4-1-2013

Recruiting the Host's Immune System to Target Helicobacter pylori's Surface Glycans

Pornchai Kaewsapsak
Bowdoin College

Onyinyechi Esonu
Bowdoin College

Danielle H. Dube
Bowdoin College

Published in final edited form as:
Chembiochem. 2013 April 15; 14(6): 721–726. doi:10.1002/cbic.201300006.

Recruiting the Host's Immune System To Target *Helicobacter pylori*'s Surface Glycans

Pornchai Kaewsapsak,
Department of Chemistry & Biochemistry Bowdoin College 6600 College Station, Brunswick, ME 04011 (USA)

Onyinyechi Esonu, and
Department of Chemistry & Biochemistry Bowdoin College 6600 College Station, Brunswick, ME 04011 (USA)

Danielle H. Dube[*,[a]]
[a]Department of Chemistry & Biochemistry Bowdoin College 6600 College Station, Brunswick, ME 04011 (USA)

Abstract

Due to the increased prevalence of bacterial strains that are resistant to existing antibiotics, there is an urgent need for new antibacterial strategies. Bacterial glycans are an attractive target for new treatments, as they are frequently linked to pathogenesis and contain distinctive structures that are absent in humans. We set out to develop a novel targeting strategy based on surface glycans present on the gastric pathogen Helicobacter pylori (Hp). In this study, metabolic labeling of bacterial glycans with an azide-containing sugar allowed selective delivery of immune stimulants to azide-covered Hp. We established that Hp's surface glycans are labeled by treatment with the metabolic substrate peracetylated N-azidoacetylglucosamine (Ac_4GlcNAz). By contrast, mammalian cells treated with Ac_4GlcNAz exhibit no incorporation of the chemical label within extracellular glycans. We further demonstrated that the Staudinger ligation between azides and phosphines proceeds under acidic conditions with only a small loss of efficiency. We then targeted azide-covered Hp with phosphines conjugated to the immune stimulant 2,4-dinitrophenyl (DNP), a compound capable of directing a host immune response against these cells. Finally, we report that immune effector cells catalyze selective damage in vitro to DNP-covered Hp in the presence of anti-DNP antibodies. The technology reported herein represents a novel strategy to target Hp based on its glycans.

Keywords

Helicobacter pylori; glycosylation; Staudinger ligation; metabolism; dinitrophenyl

Introduction

Helicobacter pylori (Hp) is a bacterial pathogen found within the stomach of 50% of humans worldwide[1]. A small percentage of infected individuals ultimately develop duodenal ulcers and gastric cancer due to *Hp* infection[2]. Though *Hp* infection can be eradicated in many patients by treatment with triple therapy, this treatment is increasingly ineffective due to the emergence of antibiotic resistant strains of *Hp*[3]. Thus, there is an urgent need for novel

*ddube@bowdoin.edu.

Supporting information for this article is available.

antibiotic therapies to eradicate an *Hp* infection. *Hp's* glycans represent an attractive target for new treatments, as they are linked to pathogenesis and contain distinctive structures that are absent in humans. For example, the exterior of *Hp* is coated in a lipopolysaccharide (LPS) that contains an unusual glycan core composed of D-glycero-D-mannoheptose (DD-heptose), fucose, and *N*-acetylglucosamine (GlcNAc)[4]. This LPS core is critical for colonization of a host by *Hp*[5] and is not synthesized by human cells. Further, *Hp's* surface is covered with glycoproteins containing exclusively bacterial sugars such as bacillosamine, pseudaminic acid, and legionamic acid[6]. Here we describe a novel chemical strategy for targeting *Hp* based on these surface structures.

Our approach employs the chemical technique known as metabolic oligosaccharide engineering (MOE)[7], which was pioneered by Bertozzi, Reutter and colleagues[8]. In MOE, cells are supplemented with an unnatural sugar that can be metabolically incorporated into cellular glycans in place of natural monosaccharides. This metabolic labeling enables the endowment of cell surface glycans with bio-orthogonal chemical functional groups such as azides. Azides are normally absent from biological systems, are non-toxic, and do not react with endogenous biological functional groups[9]. However, azides can undergo an exquisitely selective chemical reaction with triarylphosphines in the context of living cells and animals via Staudinger ligation with no adverse physiological effects[10]. Therefore, azide-covered cells are primed for covalent modification via Staudinger ligation with phosphine probes.

We hypothesized that MOE could form the basis of a strategy to inactivate *Hp*. Briefly, differences in surface glycans present on *Hp* versus mammalian cells could be amplified by incorporating an azide-containing sugar selectively onto *Hp* cells while leaving human surface glycans azide-free. Azide-covered *Hp* would be selectively targeted via Staudinger ligation with phosphine probes conjugated to immune stimulants (Figure 1). These immune stimulants, such as 2,4-dinitrophenyl (DNP)[11] and the galactosyl-(1,3)-galactose (alpha-Gal)[12] epitope, would trigger host immune cells to destroy labeled cells (Figure 1). Indeed, delivery of these immune stimulants to a variety of bacteria, viruses, and cancer cells by other targeting means has initiated selective immune killing both *in vitro* and *in vivo*[13]. We report here that the azide-containing sugar peracetylated *N*-azidoacetylglucosamine[14] (Ac_4GlcNAz) leads to selective labeling of glycans on *Hp* cell surfaces. Further, reaction of azide-labeled *Hp* with phosphine probes conjugated to DNP leads to the recruitment of antibodies that are capable of inducing cytotoxicity *in vitro* in an antibody-dependent, immune-mediated fashion. The technology reported herein represents a novel strategy to target *Hp* based on its glycans.

Results and Discussion

Our approach to targeting *Hp* relies upon three key factors: (1) selective labeling of *Hp's* surface glycans with azides, (2) the ability of the Staudinger ligation to proceed under acidic conditions found in the stomach, and (3) access to immunostimulant-linked phosphine probes that are capable of catalyzing damage to *Hp*.

Selective labeling of *Hp's* surface glycans with azides

We first sought to address whether *Hp's* surface glycans could be selectively labeled with azides while leaving the surfaces of host cells azide-free. We had previously demonstrated that *Hp* metabolically processes the unnatural, azide-containing sugar Ac_4GlcNAz, an analog of the common metabolic precursor *N*-acetylglucosamine (GlcNAc), into cellular glycoproteins[15]. In addition, identification of *Hp's* azide-labeled glycoproteins revealed that a subset of these azides are present in surface-associated and membrane embedded proteins (unpublished experiments). These data indicate that metabolic glycan labeling with

Ac_4GlcNAz labels *Hp's* cell surfaces with azides. On the host side, previous work by Bertozzi and others demonstrated that Ac_4GlcNAz is metabolically incorporated into nuclear and cytosolic glycoproteins in mammalian cells[16], yet cell surfaces remain essentially azide-free[17], even in the context of a mouse[18]. Based on this evidence, we surmised that Ac_4GlcNAz is a suitable metabolic substrate for selective labeling of *Hp's* surface glycans.

Therefore, we began by assessing whether *Hp's* surface glycoproteins are accessible for reaction with phosphine probes via Staudinger ligation upon treatment with Ac_4GlcNAz. To address this possibility, *Hp* were treated with Ac_4GlcNAz or the azide-free control sugar peracetylated *N*-acetylglucosamine (Ac_4GlcNAc), followed by reaction with a *cell-impermeable* phosphine probe comprising a FLAG peptide[19] (Phos-FLAG). Analysis of proteins from treated cells yielded an array of azide-labeled surface glycoproteins that are detectable by Western blot and fluorescence microscopy analyses with anti-FLAG antibody (Figure 2). In contrast, similar treatment of mammalian Madin-Darby canine kidney (MDCK) cells with Ac_4GlcNAz followed by reaction with Phos-FLAG indicated no detectable azide labeled surface glycoproteins by Western blot and fluorescence microscopy analyses (Figure 2). As a control to assess the efficacy of metabolic labeling in both cell types, azide-labeled glycoproteins in total cellular lysate, which includes intracellular as well as surface-exposed glycoproteins, were reacted with Phos-FLAG and produced strong signal that was visualized by Western blot (Figure 2A, "total"). Zinc staining of electrophoresed samples confirmed that all samples contain equivalent protein levels (Supplemental Figure S1), indicating that the lack of detectable azides in surface mammalian samples is not due to low protein levels. As a positive control for microscopy of mammalian cells, MDCK treated with the azidosugar peracetylated *N*-azidoacetylgalactosamine[20] (Ac_4GalNAz), which is metabolically processed into surface mucin-type O-linked glycans in mammalian cells[20], led to robust display of azides on mammalian cell surfaces (Supplemental Figure S2). Taken together, these data indicate that Ac_4GlcNAz is a suitable metabolic substrate for selective labeling of *Hp* surface glycans with azides.

Staudinger ligation proceeds under acidic conditions

The targeting approach we present here hinges upon the Staudinger ligation between phosphines and azide-covered *Hp* proceeding within the acidic environment of the stomach (as low as pH 1). To assess the efficiency of the Staudinger ligation at low pH, Phos-FLAG and the azidosugar *N*-azidoacetylglucosamine[14] (GlcNAz) were incubated in aqueous solution buffered at pH 7 or pH 1. Capillary electrophoresis analysis of the reaction mixtures revealed the presence of the ligation product in reactions conducted at pH 7 and at pH 1 (Figure 3). In contrast, incubation of Phos-FLAG alone at pH 7 and pH 1 yielded no product (Supplemental Figure 3). Integration of the product area relative to un-reacted phosphine revealed a 55% yield for the reaction carried out at pH 7 versus a 35% yield for the reaction carried out at pH 1. The decrease in product yield at pH 1 is likely due to accelerated formation of the oxidized Phos-FLAG conjugate (side peak adjacent to Phos-FLAG), a compound that cannot undergo Staudinger ligation (Figure 3 and Supplemental Figure 3). These data demonstrate that the Staudinger ligation proceeds under acidic conditions with only a small loss of efficiency, and therefore is likely to proceed in the stomach.

Design, synthesis and evaluation of phosphines conjugated to immune stimulants

Our next goal was to design and synthesize phosphines capable of catalyzing damage to azide-covered *Hp*. Toward this end we chose to synthesize phosphines comprising the immune stimulant DNP. Anti-DNP antibodies (including secreted sIgA antibodies) are naturally present in a high percentage of the human population[21] and are capable of recruiting immune effector cells to DNP-covered cells to mediate target-cell killing[22].

Indeed, Spiegel and coworkers have developed a series of bifunctional molecules that deliver DNP moieties to target cells, including viruses and cancer cells, to catalyze cell death by the host's immune system (see [13] for a recent review). Thus, there is considerable precedent that such an approach can be successful.

Phosphine-DNP conjugates **1-3** were accessed as shown in Scheme 1. These compounds, called Phos-Eg-DNP, Phos-nEg-DNP, and Phos-K(DNP)-FLAG, respectively, based on the identity of the linker, were tested for their reaction with *Hp's* azide-labeled proteins. As depicted in Figure 4, compounds **1-3** undergo Staudinger ligation with azide-covered *Hp*, as revealed by Western blot (Figure 4A) and microscopy analysis (Figure 4B) with anti-DNP antibodies. However, of the three compounds, only compound **3**, which contains a highly charged peptide, displayed sufficient water solubility and reacted with *Hp* proteins and cells without the addition of DMSO as a co-solvent. Therefore, given the solubility properties of compound **3**, Phos-K(DNP)-FLAG, it is the most viable candidate for our targeting strategy and was chosen for evaluation in cell killing experiments.

Since Phos-K(DNP)-FLAG possesses optimal solubility and undergoes Staudinger ligation with azide-covered *Hp*, we tested its ability to induce cell–mediated cytotoxicity of *Hp*. This process occurs via interactions between antibody receptors on cytotoxic effector cells found in peripheral blood (e.g. macrophages, dendritic cells) and the constant regions of antibodies[23]. Azide-covered *Hp* were reacted with Phos-K(DNP)-FLAG, then combined with anti-DNP antibodies and peripheral blood mononuclear cells (PBMCs). Cell death was measured using the commercially available LIVE/DEAD BacLight bacterial viability assay. As expected, Phos-K(DNP)-FLAG treatment of azide-covered *Hp* led to enhanced cell killing, while treatment without Phos-K(DNP)-FLAG led to lower levels of cell killing (Figure 5A). Further, Phos-K(DNP)-FLAG treatment of acetyl-labeled *Hp* controls did not increase cell killing relative to cells treated with PBS only (Figure 5A). These data indicate that Phos-K(DNP)-FLAG is not itself cytotoxic and that the combination of both azidosugar treatment and subsequent phosphine reaction is required to recruit immune effector cells to kill *Hp*. Finally, cell-mediated cytotoxicity was dependent upon the presence of anti-DNP antibody; treatment of *Hp* with an isotype control antibody in place of anti-DNP led to only background levels of cell killing (Figure 5B). The specific cytotoxicity observed upon treatment with both azidosugar and phosphine (~25%) is on-par with what is required for cell killing *in vivo*[24].

Conclusions

Here we report a novel two-step strategy for targeting *Hp's* surface glycans with the immune stimulant DNP to catalyze damage to these cells. This work is an important first step as it provides a proof-of-concept. Since bacterial glycans are significantly different than human glycans, due to the utilization of unusual monosaccharide building blocks[25], this approach has the potential to be widely applicable to a variety of pathogens that display distinctive glycans. Indeed, unnatural sugars have been incorporated into *Haemophilus ducreyi*[26], *Campylobacter jejuni*[27], *Escherichia coli*[28], and *Bacteroidales* sp.[29], suggesting that targeting strategies against these pathogens are possibilities.

The ability to recruit the host's immune system to selectively attack and eliminate bacterial cells is a relatively new strategy. Bednarski, Wang and others have recruited antibodies to *E. coli* by taking advantage of the endogenous mannose receptor[30]. More recently, Whitesides and co-workers directed an immune response against gram-positive bacteria (*Staphylococcus* sp.) using a vancomycin-based targeting approach[31]. The approach reported here adds a new dimension to this field by targeting distinctive bacterial glycans that may be present on a broad variety of pathogenic bacterial strains, and also has the

potential to exploit anti-DNP antibodies for bacterial cell destruction. Although we focus here on targeting one type of glycan structure on one bacterial strain, *Hp*, this strategy has the potential to be applied to target any number of distinct bacterial glycans. Moreover, more than one antibody population (e.g. alpha-Gal in addition to anti-DNP) can be recruited to target infectious agents. Thus, this strategy offers a new approach to glycosylation-based therapeutics.

Experimental Section

Materials and Chemical Synthesis

Organic chemicals were purchased from Sigma-Aldrich. Antibody reagents were purchased from Sigma-Aldrich, Millipore, or Becton-Dickinson. *Hp* strain 26695 was a gift of Manuel Amieva (Stanford University). MDCK cells were purchased from ATCC and grown according to the supplier's instructions. PBMCs were purchased from All Cells and used according to the supplier's instructions. Ac_4GlcNAz, Ac_4GlcNAc, GlcNAz and Phos-FLAG were synthesized as previously described[8b, 14]. Molecules **1-3** were synthesized using standard organic chemistry procedures and characterized by standard techniques including ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry. Molecules **1-3** were purified using flash silica gel chromatography.

Metabolic Labeling

Hp strain 26695 was grown in Brucella Broth containing 500 μM or 1 mM of Ac_4GlcNAz or the azide-free control Ac4GlcNAc for 3-5 days under microaerophilic conditions (14% CO2, 37 °C). MDCK were grown in Dulbecco's Modified Eagles Medium supplemented with 500 μM of Ac_4GlcNAz or Ac_4GlcNAc for 3 days. Cells were then harvested, rinsed with phosphate buffered saline (PBS), and reacted with 500 μM phosphine probe for 5 hours at 37 °C. Whole cells were then analyzed by microscopy or subjected to ADCC, as described below. Protein lysates were analyzed by Western blot.

Western Blot

Lysates from metabolically labeled *Hp* or MDCK were standardized to a protein concentration of 3 mg/mL, loaded onto a 4-15% Tris–HCl SDS-PAGE gel, separated by electrophoresis, and transferred to nitrocellulose paper. Anti-FLAG-HRP was employed to visualize FLAG-tagged proteins, and anti-DNP followed by anti-mouse IgG_1-HRP was used to visualize DNP-modified proteins.

Microscopy

Metabolically labeled *Hp* or MDCK were treated with anti-FLAG-FITC to visualize FLAG-tagged proteins, anti-DNP followed by anti-mouse IgG1-FITC to visualize DNP-modified proteins, and Sytox orange to stain cellular DNA. Mounted cells were analyzed using a Zeiss 510 Meta confocal microscope by exciting at 488 nm and 543 nm and detecting emission at 520 nm and 570 nm.

Staudinger ligation at varied pH

GlcNAz (125 μM) and Phos-FLAG (250 μM) were incubated overnight at room temperature at pH 7.4 (in PBS) or pH 1 (in HCl/KCl buffered solution). Reaction mixtures were then analyzed using a High Performance Capillary Electrophoresis system (Agilent) with diode array detection set to 210 nm. Reaction yields were calculated as follows: 100*(integration of the product area)/(integration of the un-reacted phosphine) = % yield.

ADCC Assay

Metabolically labeled *Hp* were incubated with anti-DNP antibody or an isotype control antibody, followed by five hours with human peripheral blood mononuclear cells (PBMC; 10:1 effector:target) at 37 °C in 5% CO_2. The cell mixture was then incubated with Baclight Bacterial Viability solution (Invitrogen) and mounted for analysis by Zeiss 510 Meta confocal laser scanning microscopy. The numbers of dead (red) and live (green) *Hp* cells were counted using ImageJ to determine the percentage of dead *Hp*; PBMC cells were excluded from the counting using size threshold.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank J. Prescher, S. Longwell, A. Eldridge, V. Tra, and A. Koenigsberg for helpful comments. P.K. acknowledges a Surdna undergraduate research fellowship from Bowdoin College. This work was funded by the National Institutes of Health (award R15GM093867), Research Corporation, Camille and Henry Dreyfus Foundation, and Bowdoin Faculty Research Fellowship. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

References

[1]. Graham D, Malaty H, Evans D, Evans D, Kelin P, Adam E. Gastroenterology. 1991; 100:1495–1501. [PubMed: 2019355]

[2]. Marshall BJ. Am. J. Gastroenterol. 1994; 89:S116–S128. [PubMed: 8048402]

[3]. Megraud F. Gut. 2007; 56:1502. [PubMed: 17938430]

[4]. Altman E, Chandan V, Li J, Vinogradov E. FEBS J. 2011; 278:3484–3493. [PubMed: 21790998]

[5]. Altman E, Smirnova N, Li J, Aubry A, Logan SM. Glycobiology. 2003; 13:777–783. [PubMed: 12907691]

[6]. Hopf PS, Ford RS, Zebian N, Merkx-Jacques A, Vijayakumar S, Ratnayake D, Hayworth J, Creuzenet C. PLoS ONE. 2011; 6:e25722–e25722. [PubMed: 21984942]

[7]. Dube DH, Bertozzi CR. Curr. Opin. Chem. Biol. 2003; 7:616–625. [PubMed: 14580567]

[8] a). Keppler OT, Horstkorte R, Pawlita M, Schmidts C, Reutter W. Glycobiology. 2001; 11:11R–18R. [PubMed: 11181557] b) Laughlin ST, Bertozzi CR. Nat. Prot. 2007; 2:2930–2944.

[9]. Prescher JA, Bertozzi CR. Nature Chemical Biology. 2005; 1:13–21.

[10] a). Saxon E, Bertozzi CR. Science. 2000; 287:2007–2010. [PubMed: 10720325] b) Prescher JA, Dube DH, Bertozzi CR. Nature. 2004; 430:873–877. [PubMed: 15318217]

[11]. Six HR, Uemura KI, Kinsky SC. Biochemistry. 1973; 12:4003–4011. [PubMed: 4795683]

[12]. Galili U, Rachmilewitz EA, Peleg A, Flechner I. J. Exp. Med. 1984; 160:1519–1531. [PubMed: 6491603]

[13]. McEnaney PJ, Parker CG, Zhang AX, Spiegel DA. ACS Chem. Biol. 2012; 7:1139–1151. [PubMed: 22758917]

[14]. Luchansky SJ, Hang HC, Saxon E, Grunwell JR, Yu C, Dube DH, Bertozzi CR. Meth. Enz. 2003; 362:249–272.

[15]. Koenigs MB, Richardson EA, Dube DH. Mol. Biosyst. 2009; 5:909–912. [PubMed: 19668851]

[16]. Vocadlo DJ, Hang HC, Kim E-J, Hanover JA, Bertozzi CR. Proc. Natl. Acad. Sci. U.S.A. 2003; 100:9116–9121. [PubMed: 12874386]

[17]. Saxon E, Luchansky SJ, Hang HC, Yu C, Lee SC, Bertozzi CR. J. Am. Chem. Soc. 2002; 124:14893–14902. [PubMed: 12475330]

[18]. Dube, DH. PhD Thesis. University of California; Berkeley: 2005.

[19]. Kiick KL, Saxon E, Tirrell DA, Bertozzi CR. Proc. Natl. Acad. Sci. U.S.A. 2002; 99:19–24. [PubMed: 11752401]

[20]. Hang HC, Yu C, Kato DL, Bertozzi CR. Proc. Natl. Acad. Sci. U.S.A. 2003; 100:14846–14851. [PubMed: 14657396]

[21]. Karjalaninen K, Mäkelä O. Eur. J. Immunol. 1976; 6:88–93. [PubMed: 964298]

[22] a). Muller-Eberhard HJ. Annu. Rev. Biochem. 1988; 57:321–347. [PubMed: 3052276] b) Saini MS, Diebel LN, Liberati DM, Albaran RG, Dulchavsky SA. Am. Surgeon. 1999; 65:637–642. [PubMed: 10399972]

[23]. Hale G, Clark M, Waldmann H. J. Immunol. 1985; 134:3056–3061. [PubMed: 3980990]

[24]. Dubrovska A, Kim C, Elliott J, Shen W, Kuo T-H, Koo D-I, Li C, Tuntland T, Chang J, Groessl T, Wu X, Gorney V, Ramirez-Montagut T, Spiegel DA, Cho CY, Schultz PG. ACS Chem. Biol. 2011; 6:1223–1231. [PubMed: 21936526]

[25]. Dube DH, Champasa K, Wang B. Chem. Commun. 2011; 47:87–101.

[26]. Goon S, Schilling B, Tullius MV, Gibson BW, Bertozzi CR. Proc. Natl. Acad. Sci. U.S.A. 2003; 100:3089–3094. [PubMed: 12615992]

[27]. Liu F, Aubry AJ, Schoenhofen IC, Logan SM, Tanner ME. ChemBioChem. 2009; 10:1317–1320. [PubMed: 19422007]

[28]. Dumont A, Malleron A, Awwad M, Dukan S, Vauzeilles B. Ang. Chem. Int. Ed. 2012; 51:3143–3146.

[29]. Besanceney-Webler C, Jiang H, Wang W, Baughn AD, Wu P. Bioorg. Med. Chem. Lett. 2011; 21:4989–4992. [PubMed: 21676614]

[30] a). Bertozzi CR, Bednarski MD. J. Am. Chem. Soc. 1992; 114:2242–2245.b) Li J, Zacharek S, Chen X, Wang J, Zhang W, Janczuk A, Wang PG. Bioorg. Med. Chem. Lett. 1999; 7:1549–1558.

[31]. Metallo SJ, Kane RS, Holmlin RE, Whitesides GM. J. Am. Chem. Soc. 2003; 125:4534–4540. [PubMed: 12683824]

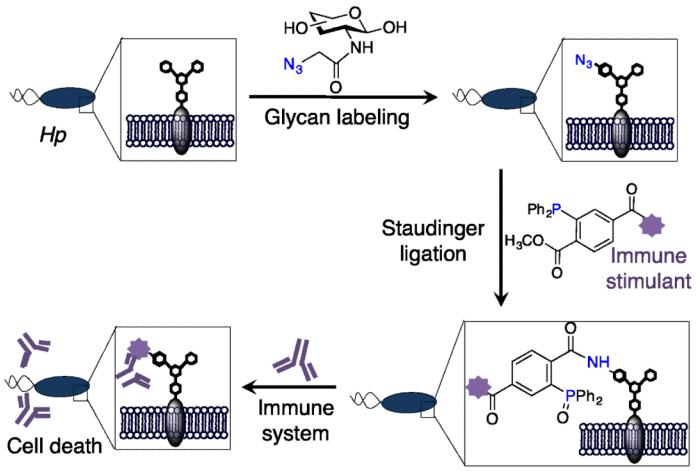


Figure 1.

Schematic overview of recruiting the host's immune system to inactivate *Hp* based on its unique glycans. First, distinctive *Hp* glycans are metabolically labeled with an unnatural azide-containing sugar. Azide-covered *Hp* then undergo Staudinger ligation with phosphine probes comprising an immune stimulant. Finally, the host's immune system is triggered to kill *Hp* covered with the immune stimulant.

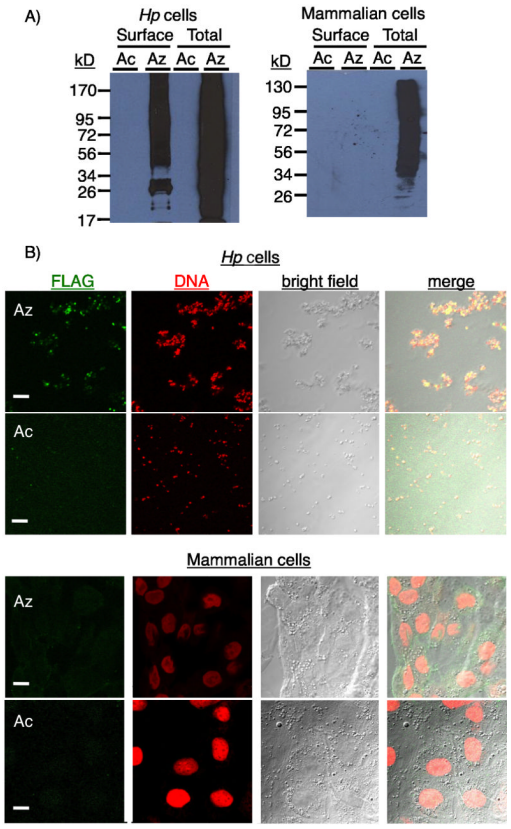


Figure 2.

Western blot and microscopy analyses indicate that Ac4GlcNAz labels *Hp's* surface glycans with azides while leaving surface glycans on mammalian cells azide-free. *Hp* and Madin-Darby canine kidney (MDCK) cells were treated with 500 μM Ac_4GlcNAz (Az) or the azide-free control sugar Ac_4GlcNAc (Ac), and surface azides were reacted with the cell-impermeable probe Phos-FLAG (500 μM) for 5 hours at 37 °C. A) Lysates from cells were analyzed directly by Western blot with anti-FLAG to detect surface azides (surface) or were treated with an extra bolus of Phos-FLAG (250 μM) prior to analysis by Western blot with anti-FLAG to detect internal as well as surface azides (total). All lanes contain equivalent protein levels. B) Cells were incubated with FITC-anti-FLAG to detect surface azides and Sytox Orange to stain cellular DNA, and then analyzed by confocal microscopy. FLAG (green), DNA (red), and brightfield images are shown, as well as the merge of all three channels. All images are scaled equivalently, and the scale bars correspond to 10 μm. The data shown are representative of replicate experiments.

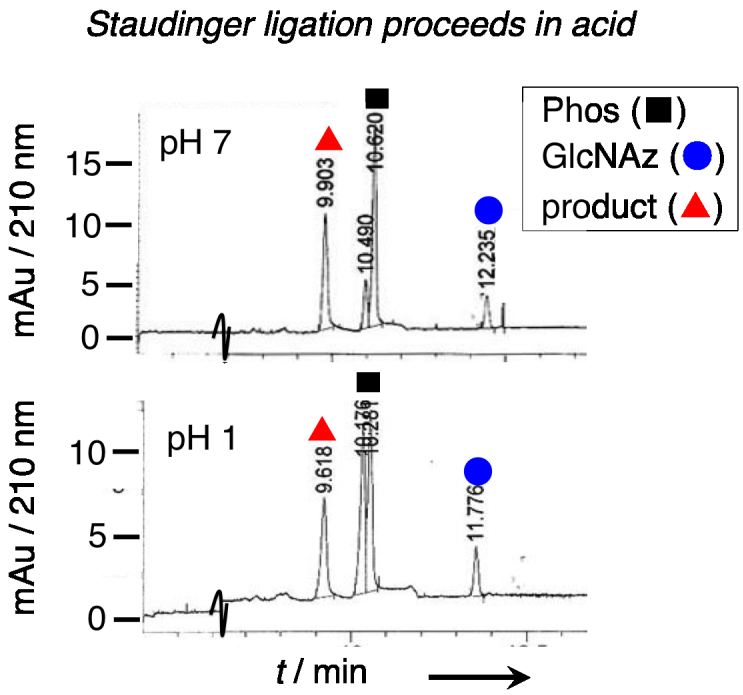


Figure 3.

Evidence that the Staudinger ligation proceeds under acidic conditions. Phos-FLAG (250 μM) and GlcNAz (125 μM) were incubated overnight at room temperature at pH 7 or pH 1, and reaction completion was monitored the following day by capillary electrophoresis (CE) with detection of absorbance at 210 nm. Elution time of Phos-FLAG and GlcNAz were determined based on synthetic standards analyzed by CE. The data shown are representative of replicate experiments.

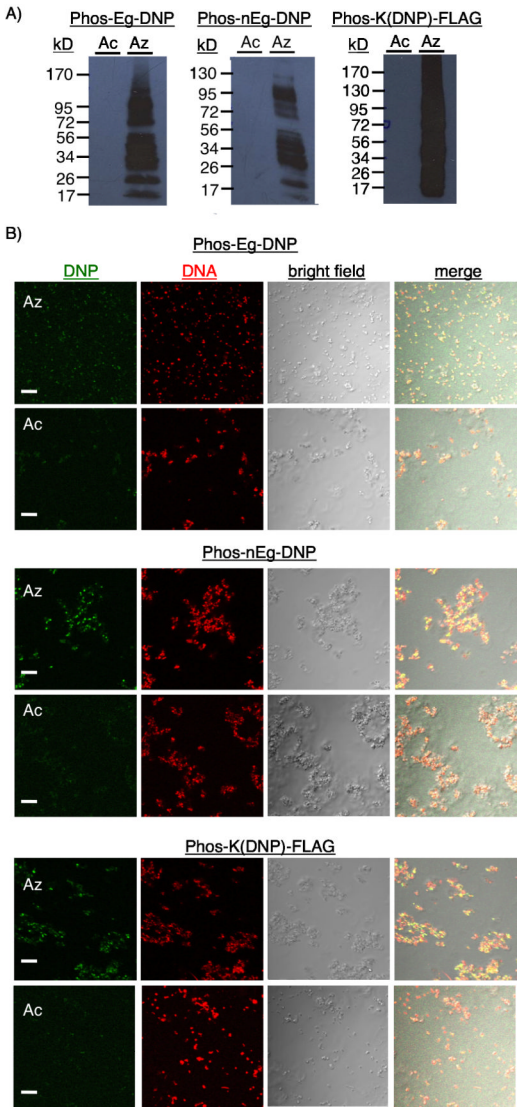


Figure 4.

Evaluation of phosphine-DNP conjugates **1-3** for reaction with azide-covered *Hp*. *Hp* were treated with 1 mM Ac_4GlcNAz (Az) or the azide-free control sugar Ac_4GlcNAc (Ac), and then reacted with the indicated Phos-DNP probes (500 μM) for 5 hours at 37 °C. A) Western blot analysis of lysates with anti-DNP antibody indicates that Phos-DNP probes react with azide-covered *Hp*. All lanes contain equivalent protein levels. B) Microscopy analysis confirms that Phos-DNP probes react with azide-covered *Hp*. Treated *Hp* were incubated with FITC-anti-DNP to detect surface DNP and Sytox Orange to stain cellular DNA, then analyzed by confocal microscopy. FLAG (green), DNA (red), and brightfield images are shown, as well as the merge of all three channels. All images are scaled equivalently, and the scale bars correspond to 10 μm. The data shown are representative of replicate experiments.

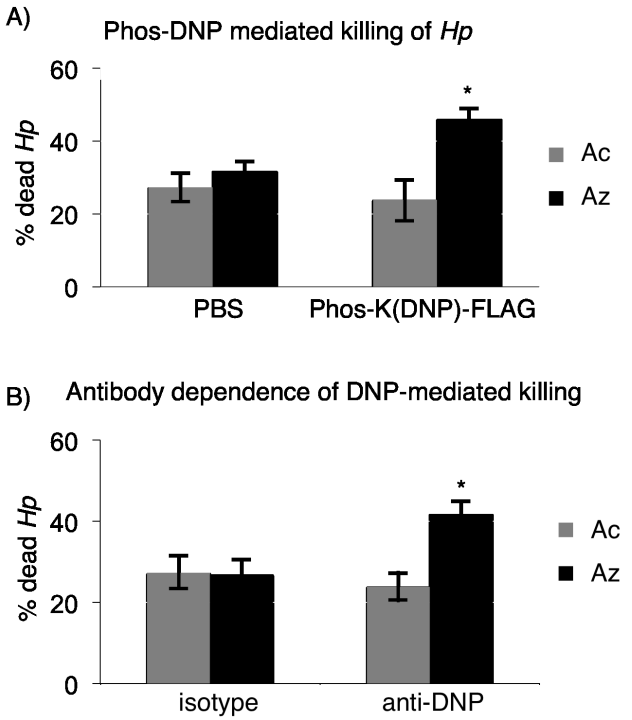
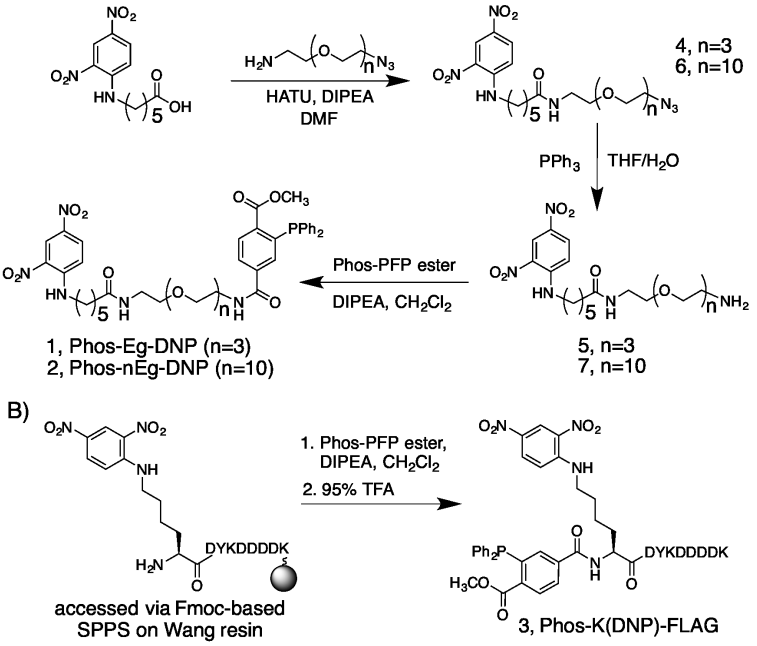


Figure 5.

Phos-K(DNP)-FLAG is capable of recruiting anti-DNP antibodies to azide-covered *Hp* and eliciting cellular cytotoxicity via immune effector cells. A) *Hp* were treated with 1 mM Ac4GlcNAz (Az) or the azide-free control sugar Ac4GlcNAc (Ac), then reacted with Phos-K(DNP)-FLAG (500 μM) or PBS for 5 hours at 37 °C. *Hp* were then labeled with anti-DNP antibody for 1 hour, followed by a four hour incubation with peripheral blood mononuclear cells (PBMCs). Percent dead *Hp* was assessed using Baclight Bacterial Viability solution. Error bars represent the standard error of replicates ($n \geq 5$). Between 1,500-10,000 cells were analyzed per treatment. B) As in (A), except following treatment with Phos-K(DNP)-FLAG, *Hp* were incubated with anti-DNP antibody or an isotype control antibody. Asterisks indicate statistical significance ($p < 0.05$) as determined by a Student's t-test. The data shown are representative of replicate experiments.



Scheme 1.

Synthesis of phosphine-DNP conjugates **1**-**3**. A) A triarylphosphine was conjugated to the immune stimulant 2,4-dinitrophenyl (DNP) via ethylene glycol-based linkers (**1** and **2**) or B) via an amino acid-based linker (**3**).