Parasite-Specific Inhibition of the Glycosylphosphatidylinositol Biosynthetic Pathway by Stereoisomeric Substrate Analogues †

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Parasite-Specific Inhibition of the Glycosylphosphatidylinositol Biosynthetic Pathway by Stereoisomeric Substrate Analogues†

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ABSTRACT: The natural substrate for the first α-D-mannosyltransferase of glycosylphosphatidylinositol biosynthesis in the protozoan parasite *Trypanosoma brucei* is D-GlcNα1−6-D-*myo*-inositol-1-*P*-*sn*-1,2-diacylglycerol. Here we show that a diastereoisomer, D-GlcNα1−6-L-*myo*-inositol-1-*P*-*sn*-1,2-diacylglycerol, is an inhibitor of this enzyme in a trypanosomal cell-free system. Tests with other L-*myo*-inositol-containing compounds revealed that L-*myo*-inositol-1-phosphate is the principal inhibitory component and that methylation of the 2-OH group of the L-*myo*-inositol residue abolishes any inhibition. Comparisons between the natural substrate and the inhibitors suggested that the inhibitors bind to the first α-D-mannosyltransferase by means of charge interactions with the 1-phosphate group and/or hydrogen bonds involving the 3-, 4-, and 5-OH groups of the L-*myo*-inositol residue, which are predicted to occupy orientations identical to those of the 1-phosphate and 5-, 4-, and 3-OH groups, respectively, of the D-*myo*-inositol residue of the natural substrate. However, additional experiments indicated that the 4-OH group of the D-*myo*-inositol residue is unlikely to be involved in substrate recognition. None of the L-*myo*-inositol-containing compounds that inhibited glycosylphosphatidylinositol (GPI) biosynthesis in a parasite cell-free system had any effect on GPI biosynthesis in a comparable human (HeLa) cell-free system, suggesting that other related parasite-specific inhibitors of this essential pathway might be developed.

A significant proportion of eukaryotic cell-surface glycoproteins are attached to the plasma membrane by covalent linkage to a glycosylphosphatidylinositol (GPI)[1] membrane anchor. The structure, function, and biosynthesis of GPI membrane anchors and related molecules have been extensively reviewed (*1*−*7*). The basic GPI core structure attached to protein comprises $NH_2CH_2CH_2PO_4H$-6Manα1−2Manα1−6Manα1−4GlcNα1−6 D-*myo*-inositol-1-HPO_4-lipid (EtNP-Man_3GlcN-PI), where the lipid can 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 (*1*, *7*).

Protozoa tend to express significantly higher densities of cell-surface GPI-anchored proteins than do higher eukaryotes (*8*). For example, *Trypanosoma brucei*, which is transmitted by the tsetse fly and which is the causative agent of African sleeping sickness, expresses a dense cell-surface coat consisting of approximately 5×10^6 dimers of a GPI-anchored variant surface glycoprotein (VSG). This protects the parasite from the alternative complement pathway of the host and, through antigenic variation, from specific immune responses (*9*, *10*).

A variety of GPI-related structures, such as lipophosphoglycans (LPGs), glycoinositolphospholipids (GIPLs), and mucin-like structures, are expressed by other trypanosomatid parasites, including *Leishmania*, *Trypanosoma cruzi*, *Herpetomonas*, *Leptomonas*, and *Phytomonas* (*1*, *11*−*15*, and references therein). Nontrypanosomatid protozoan parasites, such as *Plasmodium* (*16*, *17*), *Toxoplasma gondii* (*18*, *19*), *Trichomonas* (*20*), and *Entamoeba* (*21*), also have abundant GPI-anchored glycoproteins and/or GIPLs. Inhibitors able to arrest the formation of GPI-anchored proteins and/or GPI-related molecules on the plasma membrane of parasitic protozoa should prove useful in the development of antiparasitic agents.

The sequence of events underlying GPI biosynthesis has been studied in *T. brucei* (*22*−*26*), *T. cruzi* (*27*), *To. gondii* (*28*, *29*), *Plasmodium falciparum* (*30*, *31*), *Saccharomyces cerevisiae* (*32*, *33*), and mammalian cells (*34*−*37*, and references therein). Several features of the biosynthesis of *Leishmania* GIPLs and LPG have also been described (*38*−*40*). In all cases, GPI biosynthesis involves the addition of

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[1] Abbreviations: GPI, glycosylphosphatidylinositol; GlcN-PI, D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcN-[L]-PI, D-GlcNα1−6-L-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcN-[L]-(2-*O*-methyl)-PI, D-GlcNα1−6-L-(2-*O*-methyl)-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol; GlcN-Ino-*P*-Gro, D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-glycerol; GlcN-[L]-Ino-*P*-Gro, D-GlcNα1−6-L-*myo*-inositol-1-HPO_4-glycerol; Ino-*P*-dipalmitoyl-Gro, D-*myo*-inositol-1-PO_4-*sn*-1,2-dipalmitoylglycerol; [L]-Ino-*P*-dipalmitoyl-Gro, L-*myo*-inositol-1-PO_4-*sn*-1,2-dipalmitoylglycerol; Ino-*P*-Gro, D-*myo*-inositol-1-HPO_4-glycerol; [L]-Ino-*P*-Gro, L-*myo*-inositol-1-HPO_4-glycerol; GlcN-Ino, D-GlcNα1−6-D-*myo*-inositol; GlcN-[L]-Ino, D-GlcNα1−6-L-*myo*-inositol; GlcN-[L]-(2-*O*-methyl)-Ino, D-GlcNα1−6-L-(2-*O*-methyl)-*myo*-inositol; GlcN-[L]-(2-deoxy)-Ino, D-GlcNα1−6-L-2-deoxy-*myo*-inositol; Ino-*P*, D-*myo*-inositol-1-phosphate; [L]-Ino-*P*, L-*myo*-inositol-1-phosphate.

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GlcNAc to phosphatidylinositol (PI) to give GlcNAc-PI, which is then de-N-acetylated to form GlcN-PI (*41*−*45*). De-N-acetylation is a prerequisite for the mannosylation of GlcN-PI to form later GPI intermediates (*46*, *48*, *49*). The GlcNAc-PI de-N-acetylases from protozoan and mammalian sources are similar with regard to their specificities for the acyl R group removed from GlcNR-PI substrates (*49*) but differ with regard to their specificity for the *myo*-inositol residue. Thus, the trypanosomal enzyme can de-N-acetylate GlcNAc-PI containing either D- or L-*myo*-inositol, whereas the human (HeLa) enzyme requires D-*myo*-inositol (*50*).

Notable differences between the *T. brucei* and mammalian GPI biosynthetic pathways occur from GlcN-PI onward, including the timing of inositol acylation and deacylation (*26*), the addition of extra ethanolamine phosphate groups to mammalian GPI anchors (*34*, *35*, *51*), and fatty acid remodeling of *T. brucei* GPI anchors (*23*). The processing of GlcN-PI is a key step in the GPI biosynthetic pathway; inositol acylation (the transfer of fatty acid to the 2-OH group of the D-*myo*-inositol residue) of GlcN-PI either precedes or follows the first mannosylation, as in mammalian cells (*48*, *52*) and *T. brucei* (*26*, *48*, *52*), respectively. This difference was exploited in the discovery of the first generation of specific inhibitors of the parasite GPI biosynthetic pathway in vitro (*53*). Neither GlcN-PI analogues with the 2-OH group of the D-*myo*-inositol residue substituted with a methyl [GlcN-(2-*O*-methyl)-PI], octyl [GlcN-(2-*O*-octyl)-PI], or hexadecyl [GlcN-(2-*O*-hexadecyl)-PI] group nor the corresponding *N*-acetyl derivatives were substrates or inhibitors of the GPI biosynthetic enzymes in a HeLa cell-free system (*54*, *55*). However, a number of these 2-O-alkylated analogues were potent inhibitors of GPI biosynthesis in a *T. brucei* cell-free system; GlcN-(2-*O*-hexadecyl)-PI was shown to inhibit the first mannosyltransferase (MT-1), whereas GlcN-(2-*O*-octyl)-PI and its N-acetylated version inhibited inositol acylation of Man_{1-3}GlcN-PI and prevented the subsequent addition of an ethanolamine phosphate bridge (*53*).

Here, we describe another series of parasite-specific GPI pathway inhibitors that contain L-*myo*-inositol and discuss the mechanism by which they may inhibit *T. brucei* MT-1.

EXPERIMENTAL PROCEDURES

Materials. GDP-[2-^{3}H]mannose (14.9−17.8 Ci/mmol) and En^{3}Hance were purchased from NEN; jack bean α-mannosidase (JBAM) and glycosylphosphatidylinositol-specific phospholipase D (GPI−PLD) were from Boehringer Mannheim, and *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) was from Oxford Glycosystems. All other reagents were purchased from Merck-BDH or Sigma.

Substrates and Substrate Analogues. D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol (GlcN-PI) and D-GlcNα1−6-L-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol (GlcN-[L]-PI) were synthesized as previously described (*54*). D-GlcNα1−6-L-(2-*O*-methyl)-*myo*-inositol-1-HPO_4-*sn*-1,2-dipalmitoylglycerol [GlcN-[L]-(2-*O*-methyl)-PI] was prepared in a manner similar to that of the corresponding D-*myo*-inositol analogue (*55*).

D-GlcNα1−6-D-*myo*-inositol-1-HPO_4-glycerol (GlcN-Ino-*P*-Gro) and D-GlcNα1−6-L-*myo*-inositol-1-HPO_4-glycerol (GlcN-[L]-Ino-*P*-Gro) were prepared by basic hydrolysis of GlcN-PI and GlcN-[L]-PI, as already described (*46*).

D-*myo*-Inositol-1-PO_4-*sn*-1,2-dipalmitoylglycerol (Ino-*P*-dipalmitoyl-Gro) and L-*myo*-inositol-1-PO_4-*sn*-1,2-dipalmitoylglycerol ([L]-Ino-*P*-dipalmitoyl-Gro) were obtained by nitrous acid deamination (*46*) of GlcN-PI and GlcN-[L]-PI, respectively. D-*myo*-Inositol-1-HPO_4-glycerol (Ino-*P*-Gro) and L-*myo*-inositol-1-HPO_4-glycerol ([L]-Ino-*P*-Gro) were prepared by treatment of the deaminated compounds with base (*46*).

D-GlcNα1−6-D-*myo*-inositol (GlcN-Ino), D-GlcNα1−6-L-*myo*-inositol (GlcN-[L]-Ino), and D-GlcNα1−6-L-(2-*O*-methyl)-*myo*-inositol [GlcN-[L]-(2-*O*-methyl)-Ino] were prepared by aqueous HF dephosphorylation (*46*) of GlcN-PI, GlcN-[L]-PI, and GlcN-[L]-(2-*O*-methyl)-PI, respectively.

L-*myo*-Inositol-1-phosphate ([L]-Ino-*P*) was prepared, with minor modifications, from [L]-PI as previously described (*47*). Thus, [L]-PI was subjected to alkaline hydrolysis (2 M KOH at 100 °C for 1 h), and the products were partitioned between water and butan-1-ol. The aqueous phase was neutralized with Dowex AG50(H^+) and concentrated to dryness. The residue was subjected to descending paper chromatography using Whatman No. 1 paper with propan-2-ol, concentrated ammonia, and water (70:10:20, v/v/v) as the solvent for 36 h at 37 °C. The positions of L-*myo*-inositol-1-phosphate and *myo*-inositol-2-phosphate were identified by cochromatography with an adjacent sample of [^{3}H]-D-*myo*-inositol-1-phosphate and [^{3}H]-*myo*-inositol-2-phosphate generated from yeast PI labeled with [^{3}H]-D-*myo*-inositol. [L]-Ino-*P* was eluted from the dried paper chromatogram with 100 mM ammonia; the eluent was concentrated, and the residue was dissolved in water to provide a stock solution.

D-GlcNα1−6-L-2-deoxy-*myo*-inositol [GlcN-[L]-(2-deoxy)-Ino] was kindly donated by K. M. Wilmot (University of Georgia, Athens, GA).

N-Acetyl derivatives of all of the compounds described above were prepared as previously described (*46*).

The purity of each of the synthetic substrates was assessed by negative-ion electrospray mass spectrometry, and the concentration of each stock solution was ascertained by measuring the inositol content by selected ion-monitoring gas chromatography−mass spectrometry (*46*).

Preparation of Trypanosomal and HeLa Membranes. Bloodstream forms of *T. brucei* (variant MITat.1.4) were isolated and membranes (cell-free system) prepared as previously described (*22*, *46*). HeLa cells were grown and membranes prepared as previously described (*48*).

Assays of the Trypanosomal and HeLa Cell-Free Systems. Trypanosome membranes were washed twice and suspended at a density of 5×10^8 cell equivalents/mL in 2-fold concentrated incorporation buffer supplemented with *N*-ethylmalemide and *n*-octyl β-D-glucopyranoside (*46*, *48*). The lysate was briefly sonicated and added to dry GDP-[^{3}H]Man (0.3 μCi per 10^7 cell equivalents). After brief sonication in ice/water, aliquots of 20 μL (2.5×10^7 cell equivalents) were added to reaction tubes containing an equal volume of the various GlcN-PI analogues in 10 mM *n*-octyl β-D-glucopyranoside and therafter incubated at 30 °C for 1 h. The glycolipid products were recovered and analyzed by HPTLC, before and after enzymatic and chemical treatments.

HeLa cell lysate was thawed and supplemented as described previously (*48*). Aliquots of 100 μL (10^6 cell

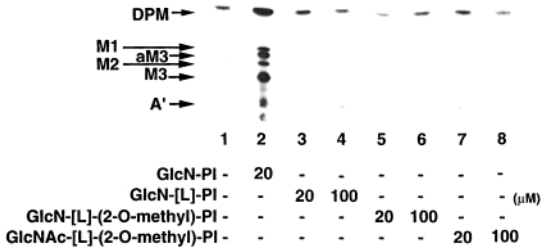

FIGURE 1: Neither GlcN-[L]-PI nor GlcN-[L]-(2-*O*-methyl)-PI is a substrate for GPI biosynthesis in the trypanosomal cell-free system. The trypanosomal cell-free system was incubated with GDP-[^{3}H]-Man and NEM alone (lane 1) or together with GlcN-PI (lane 2), GlcN-[L]-PI (lanes 3 and 4), GlcN-[L]-(2-*O*-methyl)-PI (lanes 5 and 6), or GlcNAc-[L]-(2-*O*-methyl)-PI (lanes 7 and 8) at the indicated concentrations. The radiolabeled glycolipid products were analyzed by HPTLC and fluorography. The products are as follows: DPM, dolicholphosphate mannose; M1−3, Man_{1-3}GlcN-PI; aM3, Man_3GlcN-(acyl)PI; and A′, EtNP-Man_3GlcN-PI.

equivalents) were added to tubes containing dry GDP-[^{3}H]Man (1.0 μCi) and the synthetic GlcN-PI analogues. Samples were incubated at 35 °C for 1.5 h, whereafter the glycolipids were extracted and processed as described above.

In both cases, inhibition studies were conducted in exactly the same way except that the membranes were preincubated with potential inhibitors for 5 min prior to being added to GlcN-PI.

HPTLC. Samples and glycolipid standards were applied to 10 cm aluminum-backed silica gel 60 HPTLC plates which were developed with chloroform, methanol, 1 M ammonium acetate, 13 M ammonium hydroxide, and water (180:140:9:9:23, v/v). Radiolabeled components were detected by fluorography at −70 °C after spraying the plates with En^{3}Hance and using Kodak XAR-5 film with an intensifying screen.

Enzymatic and Chemical Treatments of Radiolabeled Glycolipids. Digestions with JBAM, PI-PLC, and GPI-PLD and basic hydrolysis, deamination, and N-acetylation were performed as previously described (*46*, *48*).

Glycan Headgroup Analysis. Neutral glycan headgroups were obtained from radiolabeled glycolipids or soluble radiolabeled compounds produced in the trypanosomal assay as previously described (*46*). Briefly, labeled glycolipids were purified by preparative HPTLC, deacylated, deaminated, reduced, dephosphorylated with aqueous HF, and desalted by passage through AG50X12 (H^+) over AG3X4 (OH^-). Soluble radiolabeled compounds were treated in the same way except that the deacylation step was omitted. The resulting neutral glycans were mixed with an internal standard of Gal_3Man_3[^{3}H]AHM and analyzed by Bio-Gel P4 gel filtration. The amount of radioactivity eluting as Man_{1-3}AHM was measured.

RESULTS

GlcN-[L]-PI Is an Inhibitor of GPI Biosynthesis in the Trypanosomal Cell-Free System. The trypanosomal cell-free system was incubated with GDP-[^{3}H]Man in the presence of *N*-ethylmaleimide (NEM). NEM inhibits UDP-GlcNAc:PI α1−6 GlcNAc-transferase (without affecting the downstream enzymes) (*57*), thereby preventing the labeling of endogenous GPI intermediates (Figure 1, lane 1) and simplifying the interpretation of the effects resulting from

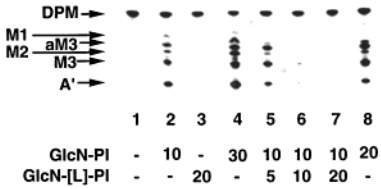

FIGURE 2: GlcN-[L]-PI is an inhibitor of GPI biosynthesis in the trypanosomal cell-free system. The trypanosomal cell-free system was incubated with GDP-[^{3}H]-Man and NEM alone (lane 1) or together with GlcN-PI (lanes 2, 4, and 8) or GlcN-[L]-PI (lane 3) or with GlcN-PI after preincubation with GlcN-[L]-PI (lanes 5−7) at the indicated concentrations. The products are as described in the legend of Figure 1.

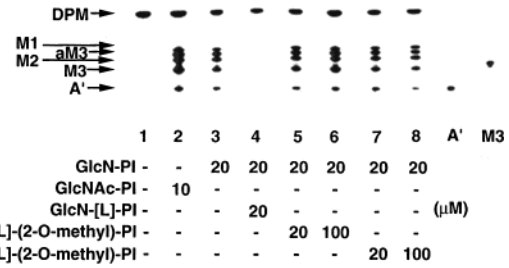

FIGURE 3: GlcN-[L]-(2-*O*-methyl)-PI is not an inhibitor of GPI biosynthesis in the trypanosomal cell-free system. The trypanosomal cell-free system was incubated with GDP-[^{3}H]-Man and NEM alone (lane 1) or together with GlcNAc-PI (lane 2) or GlcN-PI (lane 3) or with GlcN-PI after preincubation with GlcN-[L]-PI (lane 4), GlcN-[L]-(2-*O*-methyl)-PI (lanes 5 and 6), or GlcNAc-[L]-(2-*O*-methyl)-PI (lanes 7 and 8) at the indicated concentrations. The products are as described in the legend of Figure 1. Authentic M3 and A′ markers are shown on the right of the fluorograph.

the addition of the synthetic substrates and substrate analogues (*46*).

The addition of 20 μM synthetic GlcN-PI primed the production of GPI intermediates up to and including glycolipid A′ (Figure 1, lane 2), as previously reported (*46*, *48*−*50*). At the same or higher (100 μM) concentrations, GlcN-[L]-PI, GlcN-[L]-(2-*O*-methyl)-PI, and GlcNAc-[L]-(2-*O*-methyl)-PI did not produce any labeled GPI intermediates (Figure 1, lanes 3−8), indicating that they cannot prime GPI biosynthesis.

When the cell-free system was incubated with 10 or 20 μM GlcN-[L]-PI prior to the addition of 10 μM GlcN-PI, the processing of GlcN-PI was significantly inhibited (Figure 2, compare lane 2 with lanes 6 and 7). The corresponding controls, where GlcN-PI was added instead of GlcN-[L]-PI, exhibited enhanced priming rather than inhibition (Figure 2, lanes 4 and 8), ruling out the possibility that inhibition by GlcN-[L]-PI might be due to nonspecific, detergent-like effects.

The inhibition experiment was repeated using 20 μM GlcN-PI. Once again, 20 μM GlcN-[L]-PI completely inhibited the processing of GlcN-PI (Figure 3, compare lanes 3 and 4), whereas neither GlcN-[L]-(2-*O*-methyl)-PI nor GlcNAc-[L]-(2-*O*-methyl)-PI had any effect on this process at concentrations of 20 or 100 μM (Figure 3, lanes 5−8).

These results show that GlcN-[L]-PI, a diastereoisomer of GlcN-PI, is a potent inhibitor of GPI biosynthesis in the trypanosomal cell-free system and that methylation of the 2-OH group of the L-*myo*-inositol residue abolishes this inhibition.

Dissection of the Inhibitory Activity of GlcN-[L]-PI. Various fragments of GlcN-[L]-PI were prepared in an effort

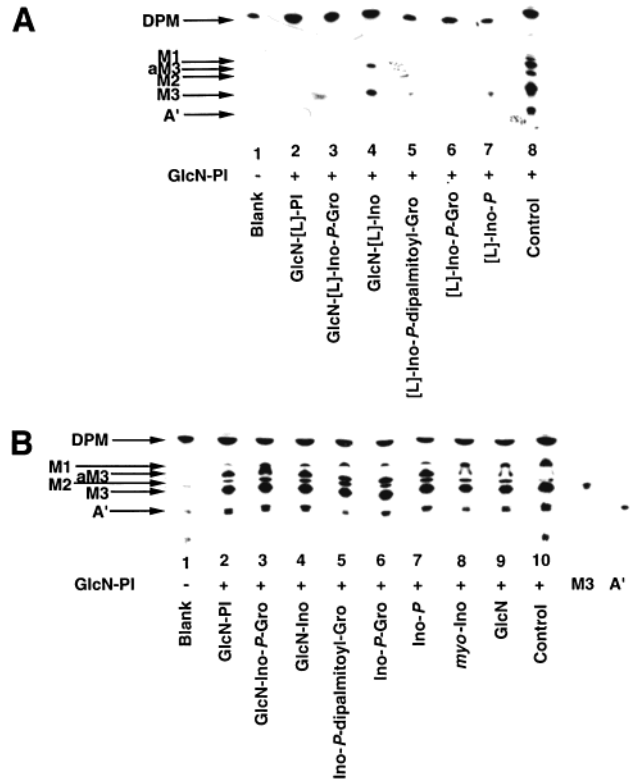

FIGURE 4: GPI biosynthesis in the trypanosomal cell-free system is inhibited by compounds containing L-*myo*-inositol-1-phosphate but not by those containing D-*myo*-inositol-1-phosphate. (A) The trypanosomal cell-free system was incubated with GDP-[^{3}H]-Man and NEM without (−) (Blank, lane 1) or with (+) 20 μM GlcN-PI before (Control, lane 8) or after preincubation with 20 μM GlcN-[L]-PI (lane 2), GlcN-[L]-Ino-*P*-Gro (lane 3), GlcN-[L]-Ino (lane 4), [L]-Ino-*P*-dipalmitoyl-Gro (lane 5), [L]-Ino-*P*-Gro (lane 6), and [L]-Ino-*P* (lane 7). The products are as described in the legend of Figure 1. (B) The trypanosomal cell-free system was incubated with GDP-[^{3}H]-Man and NEM without (−) (Blank, lane 1) or with (+) 20 μM GlcN-PI before (Control, lane 10) or after preincubation with 20 μM GlcN-PI (lane 2), GlcN-Ino-*P*-Gro (lane 3), GlcN-Ino (lane 4), Ino-*P*-dipalmitoyl-Gro (lane 5), Ino-*P*-Gro (lane 6), Ino-*P* (lane 7), *myo*-inositol (lane 8), and D-glucosamine (lane 9). The products are as described in the legend of Figure 1. Authentic M3 and A′ markers are shown on the right of the fluorograph.

to identify those structural features associated with inhibition. As already demonstrated, the processing of GlcN-PI is completely inhibited by 20 μM GlcN-[L]-PI (Figure 4A, compare lanes 2 and 8). Complete inhibition was also observed with each of the following compounds at 20 μM: GlcN-[L]-Ino-*P*-Gro (Figure 4A, lane 3), [L]-PI (Figure 4A, lane 5), [L]-Ino-*P*-Gro (Figure 4A, lane 6), and [L]-Ino-*P* (Figure 4A, lane 7). The only fragment to exhibit less than complete inhibition (approximately 25% inhibition at 20 μM) was GlcN-[L]-Ino (lane 4), but even this compound produced complete inhibition at 1 mM (see below). Significantly, none of the corresponding compounds containing D-*myo*-inositol inhibited the processing of GlcN-PI (Figure 4B, lanes 2−7), nor was any significant inhibition observed with 1 mM *myo*-inositol or D-GlcN (Figure 4B, lanes 8 and 9).[2]

These results suggest that the principal inhibitory component is [L]-*myo*-inositol-1-*P* and that neither the GlcN nor diacylglycerol components of GlcN-[L]-PI are essential for inhibition.

Effects of Diastereoisomeric Substrate Analogues Lacking the Phosphate Group. Although 20 μM GlcN-[L]-Ino only slightly inhibited the processing of GlcN-PI (Figure 4A, lane 4), inhibition was essentially complete with 1 mM GlcN-[L]-Ino or GlcNAc-[L]-Ino (Figure 5, lanes 5 and 6).[3] These effects are specific since the corresponding D-*myo*-inositol compounds had no inhibitory effect (Figure 5, lanes 3 and 4). In contrast to GlcN-[L]-Ino and GlcNAc-[L]-Ino, neither GlcN-[L]-(2-deoxy)-Ino nor GlcNAc-[L]-(2-deoxy)-Ino inhibited GlcN-PI processing by the trypanosomal cell-free system (Figure 5, lanes 7 and 8). These results show that inhibition by GlcN-[L]-Ino and GlcNAc-[L]-Ino requires the 2-OH group of the L-*myo*-inositol residue, and consistent with this view, neither GlcN-[L]-(2-*O*-methyl)-Ino nor GlcNAc-[L]-(2-*O*-methyl)-Ino was an inhibitor (data not shown).

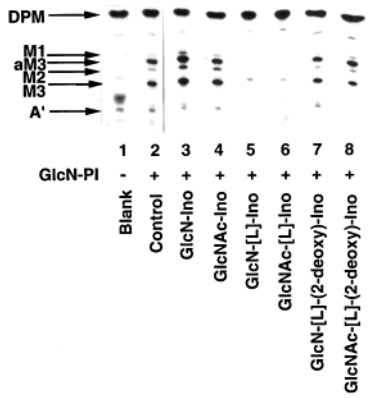

FIGURE 5: Effects of nonphosphorylated L-*myo*-inositol-containing compounds on GPI biosynthesis in the trypanosomal cell-free system. The trypanosomal cell-free system was incubated with GDP-[^{3}H]-Man and NEM without (−) (Blank, lane 1) or with (+) 20 μM GlcN-PI before (Control, lane 2) or after preincubation with 1 mM GlcN-Ino (lane 3), GlcNAc-Ino (lane 4), GlcN-[L]-Ino (lane 5), GlcNAc-Ino (lane 6), GlcN-[L]-(2-deoxy)-Ino (lane 7), and GlcNAc-[L]-(2-deoxy)-Ino (lane 8). The products are as described in the legend of Figure 1.

Since GlcN-Ino is known to be a weak acceptor substrate for MT-1 of the trypanosomal cell-free system (*46*), the transfer of [^{3}H]Man to the soluble phosphate-free GlcN-Ino analogues was examined. The results (Table 1) show that GlcN-[L]-Ino, GlcNAc-[L]-Ino, GlcN-[L]-(2-*O*-methyl)-Ino, and GlcNAc-[L]-(2-*O*-methyl)-Ino are not acceptor substrates for MT-1, unlike GlcN-[L]-(2-deoxy)-Ino and GlcNAc-[L]-(2-deoxy)-Ino (the latter undergoes de-N-acetylation prior to mannosylation, as judged by the sensitivity of the product to nitrous acid deamination). These results emphasize the significance of position 2 of the L-*myo*-inositol residue in determining the behavior (substrate or inhibitor) of such compounds with MT-1.

Inhibition of GPI Biosynthesis by L-myo-Inositol-Containing Compounds Is Parasite-Specific. Some of the foregoing compounds (and their D-*myo*-inositol diastereoisomers) were

[2] The following compounds were also found to be noninhibitory: *cis*-inositol, *epi*-inositol, *allo*-inositol, *muco*-inositol, *neo*-inositol, D-*chiro*-inositol, L-*chiro*-inositol, *scyllo*-inositol, *myo*-inositol-2-phosphate, 3-azido-3-deoxy-D-*myo*-inositol, 3-deoxy-3-fluoro-D-*myo*-inositol, 1-deoxy-1-fluoro-*scyllo*-inositol, 2-*O*-methyl-L-*chiro*-inositol, 2,2′-anhydro-2-*C*-(hydroxymethyl)-*myo*-inositol, *scyllo*-inosose, *cis*-1,2-cyclohexanediol, 1(*S*),2(*S*)-*trans*-1,2-cyclohexanediol, and 1(*R*),2(*R*)-*trans*-1,2-cyclohexanediol.

[3] Note that GlcN-[L]-Ino-*P*-Gro is also inhibitory as its *N*-acetyl derivative (data not shown), suggesting either that the GlcNAc-containing compounds are rapidly de-N-acetylated by the cell-free system or, more likely, that their inhibitory activity is independent of the state of the GlcN amino group.

Table 1: Transfer of [^{3}H]Man to Soluble Phosphate-Free Compounds

compound	transfer of [^{3}H]Man[a]
GlcN-Ino	3% (acceptor)
GlcNAc-Ino	9% (acceptor)
GlcN-[L]-Ino	0% (inhibitor)
GlcNAc-[L]-Ino	0% (inhibitor)
GlcN-[L]-(2-deoxy)-Ino	5% (acceptor)
GlcNAc-[L]-(2-deoxy)-Ino	6% (acceptor)
GlcN-[L]-(2-*O*-methyl)-Ino	0% (neither)
GlcNAc-[L]-(2-*O*-methyl)-Ino	0% (neither)

[a] Measured by scintillation counting of the Man_{1-3}GlcNAc-Ino pool isolated by gel filtration from the supernatant of the cell-free system and expressed as a percentage of the [^{3}H]Man transferred to GlcN-PI under identical conditions (see Experimental Procedures). The figures are corrected for background radioactivity generated in the absence of exogenous substrate (typically 4% of the signal generated with GlcN-PI). The mean values ($n \geq 2$) have been rounded to the nearest integer. Individual measurements were all within ±0.3% of the mean value.

tested in the human (HeLa) cell-free system. In this case, NEM cannot be used to suppress endogenous GPI biosynthesis because it inhibits several steps in the HeLa biosynthetic pathway (D. Sharma, T. K. Smith, and M. A. J. Ferguson, unpublished data). Consequently, some glycolipid H5 [EtNP-ManGlcN-(acyl)-PI] is produced from endogenous substrates in the absence of the synthetic compounds (Figure 6, lane 1). The addition of synthetic GlcN-PI increased the level of incorporation of the radiolabel into the GPI glycolipid fraction by priming the production of some H2 [ManGlcN-(acyl)-PI] and, significantly, more H5 (Figure 6, lane 2), as previously described (*48*, *49*).[4]

Preincubation of the cell-free system with either GlcNAc-[L]-PI or GlcN-[L]-PI (Figure 6, lanes 3 and 4) did not inhibit the processing of either endogenous or exogenous GlcN-PI through to H5, revealing that they are parasite-specific inhibitors.[5] Similarly, GlcN-[L]-Ino and GlcNAc-[L]-Ino failed to inhibit GPI processing in the HeLa cell-free system (Figure 6, lanes 7 and 8), as did GlcN-[L]-(2-deoxy)-PI and GlcNAc-[L]-(2-deoxy)-PI (Figure 6, lanes 9 and 10).

DISCUSSION

In the course of testing the substrate specificities of the *T. brucei* and human (HeLa cell) MT-1 enzymes of GPI biosynthesis, we were surprised to find that GlcN-[L]-PI, a diastereoisomer of the natural substrate, is a selective inhibitor of the parasite enzyme. We suggest that the 2-OH group of the L-*myo*-inositol residue is important for its inhibitory properties since GlcN-[L]-(2-*O*-methyl)-PI is not an inhibitor. The mode of inhibition was further investigated by preparing fragments of GlcN-[L]-PI. The smallest fragment that retained inhibitory properties was [L]-Ino-*P*, indicating that the GlcN and diacyglycerol components of GlcN-[L]-PI have no direct role in the inhibition of *T. brucei* MT-1. It is known that the phosphate group of GlcN-PI

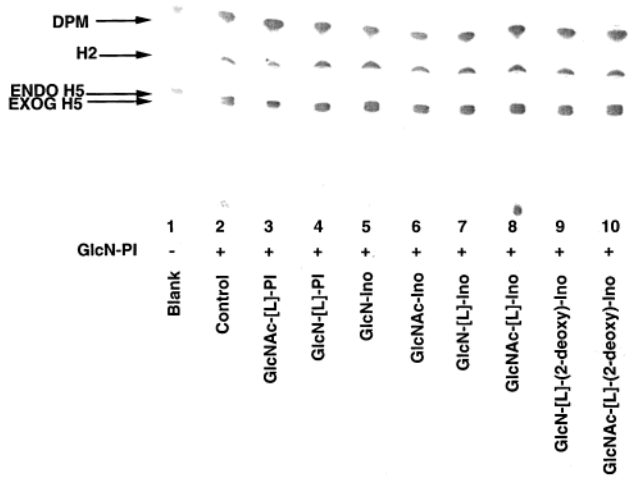

FIGURE 6: L-*myo*-Inositol-containing compounds do not inhibit GPI biosynthesis in the HeLa cell-free system. The HeLa cell-free system was incubated with GDP-[^{3}H]Man without (−) (Blank, lane 1) or with (+) 100 μM GlcN-PI alone (Control, lane 2) or after preincubation with 100 μM GlcNAc-[L]-PI (lane 3) and GlcN-[L]-PI (lane 4) or with 1 mM GlcN-Ino (lane 5), GlcNAc-Ino (lane 6), GlcN-[L]-Ino (lane 7), GlcNAc-[L]-Ino (lane 8), GlcN-[L]-(2-deoxy)-Ino (lane 9), and GlcNAc-[L]-(2-deoxy)-Ino (lane 10). The products are as follows: DPM, dolicholphosphate mannose; H2, Man_1GlcN-(acyl)PI; and ENDO and EXOG H5, endogenous and exogenous EtNP-Man_1GlcN-(acyl)PI, respectively.

greatly assists substrate recognition by *T. brucei* MT-1 (*46*), and it is likely that [L]-Ino-*P*-containing compounds bind to the enzyme with the phosphate group similarly disposed. With such a disposition, the 3-, 4-, and 5-OH groups of the L-*myo*-inositol residue would coincide with the 5-, 4-, and 3-OH groups, respectively, of the D-*myo*-inositol residue of GlcN-PI (Figure 7), thereby satisfying any relevant hydrogen bond networks necessary for substrate−inhibitor binding.[6] Although the equatorial 6-OH group of the L-*myo*-inositol residue would be in an orientation different from that of the axial 2-OH group of the D-*myo*-inositol residue, this does not appear to matter since (a) substitution at this site with GlcN or GlcNAc (as in GlcN-[L]-PI and GlcNAc-[L]-PI) does not abolish inhibition and (b) the introduction of an *O*-methyl or *O*-octyl group at the equivalent 2-OH group of the natural substrate GlcN-PI does not abolish substrate recognition (*48*, *53*). We suggest, therefore, that the inhibitory properties of [L]-Ino-*P*-containing compounds are linked to the axial orientation of the 2-OH group of the L-*myo*-inositol residue when bound to the enzyme; i.e., it has an orientation (axial rather than equatorial) different from that of the 6-*O*-substitutent of the natural substrate (Figure 7). It is possible that the 2-OH group of the L-*myo*-inositol residue engages in hydrogen bonding with adjacent donor or acceptor groups in the active site of the enzyme. The noninhibitory nature of GlcN-[L]-(2-*O*-methyl)-PI, where methylation precludes hydrogen bonding, is consistent with this notion.

The results with diastereoisomeric substrate analogues lacking a phosphate group are also informative. First, GlcN-[L]-Ino was found to be a weak inhibitor of *T. brucei* MT-1, suggesting that it binds to the enzyme in much the same

[4] The R_f of H5 produced from exogenous GlcN-PI is lower than that of the endogenous H5 (Figure 6, compare lanes 1 and 2), the dipalmitoylglycerol lipid component of the synthetic substrate being less hydrophobic than the alkylacylglycerolipid of endogenous GPI intermediates (*49*).

[5] We have previously demonstrated that GlcNAc-[L]-PI cannot be de-N-acetylated by the HeLa cell-free system (*50*) and that GlcN-[L]-PI is not a substrate for inositol acylation in the HeLa cell-free system (T. K. Smith, unpublished data).

[6] The formulas in Figure 7 are arranged in such a way that direct comparisons can be made between the synthetic analogues containing L-*myo*-inositol, whether inhibitors or substrates, and the natural substrate GlcN-PI.

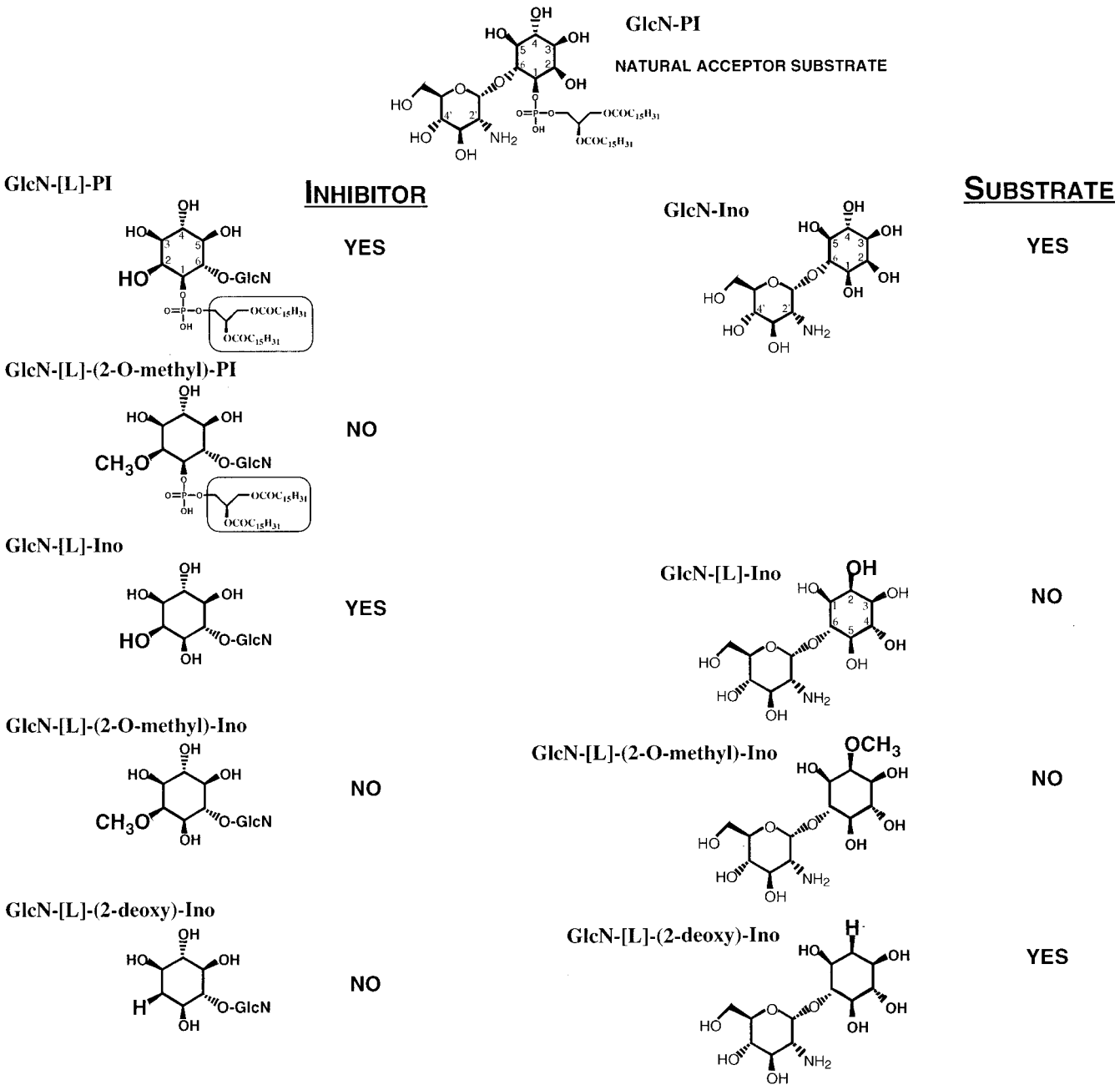

FIGURE 7: Comparison of the substrates and inhibitors. The data presented here suggest that the inhibitors bind to the active site of *T. brucei* MT-1 in the orientation shown on the left to satisfy putative electrostatic and/or hydrogen bond interactions with the enzyme by the phosphate group and the 3- and 5-OH groups of the L-*myo*-inositol residue. The inhibitors GlcN-[L]-PI and GlcN-[L]-Ino present the axial 2-OH group of the L-*myo*-inositol residue in an orientation different from that of the equatorially disposed linkage to GlcN in the natural GlcN-PI substrate, and this may be the key to their inhibitory properties. The shaded boxes indicate that the removal of the fatty acids or the entire diacylglycerol moiety did not alter the inhibitory properties of the parent molecule. Acceptor substrates presumably bind to the active site of *T. brucei* MT-1 in much the same orientation as the natural acceptor GlcN-PI; comparisons among GlcN-PI, GlcN-Ino, and GlcN-[L]-(2-deoxy)-Ino suggest that the equatorial 4-OH group of the D-*myo*-inositol residue has no significant role in substrate recognition but that an axial orientation of the substituent at the comparable position [as drawn in GlcN-[L]-Ino and GlcN-[L]-(2-*O*-methyl)-Ino] abolishes recognition, possibly resulting from unfavorable steric interactions with the enzyme.

way as GlcN-[L]-PI but without the beneficial interaction of the phosphate group. Second, GlcN-[L]-(2-deoxy)-Ino is not an inhibitor, presumably because it cannot engage in the appropiate mode of hydrogen bonding to the enzyme surface. On the other hand, it was shown to be an acceptor substrate for *T. brucei* MT-1, implying that it binds to the enzyme in an analogous way to GlcN-PI and GlcN-Ino (Figure 7). In this situation, the 1- and 3-OH groups of the L-*myo*-inositol residue would coincide with the 5- and 3-OH groups, respectively, of GlcN-PI and GlcN-Ino, and this might assist in the recognition of GlcN-[L]-(2-deoxy)-Ino as an acceptor substrate. The fact that GlcN-[L]-(2-deoxy)-Ino is a substrate for MT-1 also suggests that the 4-OH group of the D-*myo*-inositol residue is not important for substrate recognition [Figure 7; compare GlcN-Ino and GlcN-[L]-(2-deoxy)-Ino]. Finally, the inability of GlcN-[L]-(2-*O*-methyl)-Ino to act as either an inhibitor of or a substrate for MT-1 is consistent with the role of the 2-OH group of the L-*myo*-inositol residue in inhibition (cf. GlcN-[L]-Ino) and suggests that the presence of the bulkier 2-*O*-methyl group prevents GlcN-[L]-(2-*O*-

methyl)-Ino from being recognized as a substrate [cf. GlcN-[L]-(2-deoxy)-Ino].

The results reported herein, not least the identification of L-*myo*-inositol-1-phosphate as a relatively small and simple inhibitor of trypanosomal MT-1, provide valuable pointers for future inhibitor design. Furthermore, the knowledge that stereoisomeric inhibitors of the *T. brucei* GPI pathway are inactive in the HeLa cell-free system opens up a new approach to parasite-specific GPI inhibitors that may ultimately provide useful therapeutic agents.

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