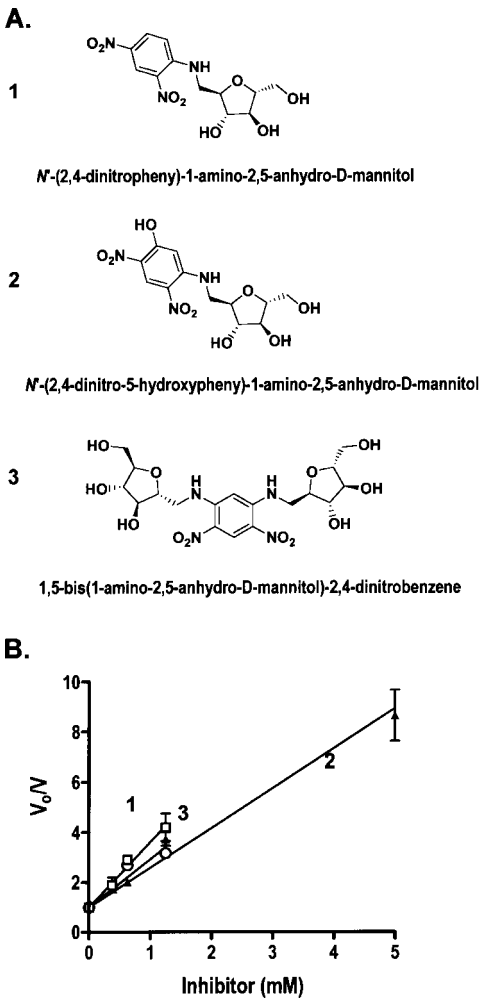


Figure 2 Dinitrophenylamine-substituted 2,5-anhydro-D-mannitol compounds have high affinity for GLUT5

(**A**) The bulky and hydrophobic nitrophenyl group was linked to a 2,5-anhydro-D-mannitol moiety by a secondary amine (compound **1**). This results in affinity for GLUT5 that is $>$ 10-times that of D-fructose. Neither an additional aryl OH group (compound **2**) nor the bulky dimeric structure (compound **3**) markedly lowered this interaction. (**B**) Uptake rate constants for D-fructose in the presence (V) and absence (V_0) of the inhibitors were used to calculate the K_i value according to the equation given in the Experimental section and are the means $\pm$ S.E.M. from three separate experiments. The numbers on the graph refer to the structures in (**A**). K_i values were: compound **1**, 0.56 $\pm$ 0.09 mM; compound **2**, 0.63 $\pm$ 0.04 mM; compound **3**, 0.52 $\pm$ 0.04 mM. Some additional inhibitor concentrations have been tested and used to calculate the K_i but these data are not shown for clarity.

Each of these compounds was tested for inhibition of D-fructose uptake into CHO cells expressing GLUT5 (Figure 1B). The affinity for D-fructose and 2,5-anhydro-D-mannitol have been previously found to be similar, with K_i values of 12–15 mM [35]. The 1-allylamine substitution into D-fructose was tolerated moderately well (K_i = 27.1 mM) in comparison with D-fructose and the affinity of this compound was higher than that of 1-*O*-allyl-D-fructose (K_i = 105 mM), described previously [35]. However, 1-allylamino-2,5-anhydro-D-mannitol was found to be a much better inhibitor (K_i = 2.66 mM). The increased affinity of this analogue may be partly due to restriction of the available ring forms (only the furanose ring form is present) and the resulting symmetry in the molecule. Thus the GLUT5 cannot distinguish between a C-1 and a C-6 substitution into this symmetrical molecule. In addition, the allyl group may increase hydrophobic interaction with GLUT5 while the NH group may maintain the H-bonding that normally occurs to the 6-OH group (and which is lost when an O-allyl substitution is made).

To further examine the potential of hydrophobic secondary amine analogues as a means of generating enhanced affinity for GLUT5, dinitrophenyl groups (Figure 2) were substituted and the resulting analogues were investigated for inhibition of D-fructose uptake. The dinitrophenylamine (Figure 2, compound **1**) had an affinity (K_i = 0.56 mM) which was $\approx$ 28-times higher than D-fructose. This high affinity was only very slightly reduced when an additional phenolic group (Figure 2, compound **2**) or a second amino-2,5-anhydro-D-mannitol moiety (Figure 2, compound **3**) were introduced. The latter result is consistent with the postulate that the C-6 position of D-fructofuranose occupies an exposed position in the GLUT5 binding site that is adjacent to the extracellular bulk solution [35]. A similar situation occurs

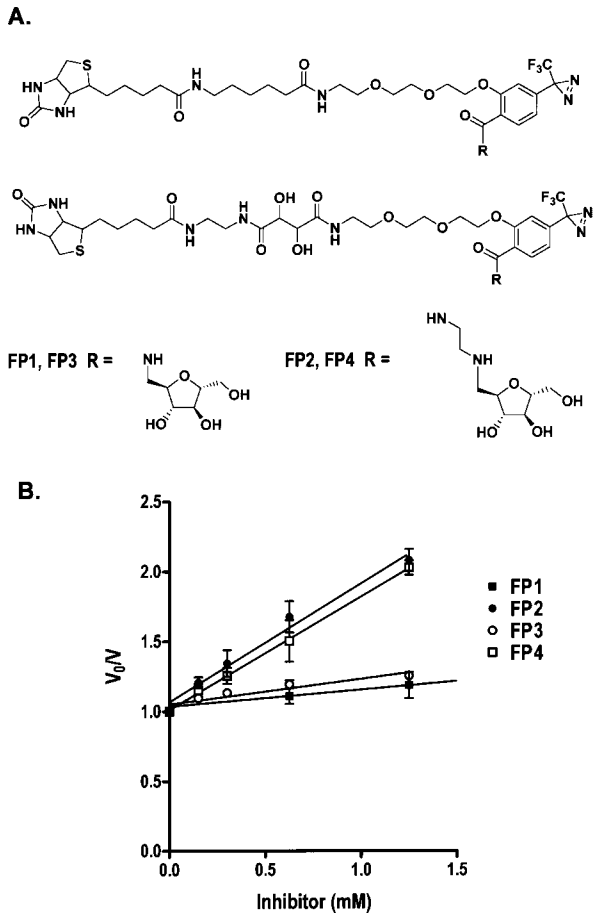


Figure 3 Inhibition of GLUT5-mediated D-fructose transport by biotinylated aryl-diazirine 2,5-anhydro-D-mannitol photolabels

(**A**) Biotin can be introduced into the photoaffinity labels either via the aminohexanoate spacer (in the top compound) or the more hydrophilic ethylenediamine-tartarate spacer (in the bottom compound). The photolabels FP1 and FP3 are amide-linked to the 2,5-anhydro-D-mannitol moiety whereas FP2 and FP4 are linked via ethylenediamine spacers. The secondary amine group in the latter compounds results in greater affinity as inhibitors of D-fructose transport. (**B**) Uptake rate constants for D-fructose in the presence (V) and absence (V_0) of the inhibitors were used to calculate the K_i value according to the equation given in the Experimental section and are the means $\pm$ S.E.M. from three separate experiments. K_i values were: FP1, 9.7 $\pm$ 2.1 mM; FP2, 1.16 $\pm$ 0.07 mM; FP3, 5.42 $\pm$ 0.34 mM; FP4, 1.29 $\pm$ 0.03 mM. Some additional inhibitor concentrations have been tested and used to calculate the K_i but these data are not shown for clarity.

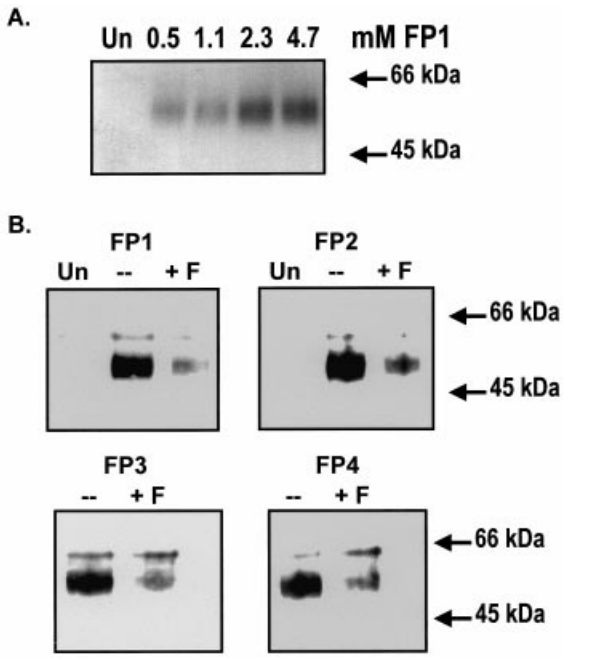


Figure 4 Photolabelling of GLUT5 by biotinylated aryl-diazirine 2,5-anhydro-D-mannitol photolabels

(**A**) The indicated concentrations of FP1 were used to photolabel GLUT5. Following irradiation to produce cross-linking of the ligand to the transporter, the cell membrane material was isolated, solubilized and the biotinylated protein was precipitated on strepatavidin–agarose beads. Protein was eluted and resolved by SDS/PAGE and then blotted with a GLUT5 antibody. A peroxidase-coupled secondary antibody and ECL was used for detection. The saturation of the signal is consistent with the observed inhibition of transport activity by this compound. (**B**) The displacements of the photolabels FP1–FP4 at 500 μM from GLUT5 by 200 mM D-fructose (+F) were examined. The lanes marked Un were from samples that were not photolabelled but subjected to the same precipitation and GLUT5-detection procedures. The data are representative of 2–3 similar experiments for each photolabel. The blots shown were not processed simultaneously.

in the case of interaction of bis-D-mannose and bis-D-glucose analogues with the class 1 GLUTs [29,31].

The affinity-enhancing effect of the secondary amine was further evident in a comparison of two potential photoaffinity analogues for GLUT5, FP1 and FP2. The structures of FP1 and FP2 are shown in Figure 3(A). FP1 has an affinity (K_i = 9.7 mM) that is similar to D-fructose. This is an important result as it indicates that a bulky photoreactive group with an extended chain and biotin tag can be introduced without loss of affinity. This result also supports the suggestion (above) that there is space around the OH-6 position of D-fructose as it binds to GLUT5 and that this space may extend to the external solution surrounding the site.

By comparison, FP2 has an additional ethylenediamine spacer between the furanose ring and the biotinyl-photolabelling moiety. This maintains the desirable feature of an NH for hydrogen bonding and has 8-fold higher affinity than FP1 (K_i = 1.16 mM; Figure 3). This increased affinity following introduction of the NH group is also observed in comparison of FP3 (K_i = 5.42 mM) and FP4 (K_i = 1.29 mM). These compounds also have amide and ethylenediamine links, respectively, between the photolabelling diazirine moiety and the fructofuranose ring (Figure 3A), but the ethylenediamine-linked compound has 4.2-fold higher affinity. FP3 and FP4 have tartarate spacers between the biotin and the diazirine moieties, which attaches by cross-linking to the GLUT5. Since the vicinal diols in the tartarate spacers are periodate cleavable, it should be possible to separate cell surface from any internalized GLUT5 using these reagents. The internalized GLUT5 should be protected from cleavage by extracellularly added periodate. In addition, and equally importantly, the introduction of the tartarate groups produces compounds that are more water soluble than those with the amino-hexanoate spacer.

We show here that the fructose photolabels FP1–FP4 can all be used to tag GLUT5. Each compound was incubated with CHO-GLUT5 cells and then irradiated. The biotinylated cell membrane protein was then isolated by centrifugation, solubilized in detergent and precipitated using streptavidin–agarose. The precipitated material was then solubilized and resolved on

SDS/PAGE gels followed by Western blot analysis using a specific GLUT5 antibody (Figure 4). The labelling by FP1 increased with concentration and the signal saturated in line with the apparent affinity constants revealed by inhibition of D-fructose transport. Furthermore, samples in which no label was present gave no detectable signal using the GLUT5 antibody and the signal was reduced by incubation in the presence of a competing high concentration of D-fructose. These data indicate that the compounds are specifically labelling GLUT5 at its binding site for D-fructose. To determine the approximate labelling efficiency, we chose to study concentrations of FP1 and FP2 that saturated half of the binding sites, as estimated from the K_i values. Following labelling at concentrations of 4.7 and 0.5 mM for FP1 and FP2, respectively, the Western blot signal from streptavidin-precipitated GLUT5 was compared with that obtained using an aliquot of total cell membranes. From this, the proportions of total cellular GLUT5 that were recovered from the streptavidin precipitates were 8.8 and 10.0% for FP1 and FP2, respectively (mean of two separate experiments in each case). These values will tend to underestimate the efficiency of the photochemical reaction itself. This is because a proportion of the total cellular GLUT5 may be present in intracellular membranes, only half of the available plasma membrane GLUT5 sites will be occupied, not all of the biotinylated GLUT5 will be precipitated by streptavidin–agarose and not all of the precipitated biotinylated GLUT5 will be eluted from the streptavidin–agarose into the gel sample buffer.

The postulated importance of hydrogen bonding and the observed enhanced affinity properties for the hydrophobic and the secondary amine analogues will aid in the development of a specific pharmacophor for the GLUT5 exofacial binding site. This will be useful for comparison with the properties of other mammalian D-fructose transporters in the GLUT family. D-Fructose is a very abundant hexose in mammalian diets and different tissues may exhibit different requirements in terms of uptake and cellular utilization of this hexose. These varying requirements may be met by a range of GLUT isoforms with different affinities and specificities for D-fructose analogues.

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