Improvement in the Properties of 3-Phenyl-3-trifluoromethyldiazirine Based Photoreactive Bis-Glucose Probes for GLUT4 Following Substitution on the Phenyl Ring

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We have developed two novel 3-phenyl-3-trifluoromethyldiazirinyl bis-glucose derivatives to investigate the properties of the adipocyte glucose transporter GLUT4. These compounds were substituted by electron-withdrawing (iodo and nitro) groups on the aromatic ring of 3-phenyl-3-trifluoromethyldiazirine photophore and were found to be more photosensitive than compounds without such substituents. The compounds were used as inhibitors of insulin-stimulated glucose transport activity in order to assess half-maximal inhibition or relative affinity values for GLUT4. The affinities were found to be 60—130 times higher than the parent compound D-glucose. Because of the increased photo-reactivity and high affinity these compounds will be useful in studies directed at further elucidation of GLUT4 function.

Key words photoaffinity label; diazirine; glucose transporter

Recent human genome sequencing has led to the realisation that the family of facilitative sugar transporters (GLUTs: glucose transporters) in mammals is more extensive than the group of five members (GLUTs 1—5) described a decade ago. Now the GLUTs are known to be a more complex family of thirteen isomeric proteins that is divisible into three subgroups based on sequence similarities.[1)] Class 1 consists of the glucose transporters (GLUTs 1—4) which preferentially transport D-glucose.[2)] Of these transporters GLUT4 is particularly important both in physiological and pathophysiological processes as it is present only in insulin-responsive tissues such as adipose, heart and skeletal muscle. Following insulin signaling, there is an increase in GLUT4 exocytosis from intracellular storage vesicles and incorporation of the protein into the surface membrane of the insulin target cells.[3)] Loss of responsiveness of this protein to insulin is one of the contributing factors to the pathogenesis of Type 2 diabetes.[4)]

To effectively examine the mechanisms involved in exposure of GLUT4 at the cell surface of adipocytes, high affinity analogues that can tag the transporter are required. Synthesizing suitable analogues is a difficult task since sugars have relatively low affinity for the transporter molecules. D-Glucose for example, when interacting with GLUT4, does so with an affinity constant (K_m) of around 8 mM. Physiologically the high K_m is important as if the affinity for D-glucose were very high then this substrate would bind very strongly to the transporter rather than being transported through it. In addition, blood glucose levels are generally 5 mM (or slightly higher following a carbohydrate rich meal) and therefore a high affinity (low K_m) system would become too easily saturated and unable to respond to the fluctuations in circulating blood glucose levels. The requirements for a GLUT4 tag are opposite to these and high affinity interaction is required in a photolabel as the ligand/protein complex has to be occupied at a high enough level to be efficiently converted to a covalent complex following UV irradiation.[5)]

We have developed a series of photoaffinity probes based on bis-hexose structures.[6)] These compounds contain hexoses linked via their 4-OH positions to a propyl-2-amine spacer. Because the bis-hexose structure renders the compounds large and hydrophilic the derivatives are impermeant and just probe those glucose transporters (GLUT4) that appear at the cell-surface in response to insulin. We have already synthesized photoreactive bis-mannose derivatives containing arylazide,[7)] benzophenone[8)] and 3-phenyl-3-trifluoromethyldiazirine[9)] substitutions for use in glucose transporter cell surface labeling. Recently, we have synthesized photoreactive bis-glucose derivatives containing the 3-phenyl-3-trifluoromethyldiazirine group.[10)] These can easily be synthesised preparative scale and have slightly higher affinity for GLUT4 than the equivalent bis-mannose compounds.

Arylazide ligands were found to be rapidly activated but the resulting nitrene derivatives show poor selectivity and specificity, whereas the benzophenone compounds were very slowly activated. This means that the latter probes are not really suited to analysis of GLUT4 cell biology because long irradiation needed for cell surface labeling can cause some intact cell damage. By contrast, the 3-phenyl-3-trifluoromethyldiazirine derivatives were rapidly incorporation into the GLUT proteins without cell damage.[11)] The time required for photoactivation is intermediate between that required to activate iodo- and nitro-phenyl azides (less than 1 min) and benzophenone derivatives (30 min or longer).

We have previously found that nitro- and iodo-phenyl derivatives of bis-mannose have higher affinity for GLUT4 than compounds that were unsubstituted in the phenyl ring.[5,6)] This information, combined with the possibility that introducing such ring substituents may also increase the rate of activation of the derivatives in response to UV irradiation, prompted us to synthesize and examine new bis-glucose compounds in which nitro and iodo groups were substituted into the aromatic ring of 3-phenyl-3-trifluoromethyldiazirine photophore.

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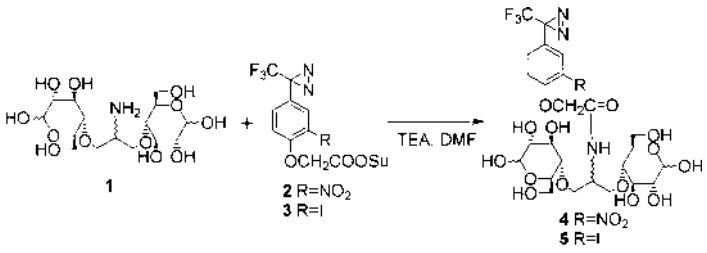

Fig. 1. Synthesis of Diazirinyl Bis-Glucose Compounds

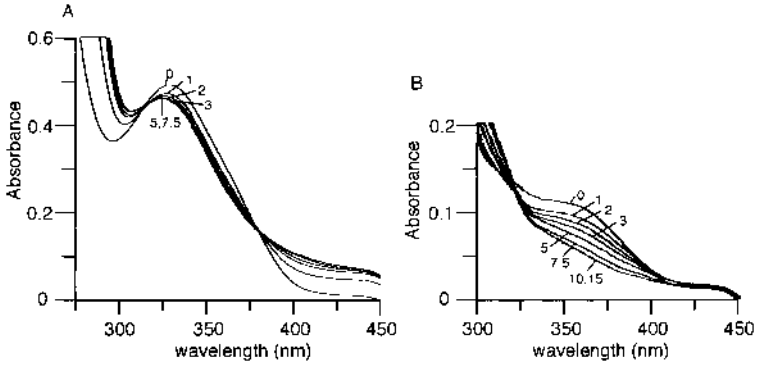

Fig. 2. Photolysis of Bis-(D-glucose)analogue **4** (A) and **5** (B) in Water

UV spectra of the photolysis reaction were recorded at times (in min) indicated.

Experimental

All chemical reagents were commercially available grade and were used without further purification. Column chromatography was performed with silica gel 60 (230—400 mesh) from Merck, pre-washed with methanol before use. NMR, UV and FAB-MS measurements were performed on JEOL GX-270, UV-160 spectrophotometer and VG 7070E spectrometers, respectively.

[2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenoxy]acetyl-1,3-bis(glucopyranosyl-4-yloxy)propyl-2-amide (4) [2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenoxy]acetic acid *N*-hydroxysuccinimide ester (**2**)[12] (8.9 mg, 22.1 μmol) and triethylamine (TEA) (20 μl) were added to 1,3-bis(D-glucopyranos-4-yloxy)-2-propylamine (BGPA, **1**)[10] (9.2 mg, 22.1 μmol) in DMF (100 μl). The reaction mixture was stirred at room temperature for 12 h in the dark and then concentrated. The residue was purified by column chromatography ($CHCl_3 : CH_3OH : H_2O = 13 : 5 : 1$) to afford a colorless oil (5.5 mg, 35%). UV λ_{max} (H_2O) nm (ε): 325 (3000), FAB-MS *m/z*: 703.1883 (Calcd for $C_{25}H_{34}F_3N_4O_{16}$: 703.1922). ^{1}H-NMR (CD_3OD) δ: 7.87 (1H, s, Ph), 7.60 (1H, d, J=8.9 Hz, Ph), 7.39 (1H, d, J=8.9 Hz, Ph), 5.07 (1H, d, J=2.6 Hz, 1-α), 4.76 (2H, s, PhOC$\underline{H}_2$), 4.44 (1H, d, J=7.6 Hz, 1-β), 4.23 (1H, m, $OCH_2CH(NH)CH_2O$), 3.98 and 3.74 (2H each, m, $OC\underline{H}_2CH(NH)C\underline{H}_2O$), 3.0—4.0 (12H, m, 2-, 3-, 4-, 5-, 6-α and β).

[2-Iodo-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenoxy]acetyl-1,3-bis(glucopyranosyl-4-yloxy)propyl-2-amide (5) To BGPA (25.5 mg, 61.4 μmol) in DMF (500 μl) were added [2-iodo-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenoxy]acetic acid *N*-hydroxysuccinimide ester (**3**)[13] (50.2 mg, 103.9 μmol) and TEA (150 μl). The reaction mixture was stirred at room temperature for 12 h and then concentrated. The residue was purified by column chromatography ($CHCl_3 : CH_3OH : H_2O = 13 : 5 : 1$) to afford colorless oil (22.3 mg, 46%). UV λ_{max} (H_2O) nm (ε) 280 (1900), 357 (360), FAB-MS *m/z*: 784.1052 (Calcd for $C_{25}H_{34}F_3IN_3O_{14}$: 784.1038). ^{1}H-NMR ($CD_3OD$) δ: 8.05 (1H, s, Ph), 7.55 (1H, d, J=8.9 Hz, Ph), 7.10 (1H, d, J=8.9 Hz, Ph), 5.27 (1H, d, J=3.0 Hz, 1-α), 4.82 (2H, s, PhOC$\underline{H}_2$), 4.66 (1H, d, J=7.9 Hz, 1-β), 4.40 (1H, m, $OCH_2C\underline{H}(NH)CH_2O$), 4.10 and 3.75 (2H each, m, $OC\underline{H}_2CH(NH)C\underline{H}_2O$), 3.0—4.0 (12H, m, 2-, 3-, 4-, 5-, 6-α and β).

Photolysis of Compounds 4 and 5 in Aqueous Solution Aqueous solutions of compound **4** (0.16 mM) and **5** (0.30 mM) were placed in quartz cuvetts. After replacing the inner atmosphere with nitrogen and cooling on ice, photolysis was carried out using a 15W (350 nm) lamp at a distance of 2 cm from the surface of the light source. The UV spectrum was measured at the intervals indicated in Fig. 2. The half-life was calculated from a semi-log plot of the decay of the absorbance at 325 nm and 357 nm, for the nitro and iodo compounds respectively.

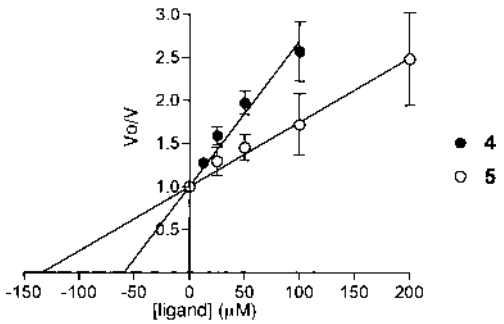

Fig. 3. Determination of Half-Maximal Inhibition Constants for Bis-Glucose Derivatives

The rates of uptake of tracer 2-deoxy-[^{14}C]-D-glucose in insulin-stimulated rat adipose cells were determined at the indicated concentrations of compounds **4** (●) and **5** (○). The rate constants (mean and S.E.M. are shown) for uptake in the presence (V) and absence (V_o) of inhibitor (I). K_i values were calculated according to the equation $V_o/V = 1 + I/K_i$. Results shown are from triplate estimates of rate in each experiment and from three independent experiments.

Adipose Cell Preparations and Glucose Transport Assays Rat adipocytes were prepared and glucose transport assays were performed as described previously.[14] Epidermal fat pads of male Wister rats were digested with collagenase, and suspended at 40% cryocrit in a KRH buffer (140 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 2.5 mM $CaCl_2$, 2.5 mM NaH_2PO_4, 10 mM HEPES, and 1% BSA, pH 7.4) at 37 °C. Cells were then stimulated by 20 nM insulin for 20 min at 37 °C. The prepared suspension (50 μl) was incubated KRH buffer (10 μl) containing 50 μM 2-[^{3}H]-D-glucose and the indicated concentrations of compound **4** or **5** for 1 min at 37 °C. Uptake was terminated by rapidly adding 3 ml of 0.3 mM pholetin in BSA free KRH buffer. Silicon oil (1 ml) was layered on top of the buffer and the samples were centrifuged at 2500 rpm for 45 s. The cell layers were recovered from the top of the oil layer and the trapped radioactivity was determined by liquid scintillation counting. The ratio of the uptake determined in the absence (V_o) or presence (V) of the compounds was plotted against the concentration of the inhibitory compounds to give the K_i value from the equation $V_o/V = 1 + I/K_i$ (where I is the inhibitor concentration). This equation applies when the concentration of substrate used is much less than the substrate K_m.

Result and Discussion

Synthesis The GLUT4 transporter has broad specificities for hexoses. Previous studies have established that bulky groups are tolerated around the 4-OH position when the hexose occupies the exofacial binding sites of the transporters.[15] The bis-hexose compounds we have developed are linked by a bridge through their C-4 positions. The remaining hexose hydroxyls are available for interaction with the GLUT4 protein. The presence of two hexose moieties makes the compounds very hydrophilic and therefore they do not penetrate through cell membrane lipid. Instead they act specifically at the exofacial binding site of the transporter. Recently, we have found bis-glucose skeleton can be synthesized on a preparative scale and that the bis-glucose series of compounds have slightly higher affinity for GLUT4 than the equivalent bis-mannose compounds.[10]

The complicated synthesis of the diazirinyl three membered ring has resulted in fewer general applications of diazirines in biomolecular studies than for other photophors. However, we have reported that compounds containing various substitutions are chemically available after construction of the diazirine three membered ring.[10,12,13,16—19] Comparative studies of three photophors: arylazide,[7] benzophenone[8] and 3-(trifluoromethyl) aryldiazirine[9] introduced into bis-hexoses have led to the conclusion that the diazirine is the most useful for labeling GLUT4.[11] The possibility of higher affinity and higher activation rates for the substituted compounds promoted us to synthesize and examine the interaction of bis-hexoses containing these substituents with GLUT4. Nitro groups were introduced by treatment with fuming nitric acid in H_2SO_4[19] or thallation then $NaNO_2$.[13] Iodide was introduced by use of NaI with Chloramine T or KI after thallation.[13] Both iodo and nitro of diazirinylphenoxyacetic acids were derivatized to their *N*-hydroxysuccinimide esters (compounds **2**, **3**) before coupling to the amine bridge of the bis(D-glucopyranos-4-yloxy)-2-propylamine (BGPA, **1**). These condensations of the photophors with BGPA proceeded in anhydrous conditions with a moderate yield. Rates of photolysis of the BGPA derivatives were determined in aqueous solution. The typical diazirinyl adsorption around 350 nm was observed for compound **5**. Compound **4** afforded maximum adsorption around 320 nm, being affected by a hyperchromic effect of nitro group. A ten-fold hyperchromic effect was observed. Photolysis rates for both compounds were examined following irradiation with black lights (with peak emission at 350—360 nm). The half-lives were calculated as 0.85 and 1.6 min for **4** and **5**, respectively. It was therefore considered that the increased photoreactivity and high extinction coefficient (over 3000) of the nitro BGPA should be particularly useful properties for production and detection of labeled components in biological studies of GLUT4.

Biological Assays The affinities of compounds **4** and **5** for rat adipocyte GLUT4 were evaluated and K_i values were found to be 60 and 134 μM, respectively. These affinities are 130 (**4**) and 60 (**5**) times higher than that of glucose (8 mM),[20] which is the parent compound. K_i values for several phenoxy derivatives of photoreactive BGPA compound were found 140—190 μM for GLUT4.[10] Compounds **4** and **5** therefore have higher affinities for GLUT4 confirming that introduction of nitro- and iodo groups into GLUT4 ligands lead to tighter binding.[5,6] The iodide group is one of the most common radioisotopes and very useful to detect the labeled components. We have previously established that introduction of radioisotopic iodine on 3-(trifluoromethyl) aryldiazirine is possible.[13] Consequently, iodo-diazirinyl-BGPA or a related compound has potential for use in Single Photon Emitted Computed Tomography (SPECT)[21] medical imaging of glucose transporters.

Together the derivatives and biological studies described indicate that considerable improvement in the development of high affinity, high photo-sensitive bis-hexose derivatives for tagging GLUT4 have been made. The improved compounds should find application in biomedical research on this protein and pathophysiological defects in its cellular behaviour in response to insulin.

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