In Silico Identification of Dual Ability of N. gonorrhoeae ddl for Developing Drug and Vaccine Against Pathogenic Neisseria and Other Human Pathogens

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Abstract

The transmission and prevention of gonococcal infection is a global health problem and an effective drug or vaccine against the pathogen is yet to be developed. In our previous studies by analyzing metabolic pathways and membrane proteome of N. gonorrhoeae we found that four membrane associated targets could be better option in developing anti-gonorrhea drugs and vaccine. Here we showed that among these putative targets, D-alanine—D-alanine ligase (ddl) is the best candidate for development of both drug and vaccine against N. gonorrhoeae and various other human bacterial pathogens. Using in silico approaches, we developed the 3-D model of the enzyme and potential epitopes are also identified that may be helpful in peptide vaccine development against multiple pathogens including pathogenic Neisseria. Epitopes require experimental validation for their effectiveness as peptide vaccines against these pathogen.

Keywords: Computational prediction; Epitope design; Immunoinformatics; Sexually transmitted disease; Broad spectrum target

Introduction

Gonorrhea is one of the most common sexually transmitted diseases (STD) caused by Neisseria gonorrhoeae. Although the gonococcal infection and transmission is mostly prevalent in underdeveloped countries, in USA, it is the second most common STD with 358 366 cases reported in 2006 that has shown a 5.5% increase incidences in 2007 (Centers for Disease Control and Prevention, 2007; Cornelissen, 2008). The disease transmission occurs from infected man to a woman. The infected male shows symptoms of urethritis, epididymitis, and prostatitis. But infected female develops polyarthralgia, tenosynovitis, arthritis, and in severe cases pelvic inflammatory disease (PID) that leads to infertility and ectopic pregnancy due to permanent blockage of the fallopian tube (Furuya and Tanaka, 2009). Due to the immmunergic of antibiotic resistance and lack of appropriate vaccine against the pathogen, the gonococcal transmission and infection remains a global public health problem/ menace (Tapsall, 2006; Workowski et al., 2008).

N. gonorrhoeae pili proteins have been reported as possible vaccine candidates (Rothbard et al., 1985) and are already patented (US Patent: 4443431). But pilE globular domain is reported to be non-immunogenic (Hansen et al., 2007). Other reported vaccine targets are porB (Zhu et al., 2004), opa, lipo-oligosaccharides, protein-I, lactoferrin (Ibp1, Ibp2) (Barbosa-Cesnik et al., 1997), phospholipase A (pldA) (Bos et al., 2005), transferrin-binding proteins (tpbA and tpbB) (Price et al., 2005; Thomas et al., 2006; Price et al., 2007), IgA1 proteases (Barbosa-Cesnik et al., 1997), and 2C7 oligosaccharide (OS) epitope (Gulati et al., 2001). The tspA and tspB of N. meningitides have been patented for vaccination against pathogenic Neisseria (US patent: 6861507). However, in perceive none of these vaccines are effective in combating the pathogen. In our recent study, to elucidate new drug target for N. gonorrhoeae, we found that 29% drug targets are associated with or localised in the membrane, and the transferrin-binding protein - tpbA (Price et al. 2005) is the most potent vaccine candidate (Barh and Misra, 2009). But if a single target can be utilised for dual purpose in developing an effective drug as-well-as vaccine against the pathogen, it will be most effective way for suppression of growth and development of such non-responsive pathogens. With this objective, we selected four targets from our previous works (Barh and Kumar, 2009; Barh and Misra, 2009) viz. sulfate transport permease protein C, ABC transporter iron-uptake permease inner membrane protein (afuB), competence lipoprotein (Cpl), and D-alanine—D-alanine ligase (ddl) for this study to explore the ability of any of these target(s) to be used for dual purpose of drug targeting and/or vaccine against pathogenic Neisseria. These targets were selected because these are non-human homologue essential enzymes, involved in pathogen specific metabolic pathways, and localized to the cell membrane or cell wall.

Materials and Methods

Identification of epitopes of N. gonorrhoeae

The basic epitope prediction strategy was taken as described in Barh and Misra, (2009) to get minimum number of antigenic epitopes that are able to produce both the B-cell and T-cell responses. The basic epitope prediction strategy was taken as described in Barh and Misra, (2009) to get minimum number of antigenic epitopes that are able to produce both the B-cell and T-cell
mediated immunity. In addition, few other tools had also been used to confirm the method. Briefly, amino acid sequences of candidate targets from our previous studies [D-alanine—D-alanine ligase (ddl), Competence lipoprotein (NGO0277), ABC transporter iron related permease protein (NGO0216/fbpB), and Putative ABC transporter (NGO0446)] were retrieved from Swiss-Prot protein database (http://us.expasy.org/sprot) and each sequence was then analyzed for antigenicity using B-cell antigenic site prediction tool “Antigenic” (http://bio.dfci.harvard.edu/Tools/antigenic.pl). Similarly, each protein sequence was also subjected for B-cell epitope prediction using BCPreds (EL-Manzalawy et al., 2008). Both the BCPred and AAP prediction modules of BCPreds were used to identify B-cell non-overlapping epitopes and common epitopes (generated by both the prediction servers) were selected. Top scored (cut off values for “Antigenic” and BCPreds were respectively 1.2 and 1) sequences were then aligned to get an overlapping sequences to make a continuous stretch of amino acid sequence that possess both antigenic sequences as well as the B-cell binding sites. Each antigenic B-cell epitope sequences were then analyzed with ProPred 1 (Singh and Raghava, 2003) for MHC class I and ProPred (Singh and Raghava, 2001) for MHC class II epitope prediction using default parameters. The proteosomal cleavage sites of identified epitopes were also analyzed. Common epitopes for both the MHC classes that also can bind to maximum MHC alleles were selected. To confirm the parameters of epitopes, each epitope was further analyzed with VaxiJen v2.0 antigen prediction server (Doytchinova and Flower, 2007) for antigenicity. To verify MHC binding properties, less than 1000 nM IC₅₀ scores for DRB1*0101 based on MHCPred v.2 (http://193.133.255.13/mhcpred/) (Guan et al., 2003) was used. Exo-membrane localization and fold level topology of epitopes were confirmed using TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (Krogh et al., 2001). The finally selected epitopes were used for structural characterization.

Identification of common epitope for multiple pathogens

A modified comparative and subtractive genomic approach as described by Barb and Kumar, (2009) was adopted to identify a single epitope that can be used against multiple pathogens. Briefly, full length sequence of the N. gonorrhoeae ddl was subjected to blastp against Database of Essential Genes (DEG) (http://tubic.tju.edu.cn/deg) (Zhang et al., 2004). Bacterial pathogens that show significant sequence homology (essential gene for the respective pathogens) were selected based on blastp scores [cut off values: bit score (>100), E-value (<E⁻¹₀), and percentage of identity at amino acid level (>35%)]. Because of the subjected sequence is a non-human homologue essential protein and putative drug target for the N. gonorrhoeae, it is presumed that the homologous sequences also will be drug targets for the corresponding pathogens. Selected sequences of pathogens were then examined using standard human blastp in NCBI server (with same cutoff values) and pathogens that carry non-human homologs were selected. NCBI blastp was used for selection of pathogens having the common epitopes at nearly accessible region. Similarly, the selected amino acid sequences of each pathogen were aligned with multiple sequence alignment tool - COMBINE Advanced T - COFFEE (V s 7.7) (Notredame et al., 2000) to determine the sequence similarity at the epitope position among the identified pathogens. The final selection of epitopes from other human pathogen were based on TMHMM (exo-membrane localization), VaxiJen (antigenicity), and MHCpred (DRB1*0101) analysis.

Homology modelling and model validation

To carry out homology modeling, Automated, Alignment, and Project modes of Swiss model server (Arnold et al., 2006) were used. The resultant models were optimized using Swiss-PdbViewer (Guex and Peitsch, 1997) and Accelrys Discovery Studio (http://accelrys.com/). Best model was selected based on reliability assessment that was carried out using Prosa-web (Wiederstein and Sippl, 2007) and Procheck (Laskowski et al., 1993) tools at SAVS server (http://nihserver.mbi.ucla.edu/SAVS/). ProFunc (Laskowski et al., 2005) was used to predict domains, motifs, ligand binding clefts, and various other functional parameters of 3D structures.

Characterization of epitopes

Due to the short sequence (9-20 mars) of epitopes, it was difficult to find the template from PDB database and to make a homology model using Swiss-model server. Therefore, initially we used drug discovery studio to identify and determine the native site and the structure of epitopes within the protein. In another effort, the DISTILL server (Bau et al., 2006) that can predict 3D structure of small fragments of proteins based on the similarity with PDB template was also used for epitope modeling. Resultant epitopes were then validated with ProSA-web and PROCHECK. ProFunc, Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and InterProScan (Quevillon et al., 2005) were used for domain, motif, and functionality assignment of epitopes. ProteinDigest (http://db.systemsbiology.net:8080/roteomics Toolkit/proteinDigest.html) was used to determine molecular weight, pI, and enzymatic degradation site(s) of epitopes.

Results

Identification of candidate peptide vaccine

The common antigenic B-cell epitopes were identified using “Antigenic” and BCPreds. This method generates a stretch of 21 mers sequence (EYSCEPVLNGKGLPGIIHIPAT) spanning amino acid position 182 to 202 of the ddl protein that meets all selection criteria for the antigenic B-cell epitope (Table 1). But other three candidate vaccine targets [Competence lipoprotein (NGO0277), ABC transporter iron related permease protein (NGO0216/fbpB), and Putative ABC transporter (NGO0446)] did not have such type of common sequence. Therefore, in this study they were excluded from further analysis. TMHMM 2.0 based exo-membrane localization and topology of ddl shows that it is fully exposed to out side the cell (Table 3).

MHC binding T-cell epitopes were identified using ProPred 1 (for MHC I) and ProPred (for MHC II) with default parameters. The 21-mer antigenic B-cell epitope sequence was analyzed with these two servers and the common epitope(s) that can bind both the MHC classes and covers maximum MHC alleles were selected. In this way only one 9-mer sequence (LPGIHIPAT) spanning at amino acid position 193 to 202 of the ddl protein was selected. Another peptide (CPVLNGKGL) spanning at amino acid position 185 to 194 was found to bind 38 MHC I alleles. But this peptide contains proteosomal cleavage site and was not considered due to the fact that a peptide which contains a
proteosomal cleavage site may be degraded during antigen processing. So it is not suitable for peptide vaccine design (Toes et al., 2001). Thus, only the epitope peptide sequence “LPGIHIIPA” was taken for further analysis as this can potentially induce both the B-cell and T-cell mediated immunity. Predicted T-cell epitopes of ddl are represented in Table 2. VaxiJen and MHCPred v.2 analysis of the epitope (LPGIHIIPA) further confirm that the epitope is antigenic (VaxiJen score = 0.4102) and can bind to DRB1*0101 allele (MHCPred nM IC50 score = 839.46) (Table 3).

“LPGIHIIPA” is common epitope for multiple pathogens

The blastp analysis showed that the ddl protein is also a putative drug target in pathogens such as N. gonorrhoeae, N. meningitidis, Haemophilus influenzae, Salmonella typhimurium, Staphylococcus aureus, Helicobacter pylori, Burkholderia pseudomallei, Vibrio cholerae, and Acinetobacter baumannii (data not shown). Ddl was also reported as potential drug target in A. hydrophila (Sharma et al., 2008). The NCBI blastp with N. gonorrhoeae ddl and multiple alignments of ddl protein sequences of these identified pathogens show that the epitope sequence (LPGIHIIPA) is highly conserved in N. meningitidis and moderately conserved in H. influenzae, B. pseudomallei, and A. baumannii. Therefore, the identified epitope may be a candidate vaccine against these pathogens also. The NCBI blastp and T-COFFEE (Notredame et al., 2000) multiple alignments are represented in Figure 1. Further to demonstrate the possibility of the native homologous epitope sequences of “LPGIHIIPA” in these bacteria that can potentially be used as peptide vaccine, we used VaxiJen, MHCPred, and TMHMM. As shown in Table 3, all homologous epitopes have potentiality to be used as peptide vaccine against the respective bacteria.

Table 1: Antigenic B-cell epitopes of N. gonorrhoeae ddl. Epitopes are identified by “Antigenic” and two modules of BCpreds. The 21 mers sequence “EYSCPVLNGKGLPGIHIIPA” was identified following the method as described in text.

Table 2: T-cell epitopes of N. gonorrhoeae ddl. The common antigenic B-cell epitope “EYSCPVLNGKGLPGIHIIPA” was analyzed for its ability to bind MHC I and MHC II molecules using Propred I and Propred. A common epitope “LPGIHIIPA” (9 mers) that generates both TCL and HCL mediated immune response was selected.

Table 3: Epitopes from ddl of various pathogens that are homologous to N. gonorrhoeae ddl. All epitopes have potentiality to be used as vaccine for corresponding pathogen and other listed pathogens.
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Figure 1: NCBI blastp with N. gonorrhoeae ddl showing conserved epitope sequences “LPGHIIPA” in other human pathogens. (b) The multiple alignment of ddl from identified other human pathogens using T-Coffee.

(a) Main view
(b) Bottom view
(c) Right view
(d) Amino acid position specific foldings

Figure 2: 3-D model of N. gonorrhoeae ddl from various angles. (a) The secondary structure the ddl showing various foldings at corresponding amino acid positions. (b) The multiple alignment of ddl from various angles. (b) The secondary structure the ddl showing various foldings at corresponding amino acid positions.
Figure 3: Validation of the 3-D model of *N. gonorrhoeae* ddl with ProSa-web. The upper panel is template (PDB id: 1iowA) and lower is ddl. (a) Overall model quality of 1iowA ($Z=9.19$). (b) Local model quality of 1iowA. (c) Overall model quality of ddl ($Z=8.57$). (d) Local model quality of ddl.

Figure 4: The Ramachandran plot for *N. gonorrhoeae* ddl. The plot shows the acceptability of the model.
gonorrhoeae using homology modelling. Based on blast parameters, the E. coli D-alanine-D-alanine ligase (PDB id: 1iowA) having 46% sequence identity with an E-value of $2e^{-69}$ was selected as template. The X-Ray diffraction structure has 1.90 Å resolutions. There are overall 2% gaps in sequence alignment and the epitope sequence was LP+I+I+P+ against LPGIHIIPA. The optimized final 3-D model of N. gonorrhoeae ddl (Figure 2a-c) consists of 3 sheets, 1 beta alpha beta unit, 4 beta hairpins, 3 beta bulges, 13 strands (3 parallel and 10 anti-parallel barrels), 12 helices (ten alpha and two 3, 10), 8 helix-helix interacs, 20 beta turns, and 4 gamma turns (Figure 2d). The molecular weight and total energy of the model were estimated as 31.9 KD, -7391.5 KJ/mol, respectively. There are Glutathione synthetase ATP-binding domain-like motif and PreA TP-grasp domain but no potential Helix-turn-helix DNA-binding motif found. The protein contains only one enzyme active site (D-alanine-D-alanine ligase) and 12 significant ligand-binding sites (data not shown).

Validation of the model

To validate the model, initially ProSA-web was used that compares and analyzes the energy distribution in protein structure as a function of sequence position to determine a structure as native like or fault. As shown in Figure 3 and the Z-scores, the model is of good quality of structure. The Procheck of SAVA master server was used for assessment of stereochemical quality of the model. According to the Ramachandran plot, residues in most favoured regions, residues in additional allowed regions, residues in generously allowed regions, and residues in disallowed regions were respectively, 90.2%, 7.4%, 1.2%, and 1.2% that ensures the geometrically acceptable quality of the model (Figure 4).

Characterization of the epitope

The epitope position within the N. gonorrhoeae ddl protein was determined using Accelrys Discovery Studio Visualizer (v1.7). The native position of the epitope (LPGIHIIPA) from various angles is shown in Figure 5a-c (marked with a white arrow) and the amino acid position is shown in Figure 5d (marked with a circle). Combining the results from the 3D model and TMHMM 2.0 based topology analysis, it is evident that the epitope is exposed to the surface of the protein and therefore it also supports that the predicted sequence is a potential candidate peptide vaccine. Due to the very short length (9mers) of the epitope, instead of Swiss model server, the DISTILL server was used to generate 3-D structure (Figure 6). But both the validation tools (ProSA-web and Procheck) show that those models are highly unusual (data not shown) and no domain or motif could be assigned using ProFunc, Motif Scan, or InterProScan for this 9mers epitope. Calculated molecular weight and pI of the 9mers epitope are respectively, 930.16 and 6.74, and is found to be undigested by Trypsin, Chymotrypsin, Cyanogen bromide, Clostripain, Iodoso Benzoate, Staph Protease, and AspN, as determined by ProteinDigest. As the focus of this study is on N. gonorrhoeae ddl, we have excluded 3D characterization of epitopes from other pathogens. But based on sequence homol-

Figure 5: The native position of the epitope within N. gonorrhoeae ddl from various angles (a-c). The graphical view of the epitope position at corresponding amino acid sequence (d).
ogy and topology analysis, it is found that, epitopes identified from other pathogens are also antigenic, MHC II (DRB1*0101) binding, located at nearly same accessible region, and exposed to cell surface similar to the *N. gonorrhoeae* ddl. Therefore they are also potential vaccine candidates.

**Discussion**

D-alanine-alanine ligase (ddl) is an essential enzyme that acts in bacterial peptidoglycan biosynthetic pathway and is also a non-human homolog. Therefore, it is an important target for developing new antibiotics. In this current study, we demonstrated that among the selected four drug targets of *N. gonorrhoeae*, ddl is one of the potential and preferred candidates to develop either anti-gonorrhoeal drug or vaccine in isolation or in tandem. This study also showed that ddl may also be a good drug target in *N. meningitides*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Burkholderia pseudomallei*, *Vibrio cholerae*, and *Acinetobacter baumannii* along with *A. hydrophila* as identified by Sharma et al. (2008). Crystal structures of ddl from *E. coli* (Fan et al., 1997) and *Staphylococcus aureus* (Liu et al., 2006) are available. Similarly, 3-D modeling of the enzyme in *Enterococcus faecalis* (Prévost et al., 2000) and *Enterococcus faecium* (Gholizadeh et al., 2001) have been developed to identify active ligand binding sites in the enzyme for development of appropriate inhibitor and to understand the mechanism of drug resistance in respective pathogens. In this study, we characterized 3-D structure of the *N. gonorrhoeae* ddl to serve the same purpose. Using conventional drug development methods and structure based rational drug discovery strategy several ddl inhibitors namely D-cycloserine (Zawadzke et al., 1991; McCoy and Maurelli, 2005), diazenedicarboxamides (Kovac et al., 2008), quercetin, apigenin (Wu et al., 2008), Bruton's tyrosine kinase inhibitor LFM-A13 (Triola et al., 2009) etc have been identified. In our docking study, we found that apigenin is one of the best ligands for *N. gonorrhoeae* ddl (data not shown).

It is well known that two amino acid sequences are functionally similar if they share >40% sequence identity at amino acid level. In this analysis, our identified ddl sequences from pathogens that are homologous to *N. gonorrhoeae* ddl qualify this cut off value. Hence, it is presumed that those ddls may have similar structure and therefore identified inhibitors for *N. gonorrhoeae* ddl may inhibit those ddls. Hence we excluded 3D and docking studies for ddls from other pathogens.

In general, earlier studies have reported either T-cell or B-cell based epitope designing for a given pathogen (Arockiasamy and Krishnaswamy, 1995; Arévalo-Herrera et al., 2002; Sollner et al., 2008). Similarly, some vaccines can only activate helper T-lymphocytes (HTLs)/CD4+ / MHC II. But activation of CD8+ cytotoxic T-lymphocytes (CTLs)/ MHC I is also required in many cases (Pancre et al., 1996). Therefore, an epitope that can produce both the B-cell and T-cell (MHC I and MHC II) mediated immunity is highly useful in developing peptide-based vaccines. In this study, to map epitopes from *N. gonorrhoeae* ddl, we have used the strategy as described by Barh and Misra, (2009) where the identified epitopes have high probability to produce both the B- and T-cell mediated immunity. In addition to our previously described method, we have also used VaxiJen and MHCPred to support the efficacy of epitopes. Similarly, in this study, using subtractive genomics and homology analysis we have also tried to identify a single epitope within a nearly same accessible region of corresponding ddl proteins in various other human pathogens so that the identified epitope may be useful against a wide range of pathogens. The identified peptide

![Figure 6: 3-D structures of 21 mers (a), 17 mers (b), and 9 mers (c) epitopes created by DISTILL. (d-f) Folding and amino acids positions of 9 mers epitope in detail.](image-url)
(LPGIHIIPA) from *N. gonorrhoeae* ddl may induce B-cell and both the CD4+ and CD8+ T-cell mediated immunity. The sequence homology analysis demonstrates that this epitope sequence is conserved in ddls of *N. meningitides*, *H. influenzae*, *B. pseudomallei*, and *A. baumannii* and at nearly same accessible region. Therefore, this peptide might be useful in designing vaccine against all these human pathogens. Similarly, homologous and native sequences of the peptide (LPGIHIIPA) in these pathogens also can serve the same purpose in developing broad spectrum peptide vaccine for all these pathogens (Table-3).

**Conclusions**

To conclude, ddl protein can be a good target for developing effective antibiotic and vaccine for pathogenic *Neisseria, H. influenzae, B. pseudomallei*, and *A. baumannii*. Effective inhibitor screening for ddl is required. Similarly, the identified epitope(s) require proper design and subsequent validation for their uses as peptide vaccine against these human pathogens. **Financial Support**

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