JOURNAL OF ENZYME INHIBITION AND MEDICINAL CHEMISTRY, 2017
VOL. 32, NO. 1, 203–207
http://dx.doi.org/10.1080/14756366.2016.1247055

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SHORT COMMUNICATION

OPEN ACCESS

Screening assay for inhibitors of a recombinant *Streptococcus pneumoniae* UDP-glucose pyrophosphorylase

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ABSTRACT

The UDP-glucose pyrophosphorylase of *Streptococcus pneumoniae* ($GalU_{Spn}$) is absolutely required for the biosynthesis of capsular polysaccharide, the *sine qua non* virulence factor of pneumococcus. Since the eukaryotic enzymes are completely unrelated to their prokaryotic counterparts, we propose that the GalU enzyme is a critical target to fight the pneumococcal disease. A recombinant $GalU_{Spn}$ was overexpressed and purified. An enzymatic assay that is rapid, sensitive and easy to perform was developed. This assay was appropriate for screening chemical libraries for searching GalU inhibitors. This work represents a fundamental step in the exploration of novel antipneumococcal drugs.

ARTICLE HISTORY
Received 13 June 2016
Revised 16 September 2016
Accepted 22 September 2016

KEYWORDS
Antibacterial target; pneumococcus; UTP:glucose 1-phosphate uridylyltransferase; virulence inhibitors

Introduction

Streptococcus pneumoniae (the pneumococcus) is the main cause of community-acquired pneumonia and also produces meningitis, bacteremia and otitis media. Many different virulence factors have been described in *S. pneumoniae*[1,2], but the capsular polysaccharide is only absolutely required for virulence *in vivo*[3,4]. To date, at least 94 different pneumococcal capsular types have been described[5–9]. This remarkable phenotypic variability appears to be present also at the genetic level[10].

The *galU* gene encodes a uridine diphosphate glucose pyrophosphorylase (UDPG:PP; UTP:glucose 1-phosphate uridylyltransferase; EC 2.7.7.9; GalU). The enzyme UDPG:PP catalyzes the reversible formation of uridine diphosphate glucose (UDP-Glc) and inorganic pyrophosphate (PPi) from uridine 3-phosphate (UTP) and glucose-1-phosphate (Glc-lP).

Early studies showed that UDP-Glc is a key component in the biosynthetic pathway of pneumococcal capsular polysaccharides containing glucose, galactose and/or UDP-glucuronic or UDP-galacturonic acids[11]. At least one of these sugars is a component of every capsular polysaccharide of *S. pneumoniae*[12].

Although it has been reported that the pneumococcal *galU* gene is highly polymorphic, there is striking sequence conservation among bacterial GalU enzymes[13]. In addition, knockout *galU* mutants of type 1 and type 3 pneumococci are unable to synthesize a detectable capsular polysaccharide and, consequently, are highly attenuated *in vivo*[14,15]. Southern blot hybridization experiments using DNAs prepared from pneumococcal isolates belonging to different types showed that every strain tested contained a *galU* homolog[16].

Since GalU is required for the synthesis of UDP-Glc, the main glucosyl donor in lipopolysaccharide and capsule biosynthesis, a relevant role of this enzyme in virulence has also been recognized in many other bacteria such as *Klebsiella*[17,18], *Erwinia*[19], *Pseudomonas*[20,21], *Xanthomonas*[22], *Escherichia coli* O157:H7[19,23], *Francisella*[24], *Proteus*[25], *Shigella*[26,27], *Vibrio cholerae*[28], *Actinobacillus*[29,30], *Aeromonas*[31] or *Haemophilus influenzae*[32]. Eukaryotic UDPG:PPs are completely unrelated to their bacterial counterparts, suggesting the possibility that inhibitors of the bacterial enzymes would not be harmful to the host[33].

Previously, the *galU* gene of *S. pneumoniae* (designated as $galU_{Spn}$ hereafter) was cloned and expressed in *Escherichia coli*, and subsequently, the purified enzyme was biochemically characterized[16,34]. The *galU* gene was expressed mainly in the exponential phase of growth[35].

We describe here the cloning and overexpression of the $GalU_{Spn}$ enzyme and the development of a method to screen for inhibitors in small volumes with high sensitivity.

Materials and methods

Construction of the recombinant plasmid pETgalU

Escherichia coli strains XL1 Blue and BL21 (DE3) were used for cloning and expression, respectively. *Escherichia coli* strains were grown in Luria Bertani medium (LB) (Difco; Becton Dickinson and Company, Baltimore, MD). The complete $galU_{Spn}$ gene was PCR amplified from the pMMG2 plasmid[16] using oligonucleotides pet28galUF2: 5′-AGGGCTAGCATGACATCAAAAGTTAG-3′ and pet28galUR: 5′-TTAGGATCCGTAGTCTTGTTCGTAGG-3′. Restriction

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endonuclease sites were introduced in the primer sequences (these are shown underlined). PCR products were purifie after digestion with BamHI and NheI restriction enzymes from agarose gels and ligated to the expression vector pET28a previously digested with the same enzymes. Sequencing was performed to verify the recombinant plasmid (pETgalU) carrying the *galU*$_{Spn}$ gene preceded by a DNA sequence encoding for six His residues.

Expression and purification of the recombinant His6GalU$_{Spn}$

Escherichia coli BL21 (DE3) was transformed with pETgalU plasmid and grown in LB medium. The culture was incubated with shaking (200 rpm) at 37 °C in an air:medium ratio of 4:1 until the optical density at 600 nm (OD_{600}) reached 0.6–0.7. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added. The optimal expression conditions were determined by varying the incubation temperature and IPTG concentration (from 0.1 to 0.4 mM). The maximum amount of recombinant GalU was achieved after induction with 0.1 mM IPTG followed by overnight incubation at 28 °C. The expression of GalU was assessed by analysis of total cell protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

His6GalU$_{Spn}$ was purified using immobilized metal affinity chromatography (IMAC). Briefly, cells were harvested by centrifugation, resuspended 1:10 in buffer A (50 mM Tris-HCl, 0.25 M NaCl; pH 8.0) and disrupted by sonication (Sonics and Materials Inc., CT). After disruption, the crude extract was clarified by centrifugation (15 000 $\times$ *g* for 15 min) and filtered through a 0.22 μm nitrocellulose membrane. The sample was conditioned in buffer A by passing through a PD-10 column (GE Healthcare, Little Chalfont, UK). A nickel affinity column (GE HP HisTrap column), (1.0-ml bed volume) equilibrated with the same buffer was loaded with the sample. Following a washing step with buffer A containing 100 mM imidazole, step elution was performed by increasing the imidazole concentration up to 500 mM. Linear flow rate was 0.4 cm min^{-1}. Protein separation was monitored by absorbance at 280 nm and 2-ml fractions were collected.

Fractions containing the His6GalU$_{Spn}$ were immediately conditioned using a PD-10 desalting column (GE Healthcare) and stored at −20 °C with 20% of glycerol. The purified protein was analyzed by SDS-PAGE in 15% polyacrylamide gels and protein concentration was measured by the Lowry assay using bovine serum albumin as standard.

Enzyme activity assays

Determination of UDP-Glc:PP activity was performed using two different assays:

a. Standard method: the production of UDP-Glc and PPi was monitored by a reaction coupled to the reduction of NAD, determined by spectrophotometric measurement of NADH formation[34,36].
b. Screening method for inhibitors assay: the enzymatic activity was evaluated in the direction of UDP-Glc synthesis by a modification of the colorimetric method as previously described by Fusari[37]. Briefly, the production of Pi, after hydrolysis of PPi by inorganic pyrophosphatase was quantified by the formation of a phosphomolybdate–malachite green complex. The assay was performed at 37 °C in a 50 μl-reaction mixture containing (unless otherwise specified) 40 mM morpholinepropanesulfonic acid (MOPS)-NaOH buffer (pH 8.0), 5 mM $MgCl_2$, 7.5 mM UTP, 0.16 mg ml^{-1} BSA, 0.5 U/ml inorganic pyrophosphatase and purified His6GalU$_{Spn}$. The reaction was initiated by adding Glc-1P (7.5 mM, final concentration). After incubation at 37 °C for 10 min, the enzyme reaction was terminated by adding 50 μl of malachite green reagent (0.03% malachite green, ammonium molybdate and 0.05% Triton X-100 in 0.7 N HCl). The complex formed with the released Pi was measured at 650 nm with a Synergy HT Multi-Mode Microplate Reader (Biotek) detector. One unit (U) of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 mol of PPi per minute (which renders 2 mol of Pi after hydrolysis), under the conditions described previously. Controls were done to correct the interference by substrates and buffer components. A standard curve obtained by addition of various amounts of PPi (0.13–13 nmol) and 0.04 U of pyrophosphatase, was used to determine the quantity of Pi produced.

For inhibition assays, His6GalU$_{Spn}$ was preincubated 30 min at 37 °C with 7.5 mM of the following nucleoside analogs: abacavir, decitabine stavudine and zidovudine provided by Dosa SA (Argentina), capecitabine provided by Tuteur (Argentina) and didanosine provided by Filaxis SA (Argentina). Then, the assay was continued as described previously. All the chemicals used in the enzymatic assay were purchased from Sigma-Aldrich (St. Louis). Methanol was of chromatographic pure grade and water was Milli-Q grade. ÄKTA Purifier, His Spin Trap and PD-10 desalting columns were purchased from GE Healthcare.

Results and discussion

The GalU UDPG:PP from *S. pneumoniae* is a key enzyme in the biosynthesis of the capsular polysaccharide of this microorganism. In this study, *galU*$_{Spn}$ was cloned and soluble overexpressed in *E. coli*

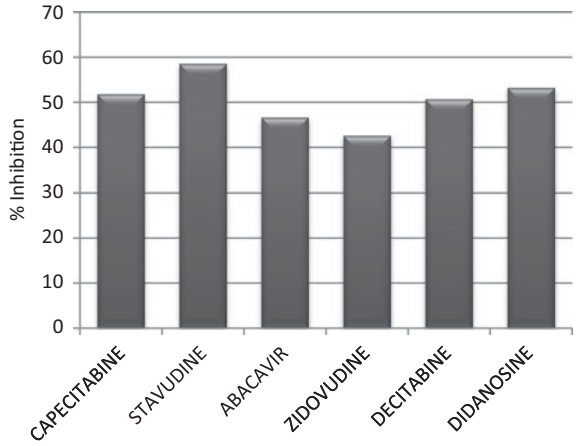

Figure 1. Percentage of inhibition activity of GalU with different nucleosides analogs.

Table 1. Purification of UDP-Glc pyrophosphorylase.

Product	Volume (ml)	Amount of protein (mg)	Total activity (U)*	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	10	66	23.8×10^7	3.6×10^6		1
GalU	1	8.8	4.4×10^7	5.05×10^6	18.69	1.40

*Enzymatic activity was assayed using the standard method described in "Materials and methods" section.

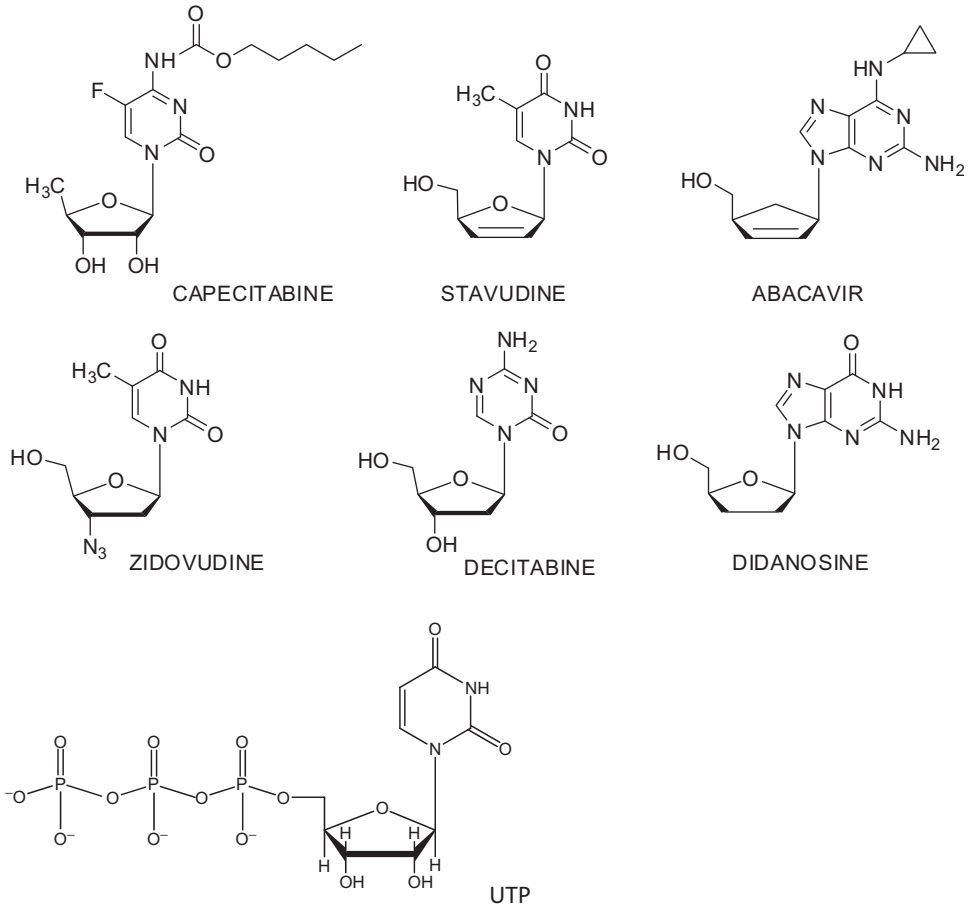

Figure 2. Structure of the nucleoside analogs and the natural substrate of UDP-glucose pyrophosphorylase, UTP.

BLE21 (DE3). Clarified bacterial protein extract was used for purification of the recombinant protein by IMAC. The purified enzyme displayed one band as judged by SDS-PAGE and Coomassie Blue staining (not shown). The apparent molecular mass was consistent with the theoretical value of the recombinant protein. On average, 1 l of culture yielded ca. 97 mg of His6GalU$_{spn}$ (specific activity $= 5.05 \times 10^6$ U mg^{-1}) (Table 1). The pure enzyme was stable for at least 6 months when stored at $-20\,^{\circ}$C in 20% glycerol. The colorimetric method described in this study was optimized for UDPG:PP activity assay in microscale grade, and rendered concordant results to the standard method[34,36].

Considering the similarity in chemical structure of natural nucleotides and nucleosides to the nucleoside analogs, we speculate that these compounds could also inhibit GalU activity and could be a useful tool to evaluate the colorimetric assay for screening small molecule libraries and identify potential inhibitors of GalU protein. In fact, this assay could detect GalU inhibition activity after 30 min of preincubation with the putative inhibitor at 7.5 mM (Figure 1).

As it was reviewed by Berbís et al.[33], in all UDPG:PPs described so far the interactions established with the nucleotide moiety of the substrate are essentially the same. In the first step, UTP is buried inside the active site pocket where it forms hydrogen bonds with certain residues of the enzyme, while the phosphate group remains fully accessible to the solvent. This initial binding induces local conformational changes near the active site prior to the subsequent steps of the reaction. We speculate that the inhibitors used herein (Figure 2) could accommodate in the cleft in a similar way as the natural substrate irrespectively of their capacity to form hydrogen bonds.

Promiscuity towards other dNTPs has been documented for UDPG:PPs from different sources[33]. The different nucleoside analogs used in this study inhibit *S. pneumoniae* UDP-glucose pyrophosphorylase, including compounds containing a purine base in their structure such as abacavir and didanosine, or even a triazine derivative such as decitabine.

Conclusions

This study describes the successful preparation of recombinant GalU from *S. pneumoniae*. It has been shown that inactivation of the *galU* gene completely abolishes the formation of the capsule, and, therefore, renders the pneumococcus completely avirulent[16]. The colorimetric test in a 96-well plate proposed herein as appropriate to screen and characterize specific inhibitors of GalU enzyme is a useful tool to be applied in the search of antipneumococcal drugs.

Furthermore, this work represents a fundamental step in the search of novel antipneumococcal drugs.

Acknowledgements

The CIBER de Enfermedades Respiratorias (CIBERES) is an initiative of the Instituto de Salud Carlos III (ISCIII). We thank A. Iglesias and C. Figueroa for providing advice in the enzymatic assay.

Disclosure statement

The authors report no competing interests.

Funding

This work has been sponsored by grants from Universidad de Buenos Aires, Agencia Nacional de Promoción Científica y Tecnológica, ANPCYT and Dirección General de Investigación Científica y Técnica (SAF2012-39444-C02-01). LB, MM, GL, MVM and AM are members of "Carrera del Investigador", CONICET, Argentina. Consejo Nacional de Investigaciones Científica y Técnicas [11220110100707CO], Agencia Nacional de Promoción Científica y Tecnológica, ANPCYT [PICT-2008- 2214], Dirección General de Investigación Científica y Técnica [SAF2012-39444-C02-01], Secretaria de Ciencia y Tecnica, Universidad de Buenos Aires [20020090300074].

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