Journal of Photochemistry & Photobiology, B: Biology 163 (2016) 352–365

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Ligand based pharmacophoric modelling and docking of bioactive pyrazolium 3-nitrophthalate (P3NP) on *Bacillus subtilis*, *Aspergillus fumigatus* and *Aspergillus niger* — Computational and Hirshfeld surface analysis

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ARTICLE INFO

Article history:
Received 20 July 2016
Accepted 27 August 2016
Available online 31 August 2016

Keywords:
Antimicrobial
MIC
DFT
Pharmacophore
Molecular docking
Hirshfeld surface analysis

ABSTRACT

Biologically active Lewis acid-base compound, pyrazolium 3-nitro phthalate (P3NP) has been synthesized and crystallized by slow evaporation - solution method at 30 °C. Spectral and single crystal X-Ray diffraction (XRD) were used to characterize the compound. The stability of the P3NP was confirmed by UV–Visible spectral analysis. P3NP crystallizes in monoclinic $P2_1/C$ space group with cell parameters, a = 13.009 (3) Å, b = 12.584 (3) Å, c = 7.529 (18) Å and β = 93.052 (4)° with Z = 4. Crystal packing was stabilized by N^+—H···O^-, O—H···O and C—H···O intermolecular hydrogen bonds. The nature of anion - cation interactions and crystal packing from various types of intermolecular contacts and their importance were explored using the Hirshfeld surface analysis. The structure was optimized by Density Functional Theory at B3LYP level with 6-311++G(d,p) basis set and the vibrational frequencies were theoretically calculated. Band gap between Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) and Electrostatic potential (ESP) were calculated. Antimicrobial activities of P3NP with targets were clinically tested and were found to exhibit antibacterial activity against gram positive and antifungal activity against pathogens with Minimum Inhibitory Concentration (MIC). Ligand based pharmacophore modelling was used to understand the potential of P3NP ligand to bind with selected target proteins. iGEM Dock was used to predict the modes of interactions of the ligand with target proteins of the microbes predicted from pharmacophore. PreADMET predicts no absorption of ligand in Human Intestinal Absorption (HIA).

1. Introduction

Pyrazoles refer to a class of heterocyclic five membered ring compounds with two nitrogen atoms in adjacent positions [1]. The synthesis of pyrazoles is of great interest to researchers due to their wide applications in pharmaceutical industry. Pyrazole and its derivatives have shown appreciable biological activities such as anti-microbial [2], analgesics [3,4], anti-inflammatory [5], antifungal [6], antibacterial [7], trypanocidal activity [8] and brain neuron inducing activity [9]. Pharmacological interactions are useful for understanding mechanisms of a therapeutic target (protein) - ligand (drug) binding. Molecular docking programs have been used for a long time to explore these relationships with reference to metabolism. However, a simple docking study is not much useful in understanding good interaction profiles such as binding sites, ligand preparation, virtual screening and post-screening analysis. An integrated approach combining these virtual screening stages and pharmacological interactions is valuable for drug discovery. An easy to use graphic environment, iGEM (*G*eneric *E*volutionary *M*ethod for molecular docking), provides biological insights by deriving the pharmacological interactions of screening pairs (Ligand–Target) compounds without relying on the long term experimental trials. iGEM dock is an integrated environment which integrates the heavily modified and enhanced in house tool GEM dock, protein-ligand profiles, pharmacological interactions and compound clusters. iGEM dock has been successfully applied to identify new inhibitors and new binding sites for some targets [10–15]. In this present investigation, computational evaluation of non-covalently interacted ligand (P3NP) with microbes especially with the help of pharmacophore prediction, electrostatic potential analysis from DFT calculations and Hirshfeld surface analysis have been carried out. All these analyses strengthen the docking studies (virtual screening) and this novel combination of ligand based pharmacological prediction with molecular docking provides an useful insight of identification and development of new medicinally important compounds. The absorption, distribution, metabolism, excretion (ADME) and toxic

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http://dx.doi.org/10.1016/j.jphotobiol.2016.08.045
1011-1344/

(TOX) properties of P3NP have been calculated with the aid of PreADMET server.

2. Experimental Details

2.1. Synthesis and Characterization of P3NP

2.1.1. Synthesis of P3NP

Analar grades of pyrazole and 3-nitro phthalic acid (E-Merck) were dissolved in 1:1 equimolar proportions using ethanol - methanol solvent mixture. The resultant mixture was stirred for 6 h and the reaction mixture was filtered off and kept aside for about three weeks without any physical disturbance in a dust free atmosphere at 30 °C. P3NP crystals were harvested after 21 days and the crystals were recrystallized several times to enhance the purity.

2.1.2. Spectral Measurements

The Fourier transform infrared (FT-IR) spectra of P3NP were recorded using a Perkin Elmer RX1 spectrometer in the region 4000–400 cm^{-1} using KBr pellet method. The FT-Raman spectrum of sample in the solid phase was recorded in the range 4000–50 cm^{-1} using Bruker RFS 27 stand-alone FT-Raman spectrometer with a 1064 nm Nd:YAG Laser source of 100 mW power. The UV–Vis spectra of P3NP were recorded using JASCO spectrometer (V-570). The ^{1}H and ^{13}C NMR spectra of the P3NP were recorded using BRUKER-DRK 50–400 MHz spectrometer instrument with tetramethyl silane (TMS) as an internal standard and D_6-DMSO as solvent.

2.1.3. Single Crystal X-Ray Diffraction

Crystal data for P3NP were collected at 30 °C on a BRUKER SMART APEX CCD X-Ray diffractometer using λ (Mo Kα) 0.71073 Å. Data reduction was carried out by Bruker SAINTPLUS [16] and absorption correction was made using SADABS [17]. The structure was solved using SHELXS-97 [18] and refined using SHELXL-97 [19]. Non-hydrogen atoms were refined with anisotropic thermal parameters. All hydrogen atoms were fixed using HFIX command in SHELX–TL.

2.2. Computational Details

Density functional theoretical (DFT) quantum chemical computations were carried out at the B3LYP level with 6-311++G(d,p) basis set using Gaussian'09 program package [20] to obtain optimized parameters, vibrational wavenumbers, IR intensities, vibrational frequencies and Raman activity in P3NP crystal structure. The optimized vibrational frequency values were multiplied by 0.978 to offset the systematic errors caused by the basis set in order to get completeness for electron correlation and vibrational anharmonicity. The predicted values were found to be in good agreement with experimental values for both FT-IR and FT-Raman and these spectra have been plotted using pure Lorentzian band shape with a bandwidth of full width and half maximum (FW-HM) of 10 cm^{-1} [21]. NBO calculations [22] were performed using NBO 3.1 program to understand inter and intramolecular delocalization or hyperconjugation. iGEMDock [23] was used to perform docking studies of P3NP. It generates protein-ligand interaction profiles of electrostatic interactions (E), hydrogen bonding (H) and van der Waals interactions (V). Trial version of Ligand Scout 3.12 was used to generate the pharmacophoric sites of ligand [24].

2.2.1. Preparation of Protein From Protein Databank

To validate the pharmacological interactions, three well-known therapeutically beneficial targets from protein data bank (RCSB PDB) such as 4D4U, 4ZA5 and 4OZ5 were selected. 4OZ5 is an important for its bacterial activity while 4D4U and 4ZA5 are important targets for fungal activity. Ligand P3NP was energy minimized (optimized) using Gaussian output file before docking. Proteins retrieved from protein data bank were cleaned by removing water molecules and previously docked ligands using UCSF CHIMERA molecular visualization software.

2.3. Experimental Antimicrobial Analysis

2.3.1. Preparation of Inoculum

The inoculums for the experiments were prepared in fresh nutrient broth from preserved slant culture. The inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards [25]. The turbidity of the culture was adjusted by the addition of sterile saline or broth [26].

The inoculums were dried at room temperature. The P3NP sample along with the standard ciprofloxacin (10 μg) antimicrobial drug was kept in a Petri dish. For effective diffusion, the Petri dishes were placed in the refrigerator at 4 °C for 1 h followed by incubation at 37 °C for 24 h. Various zones of inhibition produced by the antibiotic were measured. For antifungal screening, the inoculums were prepared in Sabouraud's broth and clotrimazole antifungal drug (10 μg) was used as the standard.

The antimicrobial activity of P3NP was screened by the method of Perez et al. [27]. Seven human pathogenic microorganisms were used. Out of which, namely, *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Salmonella paratyphi* are bacterial strains and *Candida albicans, Aspergillus niger and Aspergillus fumigatus* are fungal strains [28].

2.3.2. Minimum Inhibitory Concentration (MIC) Analysis

MIC is defined as the minimum concentration of the drug that prevents the appearance of visible growth of a microorganism within a defined period under *in-vitro* conditions.

In sterile condition, serial 2-fold dilutions of the test antimicrobial agents were made in 1 ml of Muller Hinton broth and Sabouraud's dextrose broth for bacterial and fungal growth respectively. Overnight cultures were grown at 37 °C by adopting Kirby - Bauer procedure for both diluted Muller Hinton broth and Sabouraud's dextrose broth. Two sets of eight sterile tubes were labelled as 1 to 8 and 8th tube was taken as control. Accurately, 1 ml portions of Muller Hinton broth (bacterial medium) were added in the first set of eight tubes and 1 ml of Sabouraud's Dextrose Broth (fungal medium) portions were added in the second set of eight tubes. Then, 1 ml of drug solution was transferred to 1st tube, mixed well and then 1 ml of solution was pipetted out from the 1st tube and it was transferred to the 2nd tube and this process was repeated up to 7th tube and finally 1 ml from the 7th tube was discarded and the procedure was repeated for the second set of tubes also. About 0.01 ml of *Bacillus subtilis* culture was added to all the eight test tubes of the first set and 0.01 ml of *Aspergillus fumigatus* in the second set of tubes. All the 16 tubes were incubated at 37 °C for 18–24 h. After incubation, the formation of turbidity was observed by visual method.

3. Results and Discussion

3.1. Spectral and XRD Characterization of P3NP

3.1.1. Fourier Transform Infrared Spectral Analysis

The FT-IR spectrum of P3NP crystal was recorded in the range of 4000–400 cm^{-1} in order to confirm various functional groups in the molecule. The recorded spectrum of P3NP is shown in Fig. 1. The formation of the compound is strongly evidenced by the presence of characteristic bands in the spectrum. The broad band in the range 3167–2200 cm^{-1} clearly indicates presence of intense and excessive intermolecular hydrogen bonding. The vibrational frequency of —OH of free COOH in the 3-nitrophthalate anion is observed at 2598 cm^{-1}. The absorption bands at 3167 and 3143 cm^{-1} are due to the asymmetric and symmetric stretching frequencies of N—H respectively. The band at 2363 cm^{-1} is due to the stretching vibration of tertiary amino NH^{+} cation. The presence of deprotonated carboxylate anion (COO^{-}) is confirmed by the stretching frequency at 1607 cm^{-1}. The frequency at