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Biochemical Pharmacology 67 (2004) 459–467
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Interaction of substituted hexose analogues with the *Trypanosoma brucei* hexose transporter

Laurent Azema[a], Samantha Claustre[a], Isabelle Alric[a], Casimir Blonski[a], Michèle Willson[a], Jacques Perié[a], Theo Baltz[b], Emmanuel Tetaud[b], Frédéric Bringaud[b], Dominique Cottem[c], Frederik R. Opperdoes[c], Michael P. Barrett[d,*]

[a]*Groupe de Chimie Organique Biologique, Laboratoire de Synthèse et Physico Chimie de Molécules d'Intérêt Biologique, Université Paul Sabatier, UMR-5068-CNRS, Bât IIR1 118 route de Narbonne, 31062 Toulouse Cedex, France*
[b]*Laboratoire Genomique Fonctionelle des Trypanosomatides, Université Victor Segalen Bordeaux II, UMR-CNR 5162, 146 Rue Léo Saignat, 33076 Bordeaux Cedex, France*
[c]*Research Unit for Tropical Diseases, Christian Duve Institute of Cellular Pathology and Laboratory of Biochemistry, Université Catholique de Louvain, B-1200 Brussels, Belgium*
[d]*Division of Infection & Immunity, Institute of Biomedical and Life Sciences, The Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK*

Received 28 April 2003; accepted 19 September 2003

Abstract

Glucose metabolism is essential for survival of bloodstream form *Trypanosoma brucei* subspecies which cause human African trypanosomiasis (sleeping sickness). Hexose analogues may represent good compounds to inhibit glucose metabolism in these cells. Delivery of such compounds to the parasite is a major consideration in drug development. A series of D-glucose and D-fructose analogues were developed to explore the limits of the structure–activity relationship of the THT1 hexose transporter of bloodstream form African trypanosomes, a portal that might be exploited for drug uptake. D-Glucose analogues with substituents at the C2 and C6 position continued to interact with the exofacial hexose binding site of the transporter. There was a limit to the size at C6 which still permitted recognition, although compounds carrying large groups at position C2 were still recognised. However, radiolabelled *N*-acetyl-D-[1-^{14}C] glucosamine was not internalised by trypanosomes, in spite of the ability of this compound to inhibit glucose uptake, indicating that there is a limit to the size of C2 substituent that allows translocation. Addition of an alkylating group (bromoacetyl) at position C2 in the D-glucose series and at position 6 in the D-fructose set, created two analogues which interact with the transporter and kill trypanosomes *in vitro*. This indicates that inhibition of the transporter may be a good means of killing trypanosomes.

Keywords: Human African trypanosomiasis; Sleeping sickness; Hexose transporter; Hexose analogues; Chemotherapy

1. Introduction

The chemotherapy of African sleeping sickness is at a crisis point. The disease has become resurgent in Africa [1,2] and resistance to the first-line drug in treatment of late-stage disease, melarsoprol, appears to be on the increase [3]. New drugs are urgently sought.

The compartmentalisation of glucose metabolism in trypanosomatids is unique in that the first seven enzymes of the Embden–Meyerhoff pathway reside within a unique peroxisome-like organelle termed the glycosome [4–6]. Most of the classical steps associated with regulation of the glycolytic flux do not exist in *Trypanosoma brucei* [7]. Moreover, most of the enzymes differ markedly from their mammalian counterparts. Since bloodstream form *T. brucei* are entirely dependent upon glycolysis this has lead to a belief that these enzymes may be ideal targets for selective chemotherapy [8–10].

In developing inhibitors of intracellular enzymes, however, the additional proviso of targeting a drug to the

* Corresponding author. Tel.: +44-141-330-6904; fax: +44-141-330-6904.
E-mail address: m.barrett@bio.gla.ac.uk (M.P. Barrett).
Abbreviations: 2-DOG, 2-deoxy-D-glucose; 6-DOG, 6-deoxy-D-glucose.

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doi:10.1016/j.bcp.2003.09.005

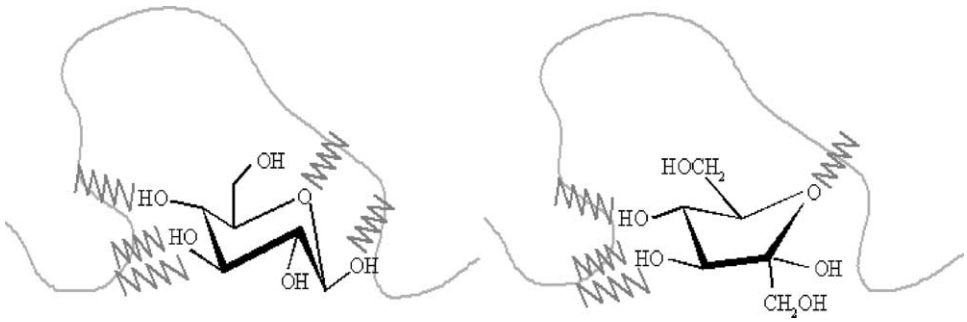


Fig. 1. Proposed model of binding of D-glucose and D-fructose to the *T. brucei* hexose transporter, THT1. D-Glucose (left) and D-fructose (right) have been proposed to interact with the *T. brucei* hexose transporter through hydrogen bonds (grey zig-zag lines) [23].

intracellular environment also needs consideration. Since African trypanosomes live free in the blood and cerebrospinal fluid of their mammalian hosts and not intracellularly, drugs can be selectively accumulated by parasites but not hosts' cells [11]. This can afford specificity as illustrated by the case of the trypanocidal melaminophenyl arsenical and diamidine drugs which enter trypanosomes via an unusual amino-purine transporter, termed P2 [12–17]. Loss of this transporter can render parasites resistant to these drugs.

The *T. brucei* hexose transporter, THT1, the principal hexose transporter expressed in bloodstream form organisms, is a candidate to deliver inhibitors of the glycolytic pathway to trypanosomes. This transporter is well characterised at both the biochemical [7,18,19] and molecular [20–22] levels; heterologous expression of THT1 in *Xenopus* oocytes yielded similar results with regard to structure–activity relationship for substrate as those of the transporter expressed in bloodstream form trypanosomes [21].

THT1 is different from all of the mammalian GLUT hexose transporters in having a relatively high affinity for D-glucose (around 1 mM compared with 5 mM for the erythrocyte transporter, GLUT1) and also being able to carry D-fructose [23]. At least two mammalian GLUT transporters can also carry D-fructose [24], although with lower affinity than THT1. It appears that the trypanosome transporter carries D-fructose in the furanose form [23].

Limited structure–activity recognition profiles of substrate recognised at the exofacial binding site of the transporter have been previously reported [18,23] (Fig. 1). The addition of relatively bulky groups at the C2 position of D-glucose could be tolerated by the transporter, as could substitutions at C6 of the pyranose. Studies on structure–activity relationships to date have involved testing the ability of analogues to inhibit uptake of D-glucose, D-fructose, 2-DOG or 6-DOG. These studies only report on the ability of analogues to inhibit uptake of known substrates from which it is frequently inferred that inhibition is due to analogues being competitive substrates of the transporter. However, the translocation process is complex, but most easily divided into four stages [25] (Fig. 2). In a first step substrate binds at the exofacial site. This is followed by transfer of substrate through the permease, associated with conformational changes, and then release at the endofacial binding site. Finally, the transporter re-orientates itself for subsequent translocation cycles.

In this study, we set out to further the understanding of the ability of the trypanosome's hexose transporter to both recognise hexose analogues (based on their ability to inhibit uptake of known substrates of the transporter), but also to translocate some of these inhibitors by directly measuring internalisation. These studies were crucial to determining the likely efficacy of the transporter as a portal for entry of new potentially trypanocidal agents and also of being a target itself.

2. Materials and methods

2.1. Chemicals

The synthesis of D-glucose analogues substituted at position C2 and C6 has been described elsewhere

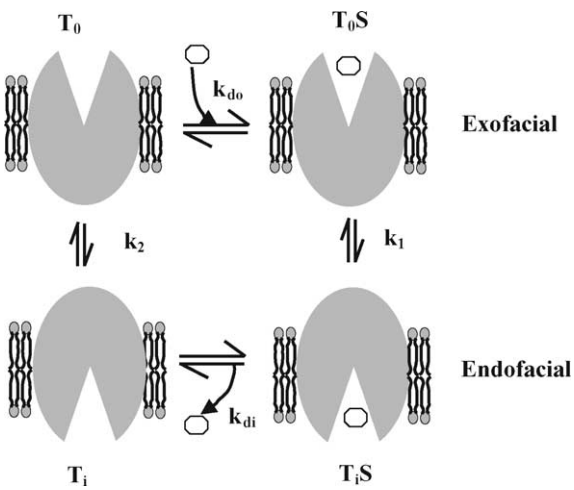


Fig. 2. A model for hexose translocation. The hexose transporter (grey) reorientates between exofacial, outward facing (To) and endofacial, inward facing (Ti) conformations. Sugar (hexagon) binds at the exofacial binding site (ToS) which causes reorientations (K_1) bringing sugar to the endofacial binding site TiS from where it is released. The transporter then returns to ground state (K_2).

[26–28], D-fructose analogues have also been described [27,29,30]. Radiolabelled D-[6-^{14}C] glucose, 2-deoxy-D-[1-^{3}H] glucose, D-[1-^{14}C] glucosamine and *N*-acetyl-D-[1-^{14}C] glucosamine were from Amersham International. All other chemicals were of the highest grade possible and purchased from Sigma.

2.2. Growth of T. brucei

Bloodstream form trypomastigotes of *T. brucei* 427 [31] were purified from rat blood using DEAE-cellulose chromatography [32], and resuspended in phosphate-buffered saline (PBS) containing 10 mM glucose (PSG) and stored on ice. Parasites were checked for viability by phase contrast microscopy.

2.3. Uptake studies

For uptake studies, cells were washed three times in 1 mL of D-glucose-free PBS per 10^9 cells at 4°, with total washing time not exceeding 5 min. Cells were resuspended to 5×10^8 mL^{-1} in PBS which had been warmed to 25° prior to uptake, at which point extracellular glucose was present only at a low sub-micromolar concentration. Aliquots were checked for viability by phase contrast microscopy with the beating of the flagellum taken as a measure of parasite viability. Transport was initiated by the addition of 0.1 mL of these parasites to 0.1 mL of PBS containing radiolabelled hexose (concentrations stated in text) without or with competitor/inhibitor such that the final concentration equalled that specified in the text. Uptake was allowed to proceed for 20 s prior to parasites being separated from uptake buffer and unincorporated label by centrifugation through a cushion of silicone oil (1-dodecylybromid, 97%, specific density 1.038, from Aldrich) as an adaptation of the centrifugation through oil technique [12,33] in a bench top microfuge. Pelleted cells were collected by freezing the tube in a dry-ice/ethanol bath then chopping the base of the tube into a scintillation vial containing 0.2 mL of 2% SDS, taking care not to disturb the frozen aqueous phase containing the uptake medium. Incorporated radioactivity was counted by liquid-scintillation after leaving tubes overnight. Apparent K_i ($K_{i\ \mathrm{app}}$) values were determined for the better inhibitors with established methods [18] using the equation $v_0/v = 1 + [\mathrm{I}]/K_i$ where v_0 and v are the uninhibited and inhibited rates, respectively and [I] is the inhibitor concentration. $K_{i\ \mathrm{app}}$ is taken as an estimate of the affinity of the transporter for each compound when using radiolabelled substrate at a concentration well below its K_m [18]. Uptake of D-[6-^{14}C] glucose, D-[1-^{14}C] glucosamine and *N*-acetyl-D-[1-^{14}C] glucosamine was carried out by incubating each of the labelled compounds at 100 μM either alone or in the presence of 25 mM of each of the other two compounds. Uptake was allowed to proceed for 10 min with aliquots measured for incorporated radioactivity (using the above protocol) at specified time points over that time-course. Kinetic constants were determined by measuring uptake rates over a range of concentrations between 25 mM and 140 μM, allowing uptake to proceed at 25° for 20 s during which time uptake is linear.

2.4. In vitro inhibition of parasite growth

Parasites were cultivated in Baltz medium [34] and sensitivity tests performed in a microtitre assay [35]. Compounds were added in doubling dilutions starting at 20 μg mL^{-1} and trypanosomes were counted by microscopy at 24 and 48 hr so that the MEC (minimal effective concentration to kill 100% of the parasites) and MTC (maximal tolerated concentration, allowing parasites to grow as effectively as untreated controls) could be determined as described [36]. Alternatively, the Alamar blue test [37] was employed to obtain LD_{50} values, derived from non-linear regression to plots of the change in fluorescence fitted to the IC_{50} algorithm of the Grafit 4.0 software (Erithacus Software).

2.5. Fluorescence studies

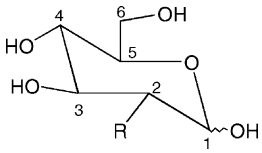
Parasites were washed three times with glucose-free phosphate buffer (0.15 M NaCl, 5 mM KH_2PO_4, pH 7.4), and resuspended in the same buffer, at a concentration of 10^9 cell mL^{-1}. After 2 min at 20°, 0.1 mL of this solution is added to two different solutions (800 μL): one containing the same buffer, one containing PBS and 200 mM of D-glucose, for a final volume of 900 μL. After 2 min at 20°, fluorescent compounds were added (0.1 mL), to a final concentration of 5 mM. After 2 min at 20°, parasites were washed several times with glucose-free PBS and observed by fluorescence microscopy, with an excitation wavelength of 340 nm and emission filter detecting fluorescence emissions of greater than 520 nm.

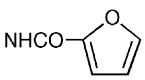
3. Results

3.1. Inhibition of hexose uptake by D-glucose analogues

As a primary screening procedure for the efficiency of the *T. brucei* bloodstream form hexose transporter, THT1, to recognise the different hexose analogues, each was tested for its ability as a 10-fold excess to inhibit the uptake of 0.5 mM [^{3}H]-radiolabelled 2-DOG using a 20-s time point. The ability of each product to inhibit uptake was compared to that of L-glucose as a negative control (L-glucose has previously been shown not to interact with the transporter [21]), and D-glucose, D-mannose, D-fructose and D-glucosamine (all of which have been shown to be substrates [18,21]) as positive controls. Table 1 ranks the C2 analogues in order of their ability to inhibit 2-DOG uptake.

Table 1
Effect of D-glucose analogues substituted at position 2 on the *Trypanosoma brucei* hexose transporter



Compounds	R	$K_{i\ app}$ (mM)
	OH (D-glucose)	1.3 ± 0.061
1	$NHC(O)CH_2Br$	6.2 ± 0.25
2	$NHC(O)CH_2CH_3$	8.5 ± 1.38
3	$NHC(O)CH_2CH_2Br$	9.8 ± 0.85
4	$NHC(O)CH_2OH$	10.5 ± 1.2
5	$NHC(O)CH_3$	11 ± 0.65
6		12.1 ± 1.7
7	$NHC(O)NHCH_2CH_3$	20.5 ± 1.97
8	$NHC(O)OCH_2CH_3$	>25
9	*N*-Acetyl-D-mannosamine	>25
10	*N*-Acetyl-D-galactosamine	>25

The structures of D-glucose analogues and $K_{i\ app}$ values (mM) are given with standard deviations from a minimum of three experiments. The $K_{i\ app}$ value gives an estimate of the affinity of the transporter for each compound as described in Section 2.

D-Glucose analogues substituted at position 2 still interact with the transporter, although the addition of aromatic groups diminishes the ability of the transporter to recognise these molecules (all compounds with a phenolic attachment failed to inhibit, data not shown). The presence of a furan ring (compound **6**) does not greatly affect recognition, and since brominated products (compounds **1** and **3**) with similar net bulk to the phenolic products are recognised, it seems that the aromatic nature of these latter compounds is important in loss of recognition rather than size. The $K_{i\ app}$ (apparent inhibition constant) values for the analogues fall in the range of 5–25 mM, somewhat higher than for D-glucose itself. Any compound with a $K_{i\ app}$ value higher than 25 mM or with no affinity was assigned a $K_m > 25$ mM.

Several C6 substituted analogues were also tested but only 6-deoxy-6-thio-D-glucose showed any interaction with a $K_{i\ app}$ of 5.6 mM. This is significantly less efficient than either 6-deoxy-D-glucose or 6-chloro-6-deoxy-D-glucose ($K_{i\,app} = 0.68$ mM) previously reported [18]. The addition of larger substituents at this position (6-deoxy-6-amino-D-glucose, 6-deoxy-6-*N*-acetyl-D-glucose, 6-deoxy-6-thiophosphate-D-glucose and 6-deoxy-6-bromo-D-glucose) greatly reduced recognition ($K_i > 25$ mM), suggesting that there is a strict limit to the size of susbtituents at position C6 which still permit recognition. 1,6-Anhydro-D-glucose has no affinity, in accordance with previous data that hydroxyl groups at positions 1 and 6 are involved in recognition [18]. Indeed, at least one of those hydroxyl groups is necessary for recognition, as 6-deoxy-D-glucose yields a $K_{i\ app}$ of 1.5 mM and 1-deoxy-D-glucose a $K_{i\ app}$ of 3.5 mM [18]. Moreover, changes at other positions gave a loss of recognition by the transporter. Indeed, the D-glucono-1,5-lactone, the *N*-acetyl-D-mannosamine (compound **9**) and the *N*-acetyl-D-galactosamine (compound **10**), corresponding to positions 1, 2 and 4 modifications, respectively, are inactive.

3.2. Inhibition of hexose uptake by D-fructose analogues

Since the *T. brucei* transporter [23], has a relatively high affinity for D-fructose—in the furanose form—a series of D-fructose (C6 modified) or 2,5-anhydro-D-mannitol (C1 modified) derivatives were synthesized. The affinity of these two series of compounds for THT1 was determined by measuring their ability to inhibit uptake of [^{3}H]-radiolabelled 2-DOG. GLUT5, a low affinity fructose transporter is expressed to low levels in erythrocytes [38] while GLUT1 the principal transporter of the erythrocyte does not recognise D-fructose.

C6 D-fructose substituents interacted with the THT1 transporter, but with low affinity. $K_{i\ app}$ values were in the range of 7.4–67 mM, as compared to D-fructose (5 mM—compound **11**) with a general trend of decreasing affinity as substituent size increased (Table 2). Figure 3 shows inhibition data for representative compounds. Surprisingly the C1 modified 2,5-anhydro-D-mannitol derivatives retained inhibitory activity even with relatively large aromatic substituents. One compound, the nitro-naphthylamino-2,5-anhydro-D-mannitol (compound **31**) (Table 2), had remarkably good apparent affinity for the transporter ($K_i = 150$ μM). Moreover, some aromatic compounds showed trypanocidal activity *in vitro* ($LD_{50} = 0.135$ mM for compound **31**). However, since simple inhibition assays

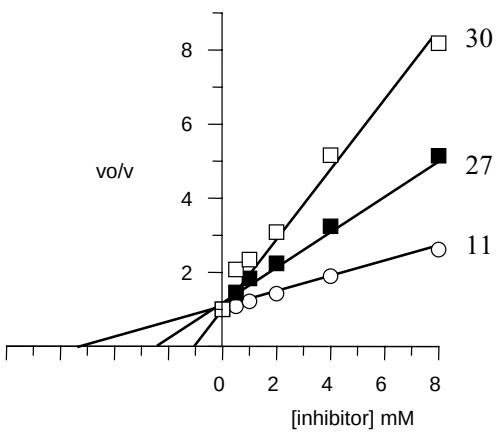
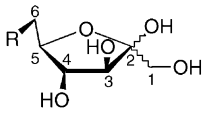
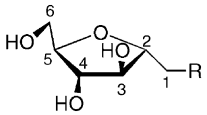
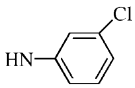
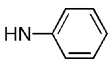
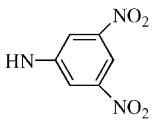
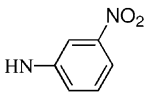
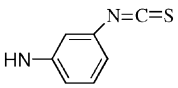
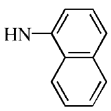
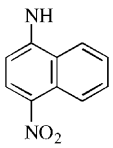


Fig. 3. Representative graphs showing inhibition of 2-deoxy-D-glucose uptake by fructose analogues. Uptake of 100 μM of 2-deoxy-D-glucose was inhibited using varying concentrations of each compound. Examples here are compounds **11**, **27** and **30** shown in Table 2. The initial rate divided by the inhibited rate was plotted against the inhibitor concentration. K_i values correspond to the point at which the lines intercept the *x*-axis. All lines had correlation coefficients, $r > 0.9$.

Table 2
Effect of D-fructose analogues on the *Trypanosoma brucei* hexose transporter

	 R	K_i (mM)		 R	K_i (mM)	LD_{50} (mM)
11	OH D-fructose	5	**22**	OH 2,5-anhydro-D-mannitol	9.7 ± 1	–
12	Cl	7.4 ± 1.2	**23**	NH_2	>100	–
13	I	51.2 ± 6.2	**24**	—=N–OH	>100	
14	NH_2	26.7 ± 0.5	**25**		0.48 ± 0.1	0.0026
15	N_3	21.4 ± 1	**26**		4.5 ± 0.8	–
16	—≡N	60.1 ± 0.1	**27**		2.1 ± 0.3	0.17
17	OCH_3	30.3 ± 1.7	**28**		1.4 ± 0.2	2.5
18	$NHC(O)CF_3$	54.2 ± 6.7	**29**		1 ± 0.15	0.8
19	$NHC(O)CH_2Br$	13.7 ± 1.2	**30**		1 ± 0.2	0.89
20	-$P(O)(OEt)_2$	67.1 ± 1.4	**31**		0.15 ± 0.05	0.135
21	$CH_2P(O)(OEt)_2$	1.8 ± 0.1				

The structures of D-fructose analogues (6-deoxy-6-alkyl-D-fructose and 2,5-anhydro-1-alkyl-D-mannitol), $K_{i\ app}$ values (mM) with standard deviations from a minimum of three experiments. The $K_{i\ app}$ value gives an estimate of the affinity of the transporter for each compound as described in Section 2. The concentration of toxic compounds that led to 50% reduction in parasite numbers (LD_{50}) are marked in the third column for the 2,5-anhydro-1-alkyl-D-mannitol series. Only compound **19** showed activity among the fructose analogues (LD_{50} = 4 μM).

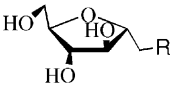
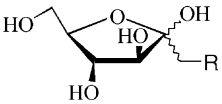
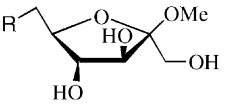
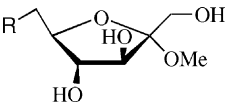
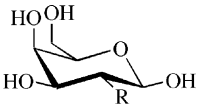
cannot provide information regarding the ability of the compounds to be internalised, nor whether internalisation necessarily depends on the trypanosome's hexose transporter, we produced fluorescently labelled derivatives of D-fructose and 2,5-anhydro-D-mannitol to monitor the accumulation of a class of compounds carrying bulky substituents.

3.3. Internalisation of hexose analogues carrying fluorescent aromatic substituents

The observation that large aromatic groups at C1 in 2,5-anhydro-D-mannitol actually improve the apparent affinity of D-fructose analogues for the trypanosome hexose transporter prompted us to synthesise a series of analogues bearing large aromatic ring structures. It is possible that hydrophobic ring structures engage in hydrophobic interactions and π-stacking within the transporter. Among these we added fluorescent groups to enable us to determine whether analogues were actually accumulated by THT1, or if they merely exerted an inhibitory activity on uptake by competition with glucose binding sites on the transporter. For this purpose, a dansyl group was added to the C1 position of 2,5-anhydro-D-mannitol (compound **32**), the C1 position of D-fructose (compound **33**) and D-fructosamine and to the C6 position of methyl-D-fructofuranoside (compounds **34** and **35**).

A dansyl group was also added to D-galactosamine (compound **36**) which in its non-dansylated state has very low affinity for the transporter, due to the importance of the stereochemistry of the C4 hydroxy group [18]. Inhibition of [^{3}H]-radiolabelled 2-DOG uptake was used to determine the ability of these compounds to interact with the exofacial binding site and the apparent inhibition constant values ($K_{i\ app}$) for both the carbohydrates and their dansylated analogues, are reported in Table 3. The dansyl group alone

Table 3
Effect of dansylated D-fructose and D-galactose on *Trypanosoma brucei* hexose transporter

		R		
		Dansyl-NH		OH
		K_i (mM)	Log *P*	K_i (mM)
32		1.6 ± 0.04	1.86 ± 0.42	8.6 ± 1.0 (2,5-anhydro-D-mannitol)
33		3.5 ± 0.05	1.69 ± 0.49	5 ± 0.5 (D-fructose)
34		4.7 ± 0.1	1.74 ± 0.58	22 ± 2.0
35		3.1 ± 0.5	1.74 ± 0.58	25 ± 2.0
36		7.5 ± 0.6	1.89 ± 0.55	>250 D-galactose

$K_{i\ app}$ values (mM) for dansylated compared with non-dansylated analogues (with SD for a minimum of three experiments) against 2-DOG uptake are given along with the lipophilic coefficients for dansylated derivatives (Log *P*), determined with the TSAR software [39] (with SD). Common names for sugars are given in parentheses in the final column.

had no inhibitory activity (data not shown) while many of the dansylated hexoses did inhibit. D-Galactose is not recognized by the transporter [18] (nor is *N*-acetyl-D-galactosamine, Table 1), however, dansylated D-galactose (compound **36**) has a $K_{i\ app}$ value close of the three dansylated D-fructose analogues (compounds **33**–**35**). For the methyl-D-fructofuranoside isomers, the affinities are identical for the α- and β-conformers (compounds **34** and **35**, respectively). The small difference observed between the $K_{i\ app}$ of the D-fructose (compound **11**—5 mM) and the $K_{i\ app}$ of the 2,5-anhydro-D-mannitol (compound **22**—8.6 mM) could be assigned to equilibrium existing between closed and open forms of the sugar.

Since the dansylated compounds fluoresce within cells, it was possible to track internalisation using a representative compound, the dansylated D-fructose (compound **33**). The compound was clearly internalised as observed by uniform cellular fluorescence—no subcellular compartments could be distinguished (data not shown). It was then possible to determine whether internalisation was dependent upon the trypanosome hexose transporter by saturating the permease with 200 mM D-glucose (40-fold excess as compared to the dansylated analogue) and measuring any inhibition of fluorescent labelling of cells incubated with the dansylated probe over 30 s. D-Glucose failed to retard the appearance of fluorescence, indicating that these compounds enter cells via routes independent of the hexose transporter. Log *P* values [39] estimated for these compounds also indicate that they are sufficiently amphipathic to cross cell membranes by passive diffusion (Table 3).

3.4. Uptake of D-glucosamine and N-acetyl-D-glucosamine by T. brucei

The assay commonly used to investigate the limits of substitution which can be added to hexoses yet still permit interaction with the *T. brucei* bloodstream form hexose transporter do not give an indication as to whether the analogues are actually internalised. Examples exist of products which interact with sugar binding sites but which do not proceed through other steps required for translocation. For example Cytochalasin B interacts with the endofacial hexose binding site of the erythrocyte transporter, GLUT1. However, this agent jams the transporter as it undergoes the conformational change initiating the translocation cycle, explaining its relatively high apparent affinity for this transporter [25,40]. Data using dansylated D-fructose analogues indicates that these compounds can inhibit the transporter and also enter cells. However, entry appears to be independent of their interaction with the hexose transporter. To test whether substituted D-glucose analogues are actually accumulated by the transporter, internalisation of radiolabelled derivatives of C2 substituents, D-[1-^{14}C] glucosamine and *N*-acetyl-D-[1-^{14}C] glucosamine was compared with that of D-[6-^{14}C] glucose (Fig. 4). Both D-glucosamine and *N*-acetyl-D-glucosamine have previously been shown to be inhibitors of 6-deoxy-D-glucose uptake in *T. brucei* [18]. D-Glucosamine and *N*-acetyl-D-glucosamine inhibited uptake of D-glucose (with $K_{i\ app}$ values of 17.1 and 8.2 mM respectively). Labelled D-glucosamine also enters trypanosomes by a

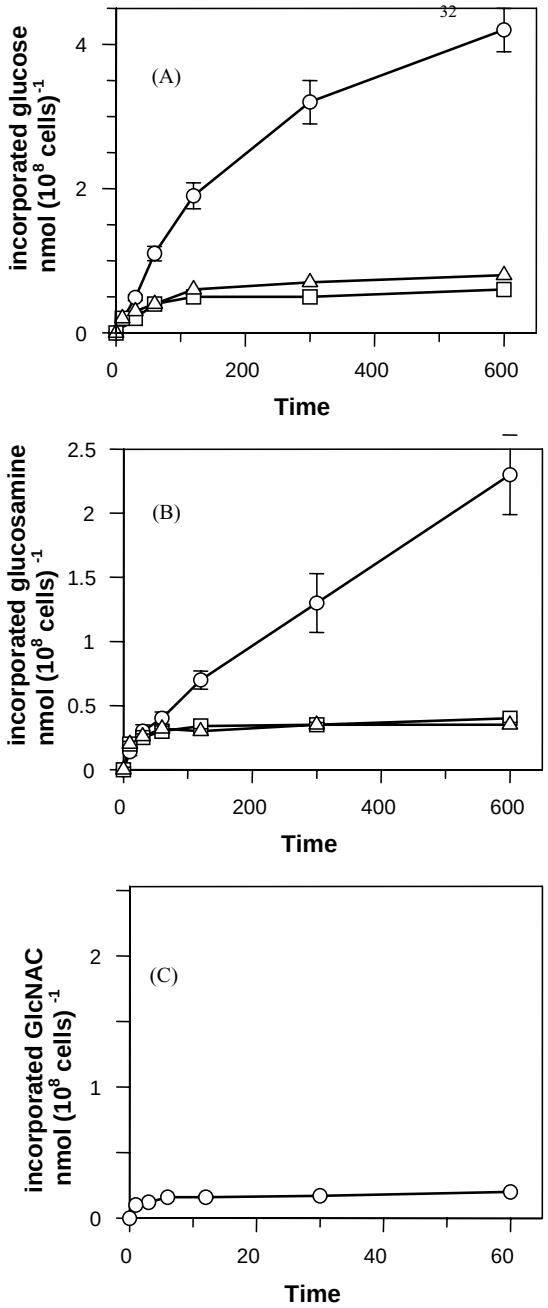


Fig. 4. Uptake of radiolabelled D-glucose, D-glucosamine and *N*-acetyl glucosamine by *T. brucei* bloodstream forms. Uptake of 100 μM of each of the radiolabelled substrates was allowed to proceed for 10 min and incorporated radioactivity was determined after separating cells from substrate using the centrifugation through oil technique. Scintillation counting was used to determine incorporated radioactivity. (A) Uptake of D-glucose in the absence of competitor (open circles) in the presence of *N*-acetyl glucosamine (triangles) or D-glucosamine (squares). (B) Uptake of D-glucosamine in the absence of competitor (circles) in the presence of *N*-acetyl glucosamine (squares) or D-glucose (triangles). (C) Uptake of *N*-acetyl glucosamine in the absence of competitor.

carrier mediated process with an apparent K_m value of 14.4 mM and its internalisation can be inhibited by D-glucose and *N*-acetyl-D-glucosamine (with $K_{i\ app}$ values of 1.9 and 7.9 mM, respectively).

By contrast there is no apparent uptake of *N*-acetyl-D-glucosamine under these conditions (Fig. 4), indicating that while this product can interact with the exofacial binding site (as judged by its ability to inhibit uptake of D-glucose and D-glucosamine), it cannot fulfil the criteria required for the remainder of the translocation cycle. This indicates that while size limits for substituents at C2 are relaxed with respect to interaction at the exofacial binding site of the transporter, the limits become much stricter in producing compounds that can actually complete the permeation pathway.

3.5. Toxicity to bloodstream form trypanosomes by hexose analogues bearing alkylated halogen groups

Doubling dilutions of each compound, starting with 20 μg mL^{-1} were used in a microtitre plate test assay, screening trypanosomes for viability by phase contrast microscopy after testing with drug. Of the D-glucose and D-fructose series, two compounds had a trypanocidal effect, acetylbromo-D-glucosamine (compound **1**) and acetylbromo-6-amino-D-fructose (compound **19**), with LD_{50} values at 4 and 8.3 μM, respectively. Both compounds possess a reactive CH_2 carbon atom, able to make a covalent bond with a nucleophilic residue such as a cysteine. Interestingly, a related compound (**3**) where the chain length is changed by two methylene groups was not toxic, suggesting that activation of the carbon centre by the carbonyl group may be important in activity. The LD_{50} values of the 2,5-anhydro-D-mannitol derivatives are also shown and the best of these (compound **25**) has an LD_{50} of 2.6 μM.

4. Discussion

The search for new drugs against human African trypanosomiasis (sleeping sickness) needs to be intensified in the wake of reports of the resurgence of several epidemics in Africa involving parasites not responding to treatment with the first-line agent for use against late-stage disease, melarsoprol [3]. Glycolysis has long been considered a potential target for chemotherapy in these parasites [8–10] since they are entirely dependent upon rapid glucose metabolism, and because many of the enzymes of the pathway are unusual when compared with their mammalian counterparts, since they reside within a peroxisome-like organelle termed the glycosome [4–6].

The present study has extended our understanding of the nature of substitutions to hexoses which can generate compounds still recognised by the principal *T. brucei* bloodstream form hexose transporter, THT1. Since THT2 is also expressed [20–22] it cannot be ruled out that this transporter plays a role in accumulation of these compounds, in spite of its contributing less than 5% of total glucose uptake in these cells. In the case of D-glucose there is a limit to substituents which can be tolerated at the C6

position. This probably relates to the orientation of sugar binding such that large groups sterically hinder binding at the exofacial binding site. In the case of C2 analogues, large substituents can be placed without preventing interaction at the C2 position although aromatic groups interfere with recognition. This indicates that the sugar is probably oriented with the C2 substituents exposed to solvent when bound to the exofacial binding site.

Using an assay where analogues are tested for their ability to inhibit the uptake of radiolabelled 2-deoxy-D-glucose or D-glucose only measures the ability of analogues to bind to the transporter without necessarily being a substrate. It was of interest, therefore, to see if limits were imposed on analogues as substrates for uptake beyond this initial step of the translocation cycle. To test directly whether D-glucose analogues substituted at the C2 position could actually enter the parasites, we measured the uptake of D-glucosamine (amino-group substituting for the hydroxyl group at position 2) and also *N*-acetyl-D-glucosamine where a methylated carbonyl group is added to this site. D-Glucosamine was accumulated by *T. brucei* (albeit with reduced efficacy compared with D-glucose). *N*-Acetyl-D-glucosamine, on the other hand, did not actually enter the cell, in spite of it being a competitive inhibitor of the uptake of both D-glucose and D-glucosamine. This result shows that substituents at the C2 position can be tolerated by the exofacial binding site, however it appears that a step in the transport pathway, subsequent to binding at the exofacial site, cannot operate when using *N*-acetyl-D-glucosamine. This data could suggest that other analogues carrying bulky groups at position C2 are unlikely to actually enter via this pathway, although further data is required to verify this.

D-Fructose analogues, including those based on 2,5-anhydro-D-mannitol, also interact with the transporter and large, aromatic groups added to C1 of 2,5-anhydro-D-mannitol, in some cases produced compounds with higher apparent affinity than D-fructose itself. We interpret this to indicate that large aromatic groups become involved in stacking interactions with an aromatic residue in close vicinity of the hexose binding site or another point in the translocation pathway. It seems unlikely that these compounds would be translocated if similar size restrictions to those identified in the *N*-acetyl-D-glucosamine experiment also applied to the D-fructose analogues. A representative compound which had a fluorescent dansyl-group added continued to interact with the transporter as judged by its ability to inhibit radiolabelled D-glucose uptake; however, the internalisation of this compound itself was not effected by D-glucose indicating that its mode of uptake was independent of the hexose transporter. Compounds in the D-mannitol series bearing aromatic rings and therefore having amphiphilic structures such as those in the dansyl series (Table 3) are likely to behave in the same way, i.e. they have affinity for the transporter but are internalised principally via passive diffusion. Since the compounds appear to be freely permeable they could also interact with the endofacial substrate binding site and inhibit activity in this way.

More promising are the results obtained with compounds **1** and **19** which are significantly toxic to trypanosomes and which both bear an alkylating group. The size of the compounds indicate that they are unlikely to be internalised via the transporter (comparing to the data obtained with *N*-acetyl glucosamine) thus the toxic effects might be accounted for by a covalent bond created between the activated carbon centre on the inhibitor and a nucleophilic residue on the transporter itself. These halogenated hexose analogues are unlikely to be of sufficient specificity to represent novel trypanocides in their own right. Our study does, however, demonstrate that the development of inhibitors of the transporter, such as compounds **1** and **19**, is a promising strategy for development of novel agents for use in trypanosomiasis chemotherapy. Several inhibitors of mammalian GLUT1 (e.g. cytochalasin B and phloretin) have an activity towards the trypanosome hexose transporter that is several orders of magnitude less than for GLUT1. Cytochalasin B appears to bind to the endofacial glucose binding site [25] and phloretin the exofacial binding site of GLUT1 [40]. Structural and functional differences between mammalian and trypanosomal transporters should therefore permit selective inhibition of the trypanosomal transporter. As predicted in metabolic flux analysis [41–43] the trypanosome hexose transporter does appear to be a good target for chemotherapy as determined using detailed SAR studies here. The combinatorial approach to drug development could be well suited to identifying compounds, not necessarily related to hexose substrates, which can interact specifically with this permease. It is noteworthy that the hexose transporter of the malaria parasite, *Plasmodium falciparum*, has recently been demonstrated to be a good target for novel compounds, exemplified by O-3 long chain hexose analogues [44]. Hexose transport in both *Plasmodium* and *Trypanosoma* thus appears to be a target for novel therapeutics.

Acknowledgments

This research was funded by the European Commission INCO DC programme

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