Inhibitors of glycosyl-phosphatidylinositol anchor biosynthesis

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Abstract

Glycosyl-phosphatidylinositol (GPI) is a complex glycolipid structure that acts as a membrane anchor for many cell-surface proteins of eukaryotes. GPI-anchored proteins are particularly abundant in protozoa such as Trypanosoma brucei, Leishmania major, Plasmodium falciparum and Toxoplasma gondii, and represent the major carbohydrate modification of many cell-surface parasite proteins. Although the GPI core glycan is conserved in all organisms, many differences in additional modifications to GPI structures and biosynthetic pathways have been reported. Therefore, the characteristics of GPI biosynthesis are currently being explored for the development of parasite-specific inhibitors. In vitro and in vivo studies using sugars and substrate analogues as well as natural compounds have shown that it is possible to interfere with GPI biosynthesis at different steps in a species-specific manner. Here we review the recent and promising progress in the field of GPI inhibition.

Keywords: Glycosyl-phosphatidylinositol; GPI; Sugars analogues; Inhibitors

1. Introduction

Many cell-surface proteins are posttranslationally modified by the addition of glycosyl-phosphatidylinositol (GPI) anchors, a process, for attaching these proteins to the outer leaflet of the plasma membrane [1–5]. They also play roles as a sorting signal of apically expressed proteins in epithelial cells [6] in signal transduction, in immune responses and pathobiology of infectious diseases [7,8] in a mechanism that confers increased lateral mobility of membrane proteins [9] and as signaling molecules with insulin-mimetic activity [10] in yeast cells. GPI synthesis and/or anchoring are essential for viability [11] and are involved in fungal cell-wall synthesis and assembly [12]. In addition, mammalian and parasite GPI-anchored proteins act as receptors for bacterial toxins aerolysin [13] and clostridial α-toxin [14]. Protozoa tend to express significantly higher densities of cell-surface GPI-anchored proteins than do higher eukaryotes. This is particularly true for Trypanosoma brucei, where the cell-surface is densely coated with GPI-anchored variant surface glycoprotein that protects the parasite from the alternative complement pathway, and through antigenic variation from specific immune responses from the host [15].

GPIs consist of a conserved core glycan (Ethanolamine-P-Man1-2Man1-6Man1-4GlcNH2) linked to the 6-position of the D-myoinositol ring of phosphatidylinositol (PI) [3,4]. GPI anchors are synthesized in a stepwise manner in the membrane of the endoplasmic reticulum beginning with the transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to PI to yield GlcNAc-PI [4,5]. The completed GPI precursor is then attached to nascent proteins via a transamidase-like reaction during which a C-terminal GPI attachment signal is released [16,17]. These GPI-anchored proteins then transit the secretory pathway to reach their final destination at the plasma membrane. About 20 genes involved in the biosynthetic pathway of GPI have been identified thus far, mainly by means of complementation [11]. Species-specific inhibitors of GPI biosynthesis should prove useful in the development of anti-parasitic agents. Especially as this pathway, at least for T. brucei, has been validated as a drug target [18]. Recent studies concerning the effects of inhibitors on GPI precursors biosynthesis and/or their transfer to proteins are discussed in this review.
2. GPI biosynthesis steps

2.1. GlcNAc-PI formation

The first step of GPI biosynthesis [Fig. 1] is catalyzed by an enzymatic multi-protein complex, the UDP-GlcNAc:PI α1-6 GlcNAc transferase (GPI-GnT), which transfers UDP-GlcNAc to PI [19–24]. This enzymatic step is inhibited irreversibly by various sulphydryl alkylating reagents such as N-ethylmaleimide (NEM) in all organisms tested thus far, probably by blocking an active site cysteine of GPI-GnT [25]. This inhibitory effect in T. brucei is abolished by pre-treatment with either UDP-GlcNAc, UMP or UDP, suggesting the involvement of a nucleotide moiety in its protection [25].

2.2. GlcN-PI formation

De-N-acetylation of D-GlcNAcα1-6D-myo-inositol-1-HPO₄²⁻,3-sn,1,2-diacylglycerol (GlcNAc-PI), the second step of GPI biosynthesis, is mediated by GlcNAc-PI de-N-acetylase in vitro in Leishmania major [26], T. brucei [27], Plasmodium falciparum [28] and human cells [29,30]. A variety of substrate analogues (GlcNR-PI) of GlcNAc-PI, were used to determine the specificity of the GlcNAc-PI de-N-acetylase in vitro in Leishmania major [26], T. brucei [27], Plasmodium falciparum [28] and human cells [29,30]. Some of these analogues include variations or modification of the D-myo-inositol; i.e. the diastereoisomer with L-myo-inositol, substitution of the 2-hydroxyl group of the D-myo-inositol with a methyl or octyl group, or GlcNAc-β-PI. All of these were not de-N-acetylated by the human (HeLa) enzyme, but were weakly processed by Trypanosoma, Leishmania and Plasmodium enzymes [28–30]. In all species, where substrate analogues of GlcNR-PI type where R is an acyl group larger than propionyl were not de-N-acetylated, except when R is a benzoyl group which is not processed by the HeLa GlcNAc-PI de-N-acetylase [26,27]. The corresponding benzyl substitution was subsequently shown to inhibit the T. brucei but not the human pathway [26,27]. Two other GlcNR-PI substrate analogues, GlcNMe₂-PI and GlcNCONH₂-PI were found to inhibit both.

Fig. 1. General overview of GPI biosynthesis and its inhibitors in T. brucei, yeast and mammalian cells. The schemes were adapted from Refs. [3,5]. Mannosylation reactions (*) can be blocked either through inhibition of Dol-P-Man biosynthesis by amphomycin, 2dGlc and 2FGlc, or by inhibition of mannosyltransferases with EDTA. Acylation (♣) steps can be inhibited by phenylmethylsulphonyl fluoride (PMSF). Deacylation reactions ([]) can be blocked in T. brucei by diisopropylfluorophosphate (DFP). The transamidation reaction can be inhibited by sulphydryl alkylating agents such as N-ethylmaleimide (NEM). PNT, phenanthroline; spe., specific.
the parasite and human enzymes. Exploiting the differences in substrate specificities of the parasite and human GlcNac de-N-acetylase, two synthetic GlcNAc-PI analogues (GlcNCONH2-β-PI and GlcNCONH2-(2-O-octyl)-PI) were shown to be potent T. brucei specific suicide inhibitors [31,32] while GalNCONH2-PI was found to be specific for the Plasmodium GlcNac-PI de-N-acetylase [28].

2.3. Inositol acylation/deacylation

The timing of the acylation/deacylation of the inositol residue in some organisms is different and determines the maturation of the GPI intermediate (mannosylation and/or addition of ethanolamine phosphate). In T. brucei, mannosylation precedes inositol acylation, which is subsequently essential for the transfer of ethanolamine phosphate to the third mannose [26,33–35]. In T. brucei the deacylation step of the acylated inositol is however required for lipid remodelling [36]. Inositol acylation in T. brucei can be inhibited in vivo as well as in vitro using a serine esterase inhibitor phenylmethylsulphonyl fluoride (PMSF) and leads to accumulation of the Man3-GlcN-PI intermediate in T. brucei [37]. This inhibition of trypanosomal inositol acylation was also observed in vitro with either GlcN-(2-O-methyl)-PI or GlcN-(2-O-octyl)-PI [26,27]. The deacylation step can also be inhibited in vivo and in vitro with diisopropylfluorophosphate (DFP), resulting in only inositol-acylated intermediates [36]. PMSF and DFP can also disrupt the dynamic equilibrium between mature acylated and non-acylated GPI precursors.

Inositol acylation of GlcN-PI is a prerequisite for GPI mannosylation and subsequent modifications in human, yeast and P. falciparum [3,34]. Once acylated, the GPI intermediate is never deacylated. However, in mammalian and yeast cells many GPI-anchored proteins are found to possess a deacylated inositol ring, suggesting that a deacylation step also occurs, but most likely after transfer of the inositol acylated precursor to protein [35,38–40]. The acylation unlike that of T. brucei is dependent upon acyl-CoA and is neither inhibited by PMSF nor by GlcN-(2-O-alkyl)PI analogues, but is inhibited by sulphhydryl alkylating agents.

All of these factors suggest that the inositol acylation in T. brucei system, compared to other GPI pathways is different, requiring distinct enzymatic reaction mechanisms [41].

2.4. Mannosylation of GlcN-PI

The trimannosyl core of GPI anchors (tetramannosyl in the case of yeast and P. falciparum) are added stepwise involving distinct mannosyltransferases encoded by different genes [42–49]. Dolichol phosphate mannosidase (Dol-P-Man), the mannose donor for the later steps of protein N-glycosylation [50] is also the donor for all GPI mannosyltransferases [51]. Dol-P-Man is synthesized from dolichol phosphate (Dol-P) and GDP-Man by an enzyme called the dolichol phosphate mannosidase. In parasitic protozoa, the lipopeptide antibiotic amphotericin forms a complex with Dol-P in the presence of Ca2+, which blocks the interaction between the Dol-P-Man synthase and Dol-P [52], thereby inhibiting GPI biosynthesis in vitro [53,54]. Therefore, Dol-P-Man biosynthesis could be an important target for the development of specific inhibitors, particularly in case of parasites possessing little or no N-glycosylation, such as the apicomplexan parasites P. falciparum and Toxoplasma gondii [55–57].

Mannosyltransferases require divalent cations (i.e. Mg2+, Mn2+) for their activity [58]; therefore, cation scavengers like EDTA are able to prevent in vitro GPI mannosylation reactions in mammalian cells, leading to the accumulation of the early GPI intermediates (GlcNAc-PI, GlcN-PI, GlcN-(acyl)-PI) [59]. Specificity of the α1-4-mannosyltransferase (first mannosyltransferase) of GPI biosynthesis was investigated in T. brucei, P. falciparum and mammalian (HeLa) cells [28,32,60,61] using various GlcN-PI analogues. 4-Deoxy-GlcN-PI was found to be a competitive inhibitor in all systems, as there is no 4-hydroxyl on the GlcN to accept the mannose. GlcN-(2-O-hexadecyl)PI inhibits specifically the first mannosyltransferase in T. brucei in a competitive manner. In the plasmodial but not the HeLa system, GlcN-(2-O-alkyl)PI were found to be a weak inhibitor, probably by competing for the active site of the first mannosyltransferase, whose natural substrate would be GlcN-(acyl)PI. The P. falciparum in vitro system was also inhibited by GlcN-[L]-PI. This diastereoisomer of the natural D-mylo-inositol containing analogue is either competitively inhibiting the inositol-acyltransferase or being inositol-acylated itself and subsequently interacting in a competitive manner with the first mannosyltransferase. The T. brucei α1-4-mannosyltransferase was also inhibited by GlcN-[L]-PI but in a different manner. The binding orientation of GlcN-[L]-PI with the 2-hydroxyl of the L-mylo-inositol residue in axial orientation above the glycosidic linkage probably forms a stabilizing interaction with the enzyme [60,61].

Synthetic mannosides acceptor substrates (thiooctyl- and octyl α-mannosides) have been shown to accept α1-2 and α1-6 mannose residues in a T. brucei in vitro system. The compound Man3-1-6-octyl-α-mannoside inhibited T. brucei GPI biosynthesis, probably by competing with the natural GPI substrate Man3-GlcN-PI for the α1-2-mannosyltransferase [62].

Mannose analogues, 2-deoxy-2-fluoro-D-glucose (2FGlc) and 2-deoxy-D-glucose (2dGlc) are known to inhibit the formation of Dol-P-Man in vivo [63–67] thus in P. falciparum cultures these analogues inhibit specifically GPI biosynthesis in the range of micromolar concentrations [67,68]. In the free living protozoan Paramecium primaurelia, 2FGlc was very useful to characterize an obligatory GPI intermediate [70]. In mouse embryonic stem cells 2FGlc allowed the identification of GPIs which are unable to incorporate ethanolamine [71]. Mannosamine (2-deoxy-2-amino-D-mannose), a known inhibitor of N-linked oligosaccharide biosynthesis [72,73] as well as glucosamine [74] were shown to affect in vivo the incorporation of GPI into a recombinant
GPI-anchored protein transfected into the polarized Madin Darby Canine Kidney (MDCK) cells, into a GPI-anchored protein (PARP) in the procyclic stage of T. brucei and into GPIs synthesized by mouse embryonic stem cells [71,75]. Further study in polarized cells, where GPI-anchored proteins are targeted to the apical membrane, inhibition by mannosamine leads to their accumulation in the secretory pathway [75]. Investigations on the effect of mannosamine in MDCK cells [74] L. mexicana [76] and T. brucei [77] showed the accumulation of a GPI intermediate that incorporates mannosamine in the second mannose position of the conserved trimannosyl core glycan, thereby preventing the addition of further GPI anchor components, i.e. the third mannose and subsequent ethanolamine-phosphate group [74]. Mannosamine treated HeLa cells and Thy-1 negative lymphoma mutants B and F, which accumulate the GPI biosynthetic intermediates Man₂-GlcN-PI and Man₃-GlcN-PI, respectively [78], were shown to accumulate a Man₃-GlcN-PI without incorporation of mannosamine into the second position, suggesting that mannosamine inhibits the addition of the third GPI mannose residue by inhibiting the respective α1-2-mannosyltransferase [78]. In contrast to mammalian cells and the procyclic form of T. brucei, mannosamine has no effect on the viability of bloodstream forms of T. brucei, suggesting that GPI precursors are synthesized in excess [77]. In Tetrahymena vorax, inhibition by mannosamine impairs drastically cell differentiation and reduces the rate of digestive vacuole formation [79]. Interestingly, in Tetrahymena pyriformis, sugar analogues were shown to interfere with GPI biosynthesis at the level of phosphatidylinositol synthesis [80].

The in vivo inhibitory effect of mannosamine in P. falciparum was first suggested to be by mannosamine becoming incorporated into the GPI intermediate GlcN-(acyl)-PI [81]. However, the effects of mannosamine in P. falciparum GPI biosynthesis are still controversial. Recent work has shown that, as is the case of L. mexicana [76] in P. falciparum epimerisation of mannosamine to glucosamine can take place but at a higher rate [69]. In P. falciparum, the inositolacylation of GlcN-PI, an essential step for the P. falciparum GPI biosynthetic pathway, was shown recently to be inhibited by glucosamine [82].

2.5. Ethanolamine phosphate transfer

The last step of the formation of GPI conserved core glycan consists on the transfer of ethanolamine phosphate to position 6 of the third mannose. In T. brucei, the addition of ethanolamine phosphate is prevented by PMSF, which inhibits the inositol acylation of the intermediate Man₂-GlcN-PI to form Man₃-GlcN-(acyl)-PI, the acceptor of ethanolamine phosphate [41]. In mammalian and yeast GPIs, the first mannose residue is modified by an additional ethanolamine phosphate molecule, and in a smaller percentage the second mannose [83–86]. A natural terpenoid lactone isolated from Codinea simplex (YW3548) causes an accumulation of the intermediate Man₂-GlcN-(acyl)-PI in yeast, Candida albicans and mammalian (lymphoma) cells, but not in parasitic protozoa [87]. Thus, it seems as if it prevents the addition of the third mannose. However, in yeast YW3548 actually blocks the addition of ethanolamine phosphate to the first mannose residue, which is a substrate requirement for the addition of the third but not second mannose, therefore, the accumulation of Man₃-GlcN-(acyl)-PI was observed [47]. Similar effects of YW3548 were observed in yeast and mammalian cells using phenanthroline, a metalloprotease inhibitor [88,89]. An accumulation of GPI intermediates that are substrates for ethanolamine phosphate transferases were identified in cells treated with phenanthroline. Therefore, both phenanthroline and YW3548 are likely to inhibit GPI-phosphoethanolamine transferases in mammalian and yeast cells, but not in protozoa. This selective inhibition is another indication of the significant differences between parasite and mammalian/yeast GPI biosynthesis.

2.6. Lipid remodelling

After the complete assembly of the GPI anchor, lipid remodelling takes place in T. brucei [90] L. mexicana [91] mammals [92] and yeast [93,94]. This reaction involves the exchange of the fatty acid components of the lipid moiety for a different fatty acids or whole lipid component. T. brucei GPI-anchored variable surface glycoproteins (VSGs) contain exclusively myristate as their lipid component [90]. Fatty acid biosynthesis is peculiar in T. brucei, since the major product is myristate and is mainly destined to be incorporated into the GPI anchor of VSG protein [95]. This fatty acid pathway could be inhibited with thiolactomycin, a fatty acid synthesis inhibitor, leading to parasite death [96]. Studies using myristic acid analogues such as 10-(propoxy)decanoic acid or its derivatives showed toxicity towards T. brucei but not to mammalian cells [96,97].

2.7. Transamidation reaction

Transfer of mature GPI precursor to protein involves a transamidation reaction, which takes place on the lumen of the ER. This multi-protein complex directly cleaves a C-terminal GPI addition signal peptide and forms a new amide linkage between the carboxyl group of the newly formed C-terminal amino acid (the transamidation site ε) and the amino group of the ethanolamine phosphate linked to the third mannose of the GPI anchor [98]. There are distinct species-specific preferences for the amino acid sequence around the ε site, these have been extensively explored through mutagenesis [99–101]. There is a variation between species in the total number and type of individual proteins in this complex, but all contain a catalytic component that has a cysteine, which can be alkylated in vitro causing loss of activity. The addition of hydrazine to the in vitro assay results in protein-hydrazide, with the hydrazide linked at the C-terminus of the protein instead of the GPI anchor [17,102].

The species differences in substrate specificity of the peptide sequence around the ε site and the structure of the GPI
anchors, as well as the differences in the protein components of the complex suggest that there may be exploitable differences between mammalian and parasite GPI-transamidase.

3. Conclusion

Here we summarize some of the specificities of the reactions involved in GPI biosynthesis and the effects of some promising inhibitors (amino sugars, protease inhibitors and substrate analogues). These inhibitors are also used as valuable tools to dissect GPI biosynthesis in vitro. All former studies suggest that despite the conserved GPI core structure, the GPI biosynthetic machinery may be different enough between mammalian and protozoa to represent a target for anti-protozoan chemotherapy. However, the exact mechanism of action of these inhibitors (sugar analogues) is still unclear and will be elucidated only by cloning and expression of the active enzymes involved in GPI biosynthesis. This first generation of GPI inhibitors provide data to design and develop a specific new anti-parasitic inhibitors of GPI anchor biosynthesis.

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