Optimization of Crystallization Conditions and Preliminary X-ray Diffraction of *Neisseria meningitidis* 1-deoxy-D-xylulose 5-phosphate Reductoisomerase (NmDXR)

Hanan A. Omar[1]* and Jennifer L. Ekstrom[2]

[1]Department of Biochemistry and Molecular Biology, Theodore Bilharz Research Institute, Imbaba, Giza, Egypt.
[2]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan, USA.
*hanan.abdelhamid.omar@gmail.com

Abstract: Invasive meningococcal disease (IMD) caused by *Neisseria meningitidis* is a major public health problem and continues to cause substantial mortality and morbidity. The *Neisseria meningitidis* 1-deoxy-D-xylulose 5-phosphate reductoisomerase (NmDXR) protein is a potential drug target for antibiotics. The DXR enzyme is inhibited by the antibiotic fosmidomycin. Crystallography has become a standard technique used for drug design. In this study, purified NmDXR was crystallized in complex with NADPH, cobalt ion and the inhibitor fosmidomycin in order to study their mode of interaction with the enzyme. SDS–PAGE of the purified NmDXR enzyme revealed a single 43 kDa protein band. The crystallization was performed using the hanging-drop and sitting-drop vapor-diffusion methods. Successful crystallization conditions were optimized by varying pH, precipitant concentration and protein concentration to determine optimal conditions. The effects of various additives on crystallization were also investigated. This strategy yielded several large crystals with dimensions up to 0.8 mm x 0.2 mm x 0.1 mm rod. Preliminary atomic-resolution data were collected. The data are 99% complete, with maximum resolution of 1.2 Å. The structure was solved by molecular replacement (MR) and the AutoMR routine within Phenix. This structural study of NmDXR should be useful for drug design development of novel NmDXR inhibitors for meningitis treatment.

[Hanan A. Omar and Jennifer L. Ekstrom. **Optimization of Crystallization Conditions and Preliminary X-ray Diffraction of *Neisseria meningitidis* 1-deoxy-D-xylulose 5-phosphate Reductoisomerase (NmDXR).** *Life Sci J* 2015;12(3):92-97]. (ISSN:1097-8135). http://www.lifesciencesite.com. 12

Keywords: *Neisseria meningitidis,* 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), Crystallography, fosmidomycin

1. Introduction

Invasive meningococcal disease (IMD) is a major public health issue due to its global distribution, potential of epidemic spread, predominant disease burden in children and adolescents, high case-fatality rates and substantial morbidity (Van Deuren *et al.,* 2000; Tunkel *et al.,* 2010 and Pace & Pollard, 2012). This disease presents a broad spectrum of manifestations and it is a neurological and clinical emergency that requires prompt recognition and initiation of therapy (Strelow & Vidal, 2013).

Neisseria meningitidis, is a bacterium that can cause meningitis (Ryan & Ray, 2004) and other forms of meningococcal disease. It is a major cause of illness and death during childhood in industrialized countries and has been responsible for epidemics in Africa and in Asia Genco & Wetzler, 2010).

DXP reductoisomerase (1-deoxy-D-xylulose 5-phosphate reductoisomerase or DXR) is an enzyme that interconverts 1-deoxy-D-xylulose 5-phosphate (DXP) and 2-C-methyl-D-erythritol 4-phosphate (MEP). It is a key enzyme in the non-mevalonate pathway of isoprenoid biosynthesis (MEP pathway). It is important for the production of molecules used in protein prenylation, cell membrane maintenance, hormones, protein anchoring and N-glycosylation (Takahashi *et al.,* 1998). Since the nonmevalonate pathway is not found in animals, it is an ideal target for the development of antibacterial drugs (Umeda *et al.,*2010). Fosmidomycin is an antibiotic that specifically inhibits DXR (Iguchi *et al.,* 1980). The fosmidomycin and derivatives such as FR900098 have already been shown to act as broad-spectrum agents against multidrug-resistant bacteria and malaria parasites (Jomaa *et al.,* 1999; Singh *et al.,* 2007; Davey *et al.,* 2011 and Pérez-Gil *et al.,* 2012).

Protein crystallography and X-ray diffraction enable us to visualize protein structures at the atomic level and enhance the understanding of protein function. Specifically enable the study of how proteins interact with other molecules, undergo conformational changes, and perform catalysis in the case of enzymes. This information helps to design novel drugs that target a particular protein, or rationally engineer an enzyme for a specific industrial process (Alberts *et al.*, 2002).

Producing crystals of biological macromolecules is often a challenging task. The crystallization of biological macromolecules can be considered as a two-stage process. The first stage, "screening," determines chemical and physical

conditions under which the sample has a propensity to crystallize. The second stage, "optimization," refines the chemical and physical parameters to produce crystals suitable for analysis by X-ray diffraction (Chayen & Saridakis, 2002). Optimization makes use of information derived from the screening experiments to produce crystals of sufficient size and quality for diffraction. Both environmental variables (such as temperature) and chemical variables (type and concentration of chemicals and the solution pH) are refined (Luft *et al.*, 2007). The aim of this study is to optimize the conditions for crystallization of NmDXR and to solve its crystal structure in complex with NADPH, cobalt ion and fosmidomycin.

2. Materials and Methods

Preparation of the enzymatic reaction:

Pure lyophilized NmDXR was dissolved in buffer A (20mM Tris-HCl, 40mM NaCl, pH 8.0) and filtered through 0.2µm spin filter. The concentration of the protein stock solution was determined using BioRad protein assay (Bradford, 1976) and adjusted by dilution with buffer A to 8.5 mg/ml. The protein was analyzed on SDS-PAGE using 4-20% gradient gel and Coomassie Brilliant Blue staining (Green & Sambrook, 2012).

Aliquots of the protein stock were combined with freshly prepared, filtered stocks of $CoCl_2$, NADPH, and the inhibitor fosmidomycin immediately before setting up crystallization plates. The protein concentration to be used in the initial screening was chosen using Hampton Research's Pre-Crystallization test (Hampton Research, USA). The enzyme mixture for the enzymatic reaction consisted of 2mg/ml NmDXR protein in buffer A, 1mM Cobalt Chloride, 3mM NADPH, 1mM fosmidomycin (Yajima *et al.*, 2007).

Preparation of the precipitant and setting up crystals:

Crystallization screens were performed by the hanging drop method, using a range of commercially available and homemade screening reagents; Qiagen PACT, ComPAS and JCSG Suites (Qiagen, USA), Emerald Biosystems Pricipitant Synergy, Cryo and Wizard screens (Emerald BioSystems, USA), and Hampton Research Crystal Screen I and II (Hampton Research, USA). The crystal setups were done by hand using optically clear 48-well pre-greased Linbro plates, and a solution volume of 200µl/well. Equal volumes of the enzyme mixture and crystallization solution (typically 1.5 µl each) were pipetted onto a siliconized glass cover slip and mixed by gentle pipetting. The cover slip was then inverted over the well containing the crystallization solution and sealed to prevent evaporation. The screening trays were held in temperature controlled incubators (at 4°C, 15°C, and 25°C) and examined under the microscope periodically (Jancarik & Kim, 1991).

Optimization of the crystallization conditions:

Successful crystallization conditions were optimized by varying pH, precipitant concentration and protein concentration to determine optimal conditions. The effects of various additives on crystallization were also investigated using the Hampton Research Additive Screen.

To obtain crystals suitable for X-ray analysis, crystallization was carried out by vapor diffusion in a sitting-drop format. A droplet was prepared by mixing 5 µl of enzyme reaction mixture and 5 µl of the crystallization solution plus the selected additive and pipetted onto the sitting drop rod of a 24-well plate containing 1 ml of crystallization solution in each well. The plate was sealed by tape and stored in the appropriate temperature controlled incubator (Cox & Weber, 1988).

Preparation of crystals for synchrotron data collection:

The crystals were transferred to mother liquors containing several distinct cryoprotectant solutions. The crystallization solution was reoptimized according to the initial diffraction data collection attempts. Additional crystallization plates were set up with various amounts of the cryoprotectant added as a component to the well solution. The largest and best formed crystals were transferred briefly into the best identified cryoprotectant solution, mounted in a cryoloop, and flash-frozen in liquid nitrogen (Xiao & Gamblin, 1996).

X-ray data collection:

Atomic-resolution data on NmDXR crystals were collected at the Advanced Light Source (Argonne National Labs, Chicago, IL) at beamline 21-ID-F of the Life Sciences Collaborative Access Team. High-resolution and low-resolution data were collected in two passes of 130 degrees each, with the detector set in the closest possible position for the high-resolution pass. The data were indexed and scaled using HKL2000 (Otwinowski & Minor, 1997).

3. Results and Discussion

DXR is a key enzyme in the non-mevalonate pathway of isoprenoid biosynthesis that is important for the synthesis of essential biological molecules (Takahashi *et al.*, 1998). NmDXR is a 43.6 kDa protein (NCBI Reference Sequence: WP_014580704.1). SDS–PAGE of the purified NmDXR enzyme revealed a single band at approximately 43 kDa by Coomassie Brilliant Blue

staining (Figure 1) revealing high purity required for the protein crystallization.

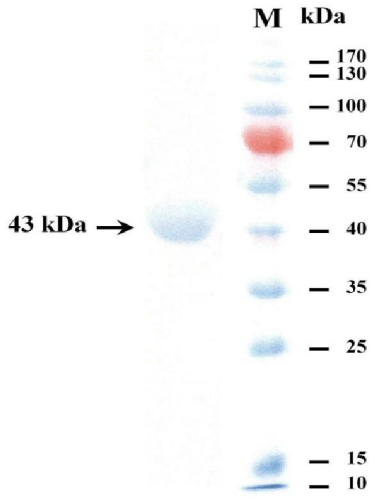

Figure 1. SDS–PAGE of the purified NmDXR enzyme revealed a single band at approximately 43 kDa by Coomassie Brilliant Blue staining. Lane M: prestained protein ladder (ThermoScientific, USA).

The enzyme mixture successfully used for crystallization contained 2 mg/ml NmDXR, 1mM $CoCl_2$, 3mM NADPH, and 1mM fosmidomycin in buffer A. The protein concentration used in the initial screening (2mg/ml) was chosen by using Hampton Research's Pre-Crystallization test.

To date, several crystal structures of DXR from *Escherichia coli* (Reuter *et. al.*, 2002; Steinbacher *et al.*, 2003; Mac Sweeney *et al.,* 2005; and Yajima *et al.*, 2002, 2004, 2007), from *Zymomonas mobilis* (Ricagno *et al.*, 2004) and from *Mycobacterium tuberculosis* (Henriksson *et al.*, 2006, 2007) have been reported. However, the crystal structure of DXR from *Neisseria meningitidis* has not yet been reported. Here, we report the crystallization of NmDXR in the presence of NADPH, cobalt ion and fosmidomycin. The structural study of NmDXR should be useful for the development of novel NmDXR inhibitors.

Many factors influence the likelihood of crystallization of a protein sample. Some of these factors include protein purity, pH, concentration of protein, temperature, precipitants and additives. The more homogeneous the protein solution is, the more likely that it will crystallize. Typically, protein samples above 97% purity are considered suitable for crystallization (Judge *et al.*, 1998), although high purity is neither necessary nor sufficient for crystallization. Solution pH can be very important and in extreme cases can result in different packing orientations. Buffers, such as Tris-HCl, are often necessary for the maintenance of a particular pH (Branden & Tooze, 1999). Precipitants, such as ammonium sulfate or polyethylene glycol, are usually used to promote the formation of protein crystals (Rhodes, 2006).

Trials to improve the crystallization conditions were performed by varying the pH, the buffer system and the concentration of the crystallizing agent. NmDXR crystals were observed in the screening trays kept at 4°C. Small crystals appeared after 4-7 days (Figure 2) in several wells, while the large crystals appeared after 6-8 weeks (Figure 3). The largest crystals with the best morphology grew from drops with well solution of 0.1M BisTris propane pH7.5, 20% PEG3350, and 0.2M NaF. It was found that the additive isopropanol at 3%v/v significantly improved crystal morphology and size.

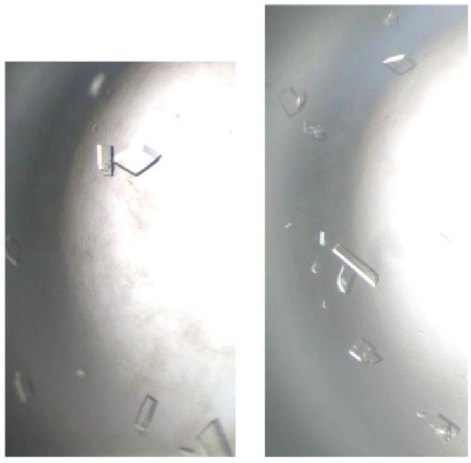

Figure 2. NmDXR small crystals appeared after 4-7 days with crystallization solution of 0.1M BisTris propane pH7.5, 20% PEG3350, and 0.2M NaF.

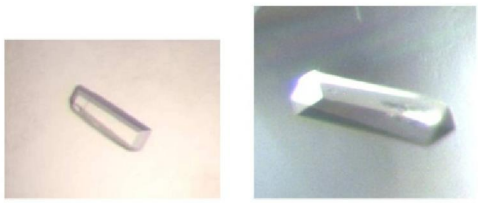

Figure 3. NmDXR large crystals appeared after 6-8 weeks with crystallization solution of 0.1M BisTris propane pH7.5, 20% PEG3350, 0.2M NaF, and 3%v/v isopropanol. Addition of isopropanol as additive improved the crystal morphology and size.

Based on the initial data collection attempts, 20%v/v PEG-400 was identified as the most promising cryoprotectant. However the crystal data quality was not yet acceptable, with high mosaicity and streaked diffraction spots caused by the crystal

transfer and freezing process. In the crystallization reoptimization, various amounts of PEG400 added as a component to the well solution. This strategy yielded several large crystals with dimensions up to 0.8 mm x 0.2 mm x 0.1 mm rod. The largest and best formed crystals came from a well containing 0.1M BisTris propane pH 7.5, 20% PEG3350, 0.2M NaF, 3% v/v isopropanol, and 10% v/v PEG400 (Figure 4). These crystals were transferred briefly into a cryoprotectant solution containing 0.1M BisTris propane pH 7.5, 20% PEG3350, 0.2M NaF, and 20% v/v PEG400, mounted in a cryoloop and flash-frozen in liquid nitrogen.

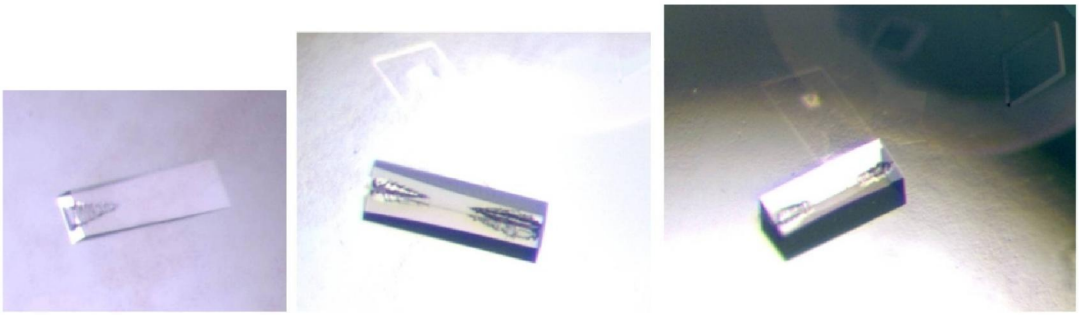

Figure 4. Reoptimization of NmDXR crystallization by adding the cryoprotectant PEG400 to the crystallization solution. NmDXR large crystals appeared after 8 weeks in crystallization solution of 0.1M BisTris propane pH7.5, 20% PEG3350, 0.2M NaF, 3%v/v isopropanol and 10%v/v PEG400.

A large (0.8mm) crystal of NmDXR (Figure 4) in complex with NADPH, Co^{2+}, and the inhibitor fosmidomycin was obtained and atomic-resolution data collected at the Advanced Light Source (Argonne National Labs, Chicago, IL) at beamline 21-ID-F of the Life Sciences Collaborative Access Team. High resolution and low resolution data were collected in two passes of 130 degrees each, with the detector set in the closest possible position for the high-resolution pass, and the crystal translated along its long axis between sets. No radiation damage was apparent. A representative diffraction image is shown in figure 5. The data were indexed and scaled using HKL2000; data scaling statistics are shown in table 1. The data are 99% complete, with maximum resolution of 1.2 Å. The space group is $P2_12_12_1$. The structure was solved by molecular replacement (MR) using the data to 3.0 Å and the AutoMR routine within Phenix. Choice of a research model was critical. None of the published DXR structures produced a clear MR solution singly, but an ensemble of three (1r0k, 2egh and 2jcv) succeeded with an initial R factor of 0.54. The structure was partially rebuilt with the AutoBuild routine, and initial refinement was carried out with Phenix refinement routines to an R/R_{free} of 0.27/0.29. The structure was manually rebuilt using O and Coot. Missing loops were modeled, the ligands and cobalt ion positioned, and ordered waters added. The structure was then refined to an R/R_{free} of 0.23/0.25 (using data to 2.0 Å) using the CNS package. The final stages of refinement (using data to 1.2 Å) are being performed using Phenix and SHELXL to model anisotropic displacement parameters, hydrogens, and the site occupancies of solvent molecules and of side chains was observed multiple conformations. The current R/R_{free} are 0.147/0.1715.

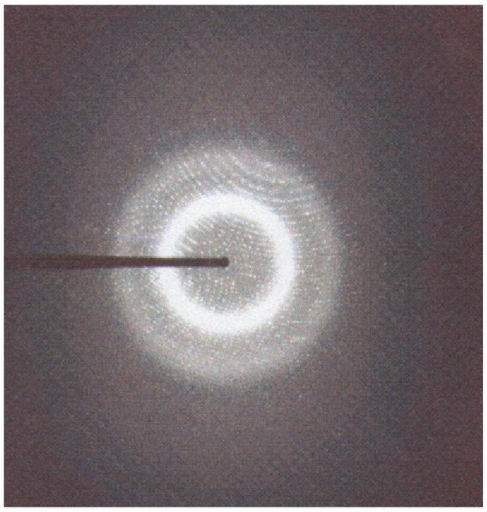

Figure 5. X-ray diffraction image from NmDXR crystal. The edge of the detector corresponds to a resolution of 1.2Å.

In the past few years macromolecular crystallography has become a standard technique used by many pharmaceutical and biotechnology companies. This methodology offers details of protein-ligand interactions at levels of resolution virtually unmatched by any other technique, and this approach promises novel, more effective, safer and

cheaper drugs (Scapin, 2006). The structural study of NmDXR presented in this research will help the development of novel NmDXR inhibitors and design new drugs for meningococcal disease.

Table 1. Data collection statistics – *N. meningitides* DXR

	Nm DXR Data
Beamline	APS21-ID-F
Space group	$P2_12_12_1$
Cell dimensions	
a, b, c (Å)	72.06, 95.25, 111.45
α, β, γ (°)	90.0, 90.0, 90.0
Resolution (Å)	50 – 1.18 (1.23 – 1.18)
R_{sym} or R_{merge}	0.047 (0.409)
$I/\sigma I$	13.32 (1.42)
Completeness (%)	99.0 (91.4)
Redundancy	2.4 (1.6)

Acknowledgment

We thank Dr. Honggao Yan, Professor of Biochemistry and Molecular Biology at Michigan State University, for providing recombinant NmDXR protein. We thank the Life Sciences Collaborative Access Team at Argonne National Labs, Chicago, IL for their help with the x-ray diffraction data collection. This study was supported by Michigan State University, USA.

References

1. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Protein Function. In: Molecular Biology of the Cell. 4th edition. New York: Garland Science, USA. 2002.
2. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem, 1976; 72:248–254.
3. Branden C, Tooze J. Introduction to Protein Structure. New York: Garland. 1999: 374–376.
4. Chayen NE and Saridakis E. Protein crystallization for genomics: towards high-throughput optimization techniques. Acta Crystallogr D Biol Crystallogr. 2002; 58:921-7.
5. Cox MJ, Weber PC. An investigation of protein crystallization parameters using successive automated grid searches (SAGS). J. Cryst. Growth 1988;90: 318–324.
6. Davey MS, Tyrrell JM, Howe RA, Walsh TR, Moser B, Toleman MA, Eberl M. A promising target for treatment of multidrug-resistant bacterial infections. Antimicrob. Agents Chemother. 2011; 55: 3635–3636.
7. Genco C, Wetzler L. Neisseria: Molecular Mechanisms of Pathogenesis. Caister Academic Press. 2010.
8. Green MR, Sambrook J. Molecular Cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA. 2012.
9. Henriksson LM, Björkelid C, Mowbray SL, Unge T. The 1.9 A resolution structure of Mycobacterium tuberculosis 1-deoxy-D-xylulose 5-phosphate reductoisomerase, a potential drug target. Acta Crystallogr D Biol Crystallogr. 2006; 62:807-13.
10. Henriksson LM, Unge T, Carlsson J, Aqvist J, Mowbray SL, Jones TA. Structures of Mycobacterium tuberculosis 1-deoxy-D-xylulose-5-phosphate reductoisomerase provide new insights into catalysis. J Biol Chem. 2007; 282(27):19905-16.
11. Iguchi E, Okuhara M, Kohsaka M, Aoki H, Imanaka H. Studies on new phosphonic acid antibiotics. II. Taxonomic studies on producing organisms of the phosphonic acid and related compounds. The Journal of antibiotics. 1980; 33 (1): 19–23.
12. Jancarik J, Kim SH. Sparse matrix sampling: a screening method for crystallization of proteins. J. Appl. Cryst. 1991; 24: 409-411.
13. Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, Türbachova I, Eberl M, Zeidler J, Lichtenthaler HK, Soldati D, Beck E. Inhibitors of the non-mevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. Science 1999; 285, 1573–1576.
14. Judge RA, Forsythe EL, Pusey ML. The effect of protein impurities on lysozyme crystal growth. Biotechnol Bioeng. 1998; 59(6):776-85.
15. Luft JR, Wolfley JR, Said MI, Nagel RM, Lauricella AM, Smith JL, Thayer MH, Veatch CK, Snell EH, Malkowski MG, DeTitta GT. Efficient Optimization of Crystallization Conditions by Manipulation of Drop Volume Ratio and Temperature. Protein Science : A Publication of the Protein Society 2007; 16.4: 715–722.
16. Mac Sweeney A, Lange R, Fernandes RP, Schulz H, Dale GE, Douangamath A, Proteau PJ, Oefner C. The crystal structure of E.coli 1-deoxy-D-xylulose-5-phosphate reductoisomerase in a ternary complex with the antimalarial compound fosmidomycin and NADPH reveals a tight-binding closed enzyme conformation. J Mol Biol. 2005; 345(1):115-27.

17. Otwinowski Z, Minor W. Methods in Enzymology: Macromolecular crystallography. Vol. 276. Edited by Charles W. Carter Jr and Robert M. Sweet. New York: Academic Press, USA. 1997; 276, 307–326.
18. Pace D, Pollard AJ. Meningococcal disease: clinical presentation and sequelae. Vaccine 2012; 30S:B3-B9.
19. Pérez-Gil J, Calisto BM, Behrendt C, Kurz T, Fita I, Rodríguez-Concepción M. Crystal structure of Brucella abortus deoxyxylulose-5-phosphate reductoisomerase-like (DRL) enzyme involved in isoprenoid biosynthesis. J Biol Chem. 2012; 287(19):15803-9.
20. Reuter K, Sanderbrand S, Jomaa H, Wiesner J, Steinbrecher I, Beck E, Hintz M, Klebe G, Stubbs MT. Crystal structure of 1-deoxy-D-xylulose-5-phosphate reductoisomerase, a crucial enzyme in the non-mevalonate pathway of isoprenoid biosynthesis. J Biol Chem. 2002; 277 (7):5378-84.
21. Rhodes G. Crystallography Made Crystal Clear, Third Edition: A Guide for Users of Macromolecular Models, 3rd Ed., Academic Press. 2006.
22. Ricagno S, Grolle S, Bringer-Meyer S, Sahm H, Lindqvist Y, Schneider G. Crystal structure of 1-deoxy-d-xylulose-5-phosphate reductoisomerase from Zymomonas mobilis at 1.9-A resolution. Biochim Biophys Acta. 2004; 1698(1):37-44.
23. Ryan KJ, Ray CG. Sherris Medical Microbiology (4th ed.). McGraw Hill. pp. 2004: 329–333.
24. Scapin G. Structural biology and drug discovery. Curr Pharm Des. 2006; 12(17):2087-97.
25. Singh N, Chevé G, Avery MA, McCurdy CR. Targeting the methylerythritol phosphate (MEP) pathway for novel antimalarial, antibacterial, and herbicidal drug discovery: inhibition of 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXR) enzyme. Curr. Pharm. Des. 2007; 13, 1161–1177.
26. Steinbacher S, Kaiser J, Eisenreich W, Huber R, Bacher A, Rohdich F. Structural basis of fosmidomycin action revealed by the complex with 2-C-methyl-D-erythritol 4-phosphate synthase (IspC). Implications for the catalytic mechanism and anti-malaria drug development. J Biol Chem. 2003; 278(20):18401-7.
27. Strelow VL, Vidal JE. Invasive meningococcal disease. Arq Neuropsiquiatr. 2013; 71(9B):653-8.
28. Takahashi S, Kuzuyama T, Watanabe H, Seto H. A 1-deoxy-D-xylulose-5-phosphate reductoisomerase catalyzing the formation of 2-C-methyl-D-erythritol 4-phosphate in the non-mevalonate pathway for terpenoid biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 1998; 95 (17): 9879–84.
29. Tunkel AR, van de Beek D, Scheld WM. Acute meningitis. In: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 7th ed. Churchill Livingstone Elsevier 2010; 1189-1229.
30. Umeda T, Tanaka N, Kusakabe Y, Nakanishi M, Kitade Y and Nakamura KT (). Crystallization and preliminary X-ray crystallographic study of 1-deoxy-D-xylulose 5-phosphate reductoisomerase from Plasmodium falciparum. Acta Cryst. 2010; F66: 330–332.
31. Van Deuren M, Brandtzaeg P, Van Der Meer JWM. Update on meningococcal disease with emphasis on pathogenesis and clinical management. Clin Microbiol Rev 2000; 13: 144-166.
32. Xiao B, Gamblin SJ. The effects of cryoprotectant on crystal stability. Journal of Crystal Growth. 1996; 168 (1–4):244–247.
33. Yajima S, Hara K, Iino D, Sasaki Y, Kuzuyama T, Ohsawa K, Seto H. Structure of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in a quaternary complex with a magnesium ion, NADPH and the antimalarial drug fosmidomycin. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2007; 63:466-70.
34. Yajima S, Hara K, Sanders JM, Yin F, Ohsawa K, Wiesner J, Jomaa H, Oldfield E. Crystallographic structures of two bisphosphonate: 1-deoxyxylulose-5-phosphate reductoisomerase complexes. J Am Chem Soc. 2004; 126(35): 10824-5.
35. Yajima S, Nonaka T, Kuzuyama T, Seto H, Ohsawa K. Crystal structure of 1-deoxy-D-xylulose 5-phosphate reductoisomerase complexed with cofactors: implications of a flexible loop movement upon substrate binding. J Biochem. 2002; 131(3):313-7.

2/28/2015