Selective inhibitors of the glycosylphosphatidylinositol biosynthetic pathway of *Trypanosoma brucei*

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Synthetic analogues of D-GlcNAc1–6D-myo-inositol-1-HPO₄-3(sn-1,2-diacylglycerol) (GlcN-PI), with the 2-position of the inositol residue substituted with an *O*-octyl ether [D-GlcNAc1–6D-(2-*O*-octyl)myo-inositol-1-HPO₄-3-sn-1,2-dipalmitoylglycerol; GlcN-(2-*O*-octyl)PI] or *O*-hexadecyl ether [D-GlcNAc1–6D-(2-*O*-hexadecyl)myo-inositol-1-HPO₄-3-sn-1,2-dipalmitoylglycerol; GlcN-(2-*O*-hexadecyl)PI], were tested as substrates or inhibitors of glycosylphosphatidylinositol (GPI) biosynthetic pathways using cell-free systems of the protozoan parasite *Trypanosoma brucei* (the causative agent of human African sleeping sickness) and human HeLa cells. Neither these compounds nor their *N*-acetyl derivatives are substrates or inhibitors of GPI biosynthetic enzymes in the HeLa cell-free system but are potent inhibitors of GPI biosynthesis in the *T.brucei* cell-free system. GlcN-(2-*O*-hexadecyl)PI was shown to inhibit the first α -mannosyltransferase of the trypanosomal GPI pathway. The *N*-acetylated derivative GlcNAc-(2-*O*-octyl)PI is a substrate for the trypanosomal GlcNAc-PI de-*N*-acetylase and this compound, like GlcN-(2-*O*-octyl)PI, is processed predominantly to Man₂GlcN-(2-*O*-octyl)PI by the *T.brucei* cell-free system. Both GlcN-(2-*O*-octyl)PI and GlcNAc-(2-*O*-octyl)PI also inhibit inositol acylation of Man₁₋₃GlcN-PI and, consequently, the addition of the ethanolamine phosphate bridge in the *T.brucei* cell-free system. The data establish these substrate analogues as the first generation of *in vitro* parasite GPI pathway-specific inhibitors.

Keywords: glycosylphosphatidylinositol/HeLa/inositol acyltransferase/mannosyltransferase/*Trypanosoma*

1995; Udenfriend and Kodukula, 1995; Medof *et al.*, 1996; Kinoshita *et al.*, 1997). The smallest GPI structure found attached to protein is NH₂CH₂CH₂PO₄H-6Man α 1–2Man α 1–6Man α 1–4GlcNAc1–6myo-inositol-1-PO₄H-lipid (EtNP-Man₃GlcN-PI), where the lipid may be diacylglycerol, alkylacylglycerol or ceramide. This minimal GPI structure may be embellished with additional ethanolamine phosphate groups and/or carbohydrate side chains in a species- and tissue-specific manner (McConville and Ferguson, 1993; Treumann *et al.*, 1998).

Although ubiquitous among the eukaryotes, the protozoa tend to express significantly higher densities of cell-surface GPI-anchored proteins than higher eukaryotes (Ferguson, 1997). For example, the tsetse-fly-transmitted parasite *Trypanosoma brucei* expresses a dense cell-surface coat consisting of $\sim 5 \times 10^6$ PI-anchored variant surface glycoprotein (VSG) dimers that protect the parasite from the alternative complement pathway and, through antigenic variation, from specific immune responses (Cross, 1996; Mehlert *et al.*, 1998). In addition to GPI-anchored glycoproteins, other trypanosomatid parasites, such as *Leishmania*, *Trypanosoma cruzi*, *Herpetomonas*, *Leptomonas* and *Phytomonas*, express a variety of GPI-related structures such as the glycoinositol phospholipids (GIPLs) (McConville and Ferguson, 1993; Redman *et al.*, 1995; Routier *et al.*, 1995; Xavier da Silveira *et al.*, 1998; Zawadzki *et al.*, 1998 and references therein). The largest GPI-related structures are the lipophosphoglycans (LPGs) of the *Leishmania* (McConville and Ferguson, 1993; McConville *et al.*, 1995), which are known to be major virulence factors for these parasites (Turco and Descoteaux, 1992). GPI-anchored glycoproteins and/or GIPLs are also abundant on non-trypanosomatid protozoan parasites such as *Plasmodium falciparum* (Gerold *et al.*, 1996), *Toxoplasma gondii* (Tomavo *et al.*, 1989; Manger *et al.*, 1998), *Trichomonas* (Singh *et al.*, 1994) and *Entamoeba* (Bhattacharya *et al.*, 1992). The sheer abundance of GPI-anchored proteins and/or GPI-related molecules on the surfaces of parasitic protozoa suggests that specific inhibitors of parasite GPI biosynthetic pathways might prove useful for the development of anti-parasitic agents.

Proteins destined to become GPI anchored are attached to a pre-assembled GPI precursor in the endoplasmic reticulum in exchange for a hydrophobic C-terminal peptide (Udenfriend and Kodukula, 1995). The basic sequence of events in GPI precursor biosynthesis has been studied in *T.brucei* (Masterson *et al.*, 1989, 1990; Menon *et al.*, 1990a,b; Güther and Ferguson, 1995), *T.cruzi* (Heise *et al.*, 1996), *T.gondii* (Tomavo *et al.*, 1992; Striepen *et al.*, 1999), *P.falciparum* (Gerold *et al.*, 1994), *Saccharomyces cerevisiae* (Sipos *et al.*, 1994) and mammalian cells (Hirose *et al.*, 1992; Puoti and Conzelmann, 1993; Mohney *et al.*, 1994; Chen *et al.*, 1998 and references therein).

Introduction

Many cell-surface proteins and glycoproteins are anchored to the plasma membrane via covalent linkage to a glycosylphosphatidylinositol (GPI) membrane anchor. The structure, function and biosynthesis of GPI membrane anchors and related molecules have been reviewed extensively (Englund, 1993; McConville and Ferguson, 1993; Stevens,

Fig. 1. GlcN-(2-*O*-hexadecyl)PI and GlcNAc-(2-*O*-hexadecyl)PI are not substrates for *T. brucei* or HeLa MT-I. (A) The trypanosomal cell-free system was incubated with GDP-[³H]Man alone (lane 1) or with 10 μ M GlcN-PI (lane 2), GlcNAc-PI (lane 3), GlcN-(hexadecyl)PI (lane 4) or GlcNAc-(hexadecyl)PI (lane 5). The labelled glycolipids were extracted and analysed by HPTLC and fluorography. The major products were Dol-P-Man (DPM) and, for GlcN-PI and GlcNAc-PI, Man₂GlcN-PI (M2), Man₃GlcN-(acyl)PI (aM3), Man₃GlcN-PI (M3) and EtNP-Man₃GlcN-PI (A'). (B) The HeLa cell-free system was incubated with GDP-[³H]Man alone (lane 1) or with 100 μ M GlcN-PI (lane 2), GlcNAc-PI (lane 3), GlcN-(2-*O*-octyl)PI (lane 4), GlcNAc-(2-*O*-octyl)PI (lane 5), GlcN-(hexadecyl)PI (lane 6) or GlcNAc-(hexadecyl)PI (lane 7). The labelled glycolipids were extracted and analysed by HPTLC and fluorography. The major products were Dol-P-Man (DPM), Man₁GlcN-(acyl)PI (H2) and EtNP-Man₁GlcN-(acyl)PI (H5). Note: H5 produced by the processing of synthetic GlcN-PI and GlcNAc-PI has a lower *R_f* than endogenous H5 due to differences in the PI glycerolipid moieties (Sharma *et al.*, 1997).

Some features of the biosynthesis of GPI-like GIPLs and the GPI-like anchor of the LPG of *Leishmania major* have also been described (Proudfoot *et al.*, 1993; Smith *et al.*, 1997a; Ralton and McConville, 1998). In all cases, GPI biosynthesis involves the addition of GlcNAc to phosphatidylinositol (PI) to give GlcNAc-PI, which is de-*N*-acetylated to form D-GlcNAc-1-6D-*myo*-inositol-1-

HPO₄-3-sn-1,2-dipalmitoylglycerol (GlcN-PI) (Doering *et al.*, 1989; Hirose *et al.*, 1991; Stevens, 1993; Milne *et al.*, 1994; Nakamura *et al.*, 1997). In *T. brucei*, *L. major* and human (HeLa) cells, de-*N*-acetylation is a prerequisite for the mannosylation of GlcN-PI to form later GPI intermediates (Smith *et al.*, 1996, 1997a,b; Sharma *et al.*, 1997). The GlcNAc-PI de-*N*-acetylases from these organisms show both similarities (Sharma *et al.*, 1997; Smith *et al.*, 1997b) and differences (Sharma *et al.*, 1999) in their substrate specificities.

From GlcN-PI onwards, there are several significant differences between the *T. brucei* and mammalian GPI biosynthetic pathways. For example: (i) inositol acylation (the transfer of fatty acid to the 2-hydroxyl of the inositol residue) of GlcN-PI precedes the first mannosylation reaction in mammalian cells (Doerrler *et al.*, 1996; Smith *et al.*, 1997b), whereas it occurs after the first mannosylation in *T. brucei* (Güther and Ferguson, 1995; Doerrler *et al.*, 1996; Smith *et al.*, 1997b); (ii) inositol acylation is inhibited by phenylmethylsulfonyl fluoride in *T. brucei* but not in HeLa cells (Güther *et al.*, 1994); (iii) inositol deacylation (the removal of the acyl group from the inositol residue) of GPI intermediates occurs throughout the trypanosomal pathway (Güther and Ferguson, 1995), whereas it does not occur until after the GPI anchor is attached to protein in mammalian cells (Chen *et al.*, 1998); (iv) additional ethanolamine phosphate groups are added to mammalian GPI intermediates during biosynthesis (Hirose *et al.*, 1992; Kamitani *et al.*, 1992; Puoti and Conzelmann, 1993), whereas no such modifications are found in *T. brucei*; (v) only *T. brucei* remodels the GPI fatty acids to produce a dimyristoyl-PI moiety (Masterson *et al.*, 1990).

From points (i), (ii) and (iii), it can be seen that there are fundamental differences between the trypanosomal and mammalian pathways with respect to the timing and roles of inositol acylation and deacylation, and between the acceptor substrate specificity of the first mannosyltransferase (MT-I), i.e. trypanosomal MT-I is a Dol-P-Man:GlcN-PI α 1-4 mannosyltransferase and mammalian MT-I is a Dol-P-Man:GlcN-(acyl)PI α 1-4 mannosyltransferase. Recently, we synthesized the analogue D-GlcNAc-1-6D-(2-*O*-methyl)*myo*-inositol-1-HPO₄-3-sn-1,2-dipalmitoylglycerol [GlcN-(2-*O*-methyl)PI], where the 2-hydroxyl group of the inositol residue is blocked by a methyl group and is unavailable for inositol acylation (Crossman *et al.*, 1997), and showed that this compound is a selective substrate for trypanosomal MT-I (Smith *et al.*, 1997b). Encouraged by these results, we have synthesized related analogues containing larger groups in ether linkage to the 2-position of the inositol residue, and report here their activities in *T. brucei* and HeLa cell-free assays of GPI biosynthesis.

Results

GlcN-(2-O-hexadecyl)PI and GlcNAc-(2-O-hexadecyl)PI are not substrates for T. brucei or HeLa MT-I

The trypanosomal cell-free system was incubated with *N*-ethyl-maleimide (NEM), which inhibits the first enzyme of GPI biosynthesis (UDP-GlcNAc:PI α 1-6 GlcNAc-transferase), without affecting the downstream enzymes (Milne *et al.*, 1992). This inhibits the labelling of endogen-

ous GPI intermediates with GDP-[³H]Man in the cell-free system (Figure 1A, lane 1) and simplifies interpretation of the effects of adding synthetic substrates and substrate analogues. The addition of synthetic GlcN-PI or GlcNAc-PI primed the production of GPI intermediates up to and including glycolipid A' (Figure 1A, lanes 2 and 3), as previously described (Smith *et al.*, 1996, 1997b; Sharma *et al.*, 1997), whereas D-GlcNα1-6D-(2-*O*-hexadecyl)myo-inositol-1-HPO₄-3-sn-1,2-dipalmitoylglycerol [GlcN-(2-*O*-hexadecyl)PI] and GlcNAc-(2-*O*-hexadecyl)PI produced no labelled GPI intermediates (Figure 1A, lanes 4 and 5). The more efficient processing of GlcNAc-PI compared with GlcN-PI is in agreement with previous reports that suggest substrate channelling between the de-*N*-acetylase and MT-I in the trypanosomal pathway (Smith *et al.*, 1996, 1997b; Sharma *et al.*, 1997).

Similar results were obtained with the HeLa cell-free system. In this case, NEM cannot be used to suppress endogenous GPI biosynthesis because it inhibits several steps in the HeLa cell GPI pathway (D.K.Sharma, T.K.Smith and M.A.J.Ferguson, unpublished data). Thus, some glycolipid H5 [EtNP-Man₁GlcN-(acyl)PI] was produced from endogenous substrates in the absence of the synthetic compounds (Figure 1B, lane 1). The addition of synthetic GlcN-PI and GlcNAc-PI increased the incorporation of radiolabel into the GPI glycolipid fraction by priming the production of some H2 [Man₁GlcN-(acyl)PI] and significantly more H5 (Figure 1B, lanes 2 and 3), as previously described (Sharma *et al.*, 1997; Smith *et al.*, 1997b). As previously noted, there is no evidence for substrate channelling between the de-*N*-acetylase and MT-I in the HeLa cell-free system because inositol acylation must occur between these steps (Smith *et al.*, 1997b). The *R_f* of the H5 produced from exogenous GlcN-PI and GlcNAc-PI is lower than that of the endogenous H5 (compare Figure 1B, lanes 1–3) because the dipalmitoylglycerol lipid component of the synthetic substrates is less hydrophobic than the alkylacyl-glycerolipid of the endogenous GPI intermediates. Furthermore, this lower *R_f* form of H5, produced from either synthetic substrate, is sensitive to glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) and nitrous acid (HONO) deamination, resistant to jack bean α-mannosidase (JBAM) and phosphatidylinositol-specific phospholipase C (PI-PLC), and has a Man₁GlcN-inositol headgroup (Sharma *et al.*, 1997). Thus, the appearance of the lower *R_f* form of H5 is synonymous with inositol acylation, MT-I mannosylation and ethanolamine phosphate addition to synthetic GlcN-PI and with de-*N*-acetylation, inositol acylation, MT-I mannosylation and ethanolamine phosphate addition to synthetic GlcNAc-PI. In contrast, the addition of GlcN-(2-*O*-hexadecyl)PI and GlcNAc-(2-*O*-hexadecyl)PI to the HeLa cell-free system did not stimulate the incorporation of additional radioactivity into the GPI glycolipid fraction nor did it result in the production of any novel glycolipids, as indicated by the HPTLC profiles (Figure 1B, compare lane 1 with lanes 6 and 7). Furthermore, HPTLC analysis of these three fractions following GPI-PLD, PI-PLC, JBAM, base hydrolysis and nitrous acid treatment gave identical results, indicating that no novel glycolipid products were hidden under the endogenous H5 band (data not shown).

These data show that GlcN-(2-*O*-hexadecyl)PI and

Fig. 2. GlcN-(2-*O*-hexadecyl)PI is a selective inhibitor of trypanosomal MT-I. (A) The trypanosomal cell-free system was incubated with GDP-[³H]Man alone (lane 1) or with GlcN-PI after pre-incubation with 0–10 μM GlcN-(2-*O*-hexadecyl)PI (lanes 3–9), as indicated. The labelled glycolipids were extracted and analysed by HPTLC and fluorography. The major products are as described in the legend to Figure 1A. (B) The HeLa cell-free system was incubated with GDP-[³H]Man alone (lane 1) or together with GlcN-PI after pre-incubation with nothing (lane 2), GlcN-(2-*O*-octyl)PI (lane 3), GlcNAc-(2-*O*-octyl)PI (lane 4), GlcN-(2-*O*-hexadecyl)PI (lane 5) or GlcNAc-(2-*O*-hexadecyl)PI (lane 6) at the concentrations indicated. The labelled glycolipids were extracted and analysed by HPTLC and fluorography. The major products are as described in the legend to Figure 1B.

GlcNAc-(2-*O*-hexadecyl)PI do not act as substrates for the trypanosomal or HeLa MT-I.

GlcN-(2-*O*-hexadecyl)PI is a selective inhibitor of trypanosomal MT-I

To assess whether GlcN-(2-*O*-hexadecyl)PI and GlcNAc-(2-*O*-hexadecyl)PI are inhibitors of GPI biosynthesis, the trypanosomal and HeLa cell-free systems were pre-incubated with these compounds and then with GlcN-PI. In the trypanosomal cell-free system, GlcNAc-(2-*O*-hexadecyl)PI had no effect but 1–4 μM GlcN-(2-*O*-hexadecyl)PI caused significant inhibition of the processing of exogenous GlcN-PI to later GPI intermediates (Figure 2A, lanes 4–7) and 5 and 10 μM GlcN-(2-*O*-hexadecyl)PI caused complete inhibition (Figure 2A, lanes 8 and 9). By contrast, neither 100 μM GlcNAc-(2-*O*-hexadecyl)PI nor 100 μM GlcN-(2-*O*-hexadecyl)PI had any effect on the processing of exogenous GlcN-PI to H2 and H5 in the HeLa cell-free system (Figure 2B, lanes 5 and 6).

These data indicate that GlcN-(2-*O*-hexadecyl)PI is a selective inhibitor of the mannosylation of exogenous GlcN-PI by MT-I in the trypanosomal cell-free system. We have not determined directly whether GlcNAc-(2-*O*-hexadecyl)PI is a substrate for the trypanosomal GlcNAc-PI de-*N*-acetylase, but since GlcNAc-(2-*O*-octyl)PI is a relatively poor substrate for this enzyme (Sharma *et al.*, 1999), it is conceivable that GlcNAc-(2-*O*-hexadecyl)PI is not a substrate. If this is the case, the inhibition of MT-I by GlcN-(2-*O*-hexadecyl)PI but not by GlcNAc-(2-

O-hexadecyl)PI would be consistent with the known requirement of the trypanosomal MT-I for recognition of the free amino group of the GlcN residue (Smith *et al.*, 1996).

GlcN-(2-O-octyl)PI is a selective inhibitor of trypanosome inositol acylation

We have already demonstrated that GlcNAc-(2-*O*-octyl)PI cannot be de-*N*-acetylated by the HeLa cell-free system (Sharma *et al.*, 1999). Here we show that the addition of synthetic D-GlcNAc1-6D-(2-*O*-octyl)*myo*-inositol-1-HPO₄-3-sn-1,2-dipalmitoylglycerol [GlcN-(2-*O*-octyl)PI] to the HeLa cell-free system did not stimulate the incorporation of additional radioactivity into the GPI glycolipid fraction nor did it result in the production of any novel glycolipids, as indicated by the HPTLC profiles (Figure 1B, compare lane 1 with lane 4). Furthermore, HPTLC analysis of these two fractions following GPI-PLD, PI-PLC, *JBAM*, base hydrolysis and nitrous acid treatment gave identical results, indicating that no novel glycolipid products were hidden under the endogenous H5 band (data not shown). Thus, we conclude that GlcN-(2-*O*-octyl)PI is not a substrate for HeLa MT-I. In addition, neither this compound nor its *N*-acetyl derivative affected the processing of exogenous GlcN-PI to glycolipid H5 (Figure 2B, lanes 3 and 4). We interpret this to mean that neither GlcN-(2-*O*-octyl)PI nor GlcNAc-(2-*O*-octyl)PI inhibit inositol acylation, MT-I α -mannosylation or ethanolamine phosphate addition to GlcN-PI in the HeLa cell-free system. Thus, GlcN-(2-*O*-octyl)PI and GlcNAc-(2-*O*-octyl)PI are neither substrates nor inhibitors of MT-I in the HeLa cell-free system.

On the other hand, we have shown previously that GlcNAc-(2-*O*-octyl)PI can be de-*N*-acetylated by the trypanosomal cell-free system (Sharma *et al.*, 1999). In the presence of GDP-[³H]Man, both GlcNAc-(2-*O*-octyl)PI and GlcN-(2-*O*-octyl)PI are mannosylated to form predominantly Man₂GlcN-(2-*O*-octyl)PI (Figure 3A, lanes 4 and 5; Table I). The de-*N*-acetylation of GlcNAc-(2-*O*-octyl)PI prior to mannosylation was demonstrated by the sensitivity of the Man₂GlcN-(2-*O*-octyl)PI product to nitrous acid deamination (Sharma *et al.*, 1999). The presence of the 2-*O*-octyl group appears to abrogate the effects of substrate channelling; thus, the more efficient processing of GlcNAc-PI versus GlcN-PI (Figure 3A, lanes 2 and 3) is not reflected in the processing of GlcNAc-(2-*O*-octyl)PI versus GlcN-(2-*O*-octyl)PI (Figure 3A, lanes 4 and 5). The de-*N*-acetylation of GlcNAc-(2-*O*-octyl)PI is slower than de-*N*-acetylation of GlcNAc-PI (Sharma *et al.*, 1999) and it is possible that this compensates for subsequent channelling between the de-*N*-acetylase and MT-I. The accumulation of Man₂GlcN-(2-*O*-octyl)PI also suggests that the third mannosyltransferase of the trypanosomal GPI recognizes this analogue as a substrate poorly.

Here we show that the presence of GlcNAc-(2-*O*-octyl)PI or GlcN-(2-*O*-octyl)PI in the trypanosomal cell-free system alters the processing of exogenous synthetic GlcN-PI (Figure 3A, compare lane 2 with lanes 6, 7 and 10) and GlcNAc-PI (Figure 3A, compare lane 3 with lanes 8 and 9) in two ways. First, GlcNAc-(2-*O*-octyl)PI and GlcN-(2-*O*-octyl)PI cause a significant reduction in the incorporation of radiolabel into the GPI glycolipid fraction

Fig. 3. Inhibition of inositol acylation in the trypanosomal cell-free system by GlcN-(2-*O*-octyl)PI and GlcNAc-(2-*O*-octyl)PI. (A) The trypanosomal cell-free system was incubated with GDP-[³H]Man alone (lane 1) or in the presence of GlcN-PI or GlcNAc-PI after pre-incubation with nothing (lanes 2 and 3) or with GlcN-(2-*O*-octyl)PI or GlcNAc-(2-*O*-octyl)PI at the concentrations indicated (lanes 4–10). The results with GDP-[³H]Man and 10 μ M GlcN-(2-*O*-octyl)PI or GlcNAc-(2-*O*-octyl)PI alone are shown in lanes 4 and 5. The characterization of the major products is given in Table I. M2-oct is Man₂GlcN-(2-*O*-octyl)PI. (B) The trypanosomal cell-free system was incubated with GDP-[³H]Man in the presence of 35 μ M GlcN-PI or 10 μ M GlcNAc-PI after pre-incubation with nothing (lanes 1 and 7) or with increasing concentrations of GlcN-(2-*O*-octyl) (lanes 2–6 and 8–12).

(more apparent in Figure 3B where a lower fluorograph exposure was used), suggesting that both compounds, or their metabolites, partially inhibit MT-I. Secondly, the products that are formed from GlcNAc-PI and GlcN-PI are exclusively PI-PLC- and *JBAM*-sensitive Man₁₋₃GlcN-PI species (see Table I for details of the structural characterizations of these bands). These results show that GlcNAc-(2-*O*-octyl)PI and GlcN-(2-*O*-octyl)PI, or their metabolites, inhibit inositol acylation in the trypanosomal cell-free system, thereby preventing the formation of the otherwise abundant Man₃GlcN(acyl)PI intermediate (Figure 3A, lanes 2 and 3) and the formation of glycolipid A' from GlcNAc-PI (Figure 3B, lane 3). The latter effect is consistent with the known requirement for inositol acylation of ethanolamine phosphate addition in the trypanosomal GPI pathway (Güther and Ferguson, 1995; Smith *et al.*, 1997b). The inhibition of inositol acylation was independent of the concentration of exogenous GlcN-PI (10 or 100 μ M) used to prime the pathway (Figure 3A, lanes 6 and 10).

The effects of 0.5–8.0 μ M GlcN-(2-*O*-octyl)PI on the processing of exogenous GlcN-PI and exogenous GlcNAc-PI were assessed (Figure 3B). In this experiment, we compensated for the greater efficiency of GlcNAc-PI processing relative to that for GlcN-PI (Smith *et al.*, 1996) by using 35 μ M GlcN-PI and 10 μ M GlcNAc-PI to prime the pathway (Figure 3B, lanes 1 and 7). In both cases, complete inhibition of inositol acylation was observed between 1 and 2 μ M GlcN-(2-*O*-octyl)PI (Figure 3B, lanes 2–6 and 8–12).

Table 1. Characterization of the radiolabelled glycolipids formed in the trypanosomal cell-free system in the absence and presence of GlcN-(2-*O*-octyl)PI

	Treatment						Glycan size ^d	Assignment
	JBAM	PI-PLC	GPI-PLD	HONO	APAM	BASE		
DPM ^{a,b}	—	—	—	—	—	—	—	Dol-P-Man
M ₁ ^{a,b}	+	+	+	+	—	+	n.d.	Man ₁ GlcN-PI
aM ₃ ^a	+	—	+	+	+ ^c	+	4.2	Man ₃ GlcN(acyl)PI
M ₂ ^{a,b}	+	+	+	+	—	+	n.d.	Man ₂ GlcN-PI
M ₃ ^{a,b}	+	+	+	+	+ ^c	+	4.1	Man ₃ GlcN-PI
A' ^a	—	+	+	+	—	+	4.2	EtN-P-Man ₃ GlcN-PI
M ₂ -oct ^b	+	—	+	+	—	+	3.3	Man ₂ GlcN(2- <i>O</i> -octyl)PI

^{a,b}Samples similar to those in Figure 3, lane 3 ^a(containing bands M₁, aM₃, M₂, M₃ and A') and Figure 3, lane 6–10 ^b(containing bands M₂-oct, M₁, M₂ and M₃) were treated as indicated and the products that partitioned into butan-1-ol were analysed by HPTLC.

^c '+' indicates a positive digestion, i.e. loss (or shift) of the radiolabelled band; '—' indicates a negative digestion, i.e. no loss of the radiolabelled band.

^dThe sizes of the neutral glycans terminating in 2,5-anhydromannitol (AHM) were determined by BioGel P4 chromatography and are expressed in glucose units. The published values for authentic Man α 1–6Man α 1–4AHM and Man α 1–2Man α 1–6Man α 1–4AHM are 3.2 and 4.2 glucose units, respectively (Ferguson, 1994). n.d., not determined.

The inhibitory effects of GlcN-(2-*O*-octyl)PI and GlcN-(2-*O*-hexadecyl)PI are not dependent on detergent

The trypanosomal and HeLa cell-free systems differ in that subcritical micellar concentrations of *n*-octyl β -D-glucopyranoside (nOG) detergent (10 mM nOG) are used in the trypanosomal system, where it stimulates the GPI pathway (Smith *et al.*, 1996), whereas nOG is not used in the HeLa system, where it inhibits the GPI pathway. While it is clear that the absence of detergent in the HeLa system does not prevent the processing of exogenous synthetic GlcN-PI and GlcNAc-PI to H2 and H5 (Sharma *et al.*, 1997, 1999; Smith *et al.*, 1997b; Figure 1B, lanes 2 and 3; Figure 2B, lanes 2–6), it could be argued that the more hydrophobic nature of GlcN-(2-*O*-octyl)PI and GlcN-(2-*O*-hexadecyl)PI might render them insoluble in the absence of nOG and, therefore, inactive in the HeLa system. Since we cannot put 10 mM nOG into the HeLa system, we addressed this by examining the effects of GlcN-(2-*O*-octyl)PI and GlcN-(2-*O*-hexadecyl)PI on the trypanosomal system in the absence of nOG.

The addition of exogenous synthetic GlcN-PI to the trypanosomal system in the absence of nOG produced the usual range of GPI intermediates (Figure 4A, lane 1) and the addition of GlcN-(2-*O*-octyl)PI produced one major product as before (Figure 4A, lanes 2 and 3), presumably Man₂GlcN-(2-*O*-octyl)PI. The addition of both compounds produced a pattern of bands (Figure 4A, lanes 4 and 5) similar to those seen in the presence of nOG (Figure 3A, lane 6; Figure 3B, lane 6). These data show that GlcN-(2-*O*-octyl)PI has similar effects on the trypanosomal system whether or not nOG is present. Similarly, the production of GPI intermediates from exogenous synthetic GlcN-PI (Figure 4B, lane 1) was inhibited by GlcN-(2-*O*-hexadecyl)PI in the trypanosomal system in the absence of nOG (Figure 4B, lane 2).

These results show that the inhibitory activities of GlcN-(2-*O*-octyl)PI and GlcN-(2-*O*-hexadecyl)PI are not dependent on the presence of detergent.

Discussion

The data reported here and elsewhere (Smith *et al.*, 1997b; Sharma *et al.*, 1999) are summarized in Figure 5. The

Fig. 4. The inhibitory effects of GlcN-(2-*O*-octyl)PI and GlcN-(2-*O*-hexadecyl)PI are not dependent on detergent. (A) The trypanosomal cell-free system (without nOG) was incubated with GDP-[³H]Man in the presence of 10 μM GlcNAc-PI (lane 1) or 10 μM GlcN-(2-*O*-octyl)PI (lanes 2 and 3). The cell-free system was also pre-incubated with 10 μM GlcN-(2-*O*-octyl)PI prior to the addition of 10 μM GlcNAc-PI (lanes 4 and 5). The presence of GlcN-(2-*O*-octyl)PI reduced the mannosylation of GlcN-PI and prevented the formation of Man₃GlcN(acyl)PI (aM3). (B) The trypanosomal cell-free system (without nOG) was incubated with GDP-[³H]Man in the presence of 10 μM GlcN-PI (lane 1) or 10 μM GlcN-(2-*O*-hexadecyl)PI (lane 3). The cell-free system was also pre-incubated with 10 μM GlcN-(2-*O*-hexadecyl)PI prior to the addition of 10 μM GlcN-PI (lanes 2). The presence of GlcN-(2-*O*-hexadecyl)PI prevented the mannosylation of GlcN-PI.

data presented in this paper support the following conclusions: (i) neither trypanosomal nor HeLa MT-I can act on GlcN-(2-*O*-hexadecyl)PI; (ii) GlcN-(2-*O*-hexadecyl)PI is an inhibitor of trypanosomal MT-I but not of HeLa MT-I; (iii) GlcN-(2-*O*-octyl)PI and GlcNAc-(2-*O*-octyl)PI are neither substrates for nor inhibitors of any of the early GPI biosynthetic enzymes in the HeLa cell-free system, whereas they, or their metabolites, cause partial inhibition

Fig. 5. Summary of the differences between mannosylation and inositol acylation in *T.brucei* and HeLa cells. The top section shows how the *T.brucei* and HeLa GPI biosynthetic pathways diverge after GlcN-PI and converge again at Man₁GlcN-(acyl)PI (Güther and Ferguson, 1995; Doerrler *et al.*, 1996; Smith *et al.*, 1997b). The tables summarize data from this paper and from Smith *et al.* (1997b) and Sharma *et al.* (1999), and show the abilities of synthetic GlcN-PI and the three 2-*O*-alkyl derivatives to be processed by the *T.brucei* and HeLa cell-free systems. The orders of MT-I and inositol acyltransferase in the tables reflect their relative orders in the two pathways. The compounds (or their products) are either substrates (S), inhibitors (I) or neither substrates nor inhibitors (-), as indicated. *For the de-*N*-acetylase data, the compounds were presented as their *N*-acetyl derivatives. ^aThe GlcN-(2-*O*-methyl)PI derivative is a weak inhibitor of the *T.brucei* inositol acyltransferase (T.K.Smith and M.A.J.Ferguson, unpublished data). n.d., not determined.

of trypanosomal MT-I and complete inhibition of trypanosomal inositol acylation but not of HeLa inositol acylation.

With respect to (i), the inability of trypanosomal MT-I to act on GlcN-(2-*O*-hexadecyl)PI is not surprising since although GlcN-(2-*O*-methyl)PI is a good substrate (Smith *et al.*, 1997b), GlcN-(2-*O*-octyl)PI is a relatively poor substrate (Sharma *et al.*, 1999). Thus, increasing the size of the 2-*O*-alkyl chain still further might be expected to prevent substrate recognition. On the other hand, GlcN-(2-*O*-hexadecyl)PI is a close analogue of GlcN-(2-*O*-hexadecanoyl)PI, the natural substrate for HeLa MT-I, and might be expected to be a good substrate for this enzyme. This is clearly not the case and the results suggest that the carbonyl group of the natural 2-*O*-hexadecanoyl ester,

which is absent in the 2-*O*-hexadecyl ether, is essential for substrate recognition by HeLa MT-I.

With respect to (ii), the apparent substrate channelling observed between the GlcNAc-PI de-*N*-acetylase and MT-I in the trypanosomal cell-free system (Smith *et al.*, 1996, 1997b; Sharma *et al.*, 1997) seems to circumvent inhibition of MT-I by GlcN-(2-*O*-hexadecyl)PI when GlcNAc-PI is present. The lack of inhibition by GlcNAc-(2-*O*-hexadecyl)PI suggests that the free amino group of the GlcN residue is important for inhibitory activity, just as it is for recognition of the natural acceptor substrate GlcN-PI (Smith *et al.*, 1996). The mechanism of inhibition of the trypanosomal MT-I by GlcN-(2-*O*-hexadecyl)PI is unknown. Nevertheless, a prediction (Smith *et al.*, 1997b)

that parasite-specific MT-I inhibitors should be attainable, based on the discovery of a selective substrate for the parasite MT-I, appears to be true. The inhibitory effects of GlcN-(2-*O*-hexadecyl)PI become apparent between 0.5 and 1 μ M (Figure 2A, lanes 3 and 4) but it is difficult to produce a meaningful IC₅₀ value with membrane-bound enzyme complexes and lipidic substrates and inhibitors. The possibility that the inhibition by GlcN-(2-*O*-hexadecyl)PI was due to non-specific effects of this compound on membrane structure can be excluded because: (i) the closely related compound GlcNAc-(2-*O*-hexadecyl)PI was inactive in the trypanosomal cell-free system; (ii) GlcN-(2-*O*-hexadecyl)PI was inactive in HeLa membranes at 100 μ M; and (iii) additional experiments in the trypanosomal cell-free system showed that GlcN-(2-*O*-hexadecyl)PI did not inhibit the mannosylation of GlcN-PI produced *in situ* by the de-N-acetylation of exogenous GlcNAc-PI (data not shown).

With respect to (iii), the partial inhibition of MT-I by GlcN-(2-*O*-octyl)PI and GlcNAc-(2-*O*-octyl)PI is similar to the effects seen with (the more potent) GlcN-(2-*O*-hexadecyl)PI analogue. Presumably, GlcNAc-(2-octyl)PI partially inhibits MT-I because it can be converted to GlcN-(2-*O*-octyl)PI. The potent inhibition of the trypanosomal, but not the HeLa, inositol acyltransferase by GlcN-(2-*O*-octyl)PI and GlcNAc-(2-*O*-octyl)PI, or their metabolites, is more significant. Differences between inositol acylation in trypanosomal and mammalian cells have been documented. For example, mammalian inositol acyltransferase(s) are coenzyme A (CoA) (Stevens and Zhang, 1994) and/or acyl-CoA (Doerrler *et al.*, 1996) dependent, whereas the washed, trypanosomal, cell-free system does not require added cofactors for inositol acylation. Furthermore, the trypanosomal inositol acyltransferase is inhibited by phenylmethylsulfonyl fluoride, whereas the mammalian activity is not (Güther *et al.*, 1994). These differences are not surprising considering the differences in timing and function of inositol acylation in the trypanosomal and mammalian GPI pathways (Güther and Ferguson, 1995; Chen *et al.*, 1998). Inositol acylation in trypanosomes occurs only after the formation of Man₁GlcN-PI and is essential for efficient ethanolamine phosphate addition to Man₃GlcN-(acyl)PI (Güther and Ferguson, 1995; Smith *et al.*, 1997b) (see Figure 3 and Table I), whereas in mammalian cells it occurs at the level of GlcN-PI and is a prerequisite for subsequent mannosylation (Doerrler *et al.*, 1996; Smith *et al.*, 1997b). The importance of inositol acylation in the trypanosomal GPI pathway, and the selective inhibition of this activity with GlcN-(2-*O*-octyl)PI and GlcNAc-(2-*O*-octyl)PI, suggest trypanosomal inositol acyltransferase as a potential target for the development of trypanosome-specific therapeutic agents.

In summary, we have produced three synthetic GlcN-PI analogues that display parasite-specific inhibition of the GPI biosynthetic pathway *in vitro*. These are GlcN-(2-*O*-hexadecyl)PI, which inhibits MT-I, and GlcN-(2-*O*-octyl)PI and GlcNAc-(2-*O*-octyl)PI, which inhibit inositol acyltransferase. These results confirm the prediction made by us, and by others, that pathogen-specific inhibitors of GPI biosynthesis should be attainable. Future work will involve the design and synthesis of cell-permeable molecules that retain these inhibitory properties for testing *in vivo*.

Materials and methods

Materials

GDP-[2-³H] mannose (14.9–17.8 Ci/mmol) and En³Hance™ were from NEN. JBAM and GPI-PLD were from Boehringer Mannheim, *Bacillus thuringiensis* PI-PLC and *Aspergillus phoenicis* (Man α 1–2Man-specific) α -mannosidase (APAM) were from Oxford GlycoSystems. nOG was from Calbiochem. Ion-exchange resins (AG-50X12 and AG-3X4) were from Bio-Rad. All the other reagents were purchased from Merck-BDH or Sigma.

Substrates and substrate analogues

D-GlcNAc1–6D-*myo*-inositol-1-HPO₄-3-sn-1,2-dipalmitoylglycerol (GlcN-PI) was synthesized according to Cottaz *et al.* (1993). GlcN-(2-*O*-methyl)PI was prepared according to Crossman *et al.* (1997). GlcN-(2-*O*-octyl)PI and GlcN-(2-*O*-hexadecyl)PI were made in a similar manner. Compounds were N-acetylated as described in Smith *et al.* (1996), and the concentration and purity of all of the synthetic substrates were checked by *myo*-inositol content by gas chromatography–mass spectrometry and negative-ion electrospray mass spectrometry, respectively (Smith *et al.*, 1996).

Preparation of trypanosome and HeLa membranes

Bloodstream forms of *T.brucei* (variant MITat.1.4) were isolated and membranes (cell-free system) prepared as described previously (Masterson *et al.*, 1989; Smith *et al.*, 1996). HeLa cells were grown and membranes prepared as described previously (Smith *et al.*, 1997b).

Trypanosome and HeLa cell-free system assays

Trypanosome membranes were washed twice and suspended at 5×10^8 cell equivalents/ml in 2 $\times$ concentrated incorporation buffer supplemented with *N*-ethylmaleimide and nOG (Smith *et al.*, 1996, 1997b). The lysate was sonicated briefly and added to dry GDP-[³H]Man (0.3 μ Ci/10⁷ cell equivalents). After brief sonication on ice–water, aliquots of 20 μ l (2.5 $\times 10^7$ cell equivalents; 47 μ g total protein, 0.34 μ g phospholipid phosphorus) were added to reaction tubes containing an equal volume of solutions of the various GlcN-PI analogues in 10 mM nOG and incubated at 30°C for 1 h. The glycolipid products were recovered and analysed by HPTLC before and after enzymatic and chemical treatments, as described previously (Smith *et al.*, 1996). HeLa cell lysate was thawed and supplemented as described previously (Smith *et al.*, 1997b). Aliquots of 100 μ l (10⁶ cell equivalents; 88 μ g total protein, 0.33 μ g phospholipid phosphorus) were added to tubes containing dry GDP-[³H]Man (1.0 μ Ci) and the synthetic GlcN-PI analogues. Samples were incubated at 35°C for 1.5 h and glycolipids were extracted and processed as described above. In both cases, inhibition studies were performed in exactly the same way except that the membranes were pre-incubated with the potential inhibitors for 5 min prior to being added to GlcN-PI or GlcNAc-PI.

HPTLC

Samples and glycolipid standards were applied to 10 cm aluminium-backed silica gel 60 HPTLC plates that were developed with chloroform:methanol:1 M ammonium acetate:13 M ammonium hydroxide:water (180:140:9:9:23, v/v). Radiolabelled components were detected by fluorography at –70°C using Kodak XAR-5 film and an intensifying screen after spraying the plates with En³Hance™.

Enzyme and chemical treatments of radiolabelled glycolipids

Enzyme digestions with APAM, JBAM, PI-PLC and GPI-PLD, and base hydrolysis, deamination and N-acetylation were performed as described in Smith *et al.* (1996, 1997b).

Glycan headgroup analysis

Neutral glycan headgroups were obtained from the radiolabelled glycolipids produced in the trypanosome assay as described in Smith *et al.* (1996). Briefly, labelled glycolipids were purified by preparative HPTLC, deacylated, deaminated, reduced, dephosphorylated with aqueous HF and desalted by passage through AG50X12(H⁺) over AG3X4 (OH[–]). The resulting neutral glycans were analysed by Bio-Gel P4 gel filtration.

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