Drug likeliness of 3,6-diisopropyl-2,5-piperazinedione - produced by marine sponge associated bacteria

*Rhodopseudomonas palustris* MSB 55

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Abstract

Drug discovery and development is an intense, lengthy and an interdisciplinary endeavour. Drug discovery is mostly portrayed as a linear, consecutive process that starts with target and lead discovery, followed by lead optimization and pre-clinical in vitro and in vivo studies to determine if such compounds satisfy a number of pre-set criteria for initiating clinical development. In this present study a sponge Axinella donani was collected from Kanyakumari coast line and their associated bacteria were isolated and identified. Various therapeutic compounds were identified by the techniques such as spectroscopic and chromatographic methods. Drug likeliness of 3,6-diisopropyl-2,5-piperazinedione - produced by *Rhodopseudomonas palustris* MSB 55 was studied by Autodock from this study, it was found to be a Neuronal Nicotinic receptor served as target for 3,6-diisopropyl-2,5- piperazinedione. ADME/TOX tool helped to find out whether the molecule is obeying Lipinski’s Rule of Five to evaluate drug likeness. From the present study it could be concluded that the sponge associated bacteria can serve as a source for therapeutic compounds.

Keywords: Axinella donani, *Rhodopseudomonas palustris*, MSB 55, 3,6-diisopropyl-2,5- piperazinedione, neuronal nicotinic receptor, autodock, ADME/TOX.

Introduction

The marine environment has been explored in the search of new bioactive molecules for the last 60 years. Oceans cover over 70% of the Earth’s surface and constitute an extremely rich source of biological and chemical diversity. Marine invertebrates have proven to be an outstanding source of active molecules, among which many are classified as indole alkaloids. Although many of these marine alkaloids closely resemble the endogenous amines (serotonin, dopamine or histamine), their potential affinity to various neurological targets and consequential impact on animal behavior is virtually unexplored. Given that depression affects approximately 18 million Americans annually, it is crucial to develop new effective treatments for this disorder. Intensive studies are being conducted in the area of new targets for antidepressant drugs, but most of these agents
still target the neurotransmitter systems, mainly serotonin, dopamine, and noradrenalin. Natural products have played a significant role in drug discovery. Over the past 75 years, natural product derived compounds have led to the discovery of many drugs to treat human disease. Drugs developed from marine sources give us this hope and also give us novel mechanisms to fight some of the most debilitating diseases encountered today, including: HIV, osteoporosis, Alzheimer’s disease and cancer. Although, the costs associated with developing drugs from marine sources have been prohibitive in the past, the development of new technology and a greater understanding of marine organisms and their ecosystem are allowing us to further develop our research into this area of drug development (Vignesh et al., 2011). This study is, an as yet untrodden path in drug discovery, for the global health benefits of humankind from marine environment.

Nicotinic acetylcholine receptors (nAChR) have been strongly implicated as therapeutic targets for treating cognitive deficits in disorders such as schizophrenia and Alzheimer’s disease (AD). In particular alpha7 and alpha4beta2 subtype-selective nAChR agonists and partial agonists have been developed as potential candidates for the treatment of schizophrenia, cognitive disorders (including Alzheimer’s disease), and inflammation. (Haydar & Dunlop, 2010). The present study was undertaken to find out whether the test compound serve as a target for nicotinic acetylcholine receptor or not.

**Materials and methods**

**Sampling and isolation of sponge-associated bacteria**

The study material Axenella donani was collected from Keelamanakudi, a coast of kanyakumari, by lobster catching technique with the help of fisherman. It was transferred to the lab using ice box. Upon collection sponge colonies were put into sterile plastic bags. The tissues were then rinsed with sterile seawater and homogenized with blender. The homogenized tissues were serially diluted up to 10^-7, spread plate method was carried out on ½ strength ZoBell marine agar medium and incubated at room temperature for 48 hours. On the basis of morphological features, colonies were pure cultured (Madigan et al., 2000).

**Morphological examination of sponge by scanning electron microscope**

Scanning electron microscope facility from IIT Chennai was used for the morphological study of sponge. A portion of sponge in ethanol was placed onto a specimen holder and optionally coated with gold in a Cressington 108 auto Sputter Coater. The specimens were examined and photographed in a Philips XL30 ESEM scanning electron microscope, high vacuum mode, using BSE detector with accelerating voltage 15 kV.

**PCR procedure**

An unknown bacterium was identified by 16S rRNA sequencing. Universal primer for eubacteria (Forward primer: 5’-AGAGTTTGATCMTGGCTCAG-3’, Reverse primer: 5’-AAGGAGGTGWTCCARCC-3’) was used for this study. The reaction mix was prepared by adding Master mix 25μl, Primer -forward(10pmoles/μl) 1μl, Primer-reverse(10pmoles/μl) 1μl, Bacterial Genomic DNA 2μl, Water, nuclease free 21μl, Total volume 50μl. It was mixed gently and spun down briefly. It was placed into PCR machine and programmed as follows; (Initial Denaturation : 94°C for 3 minutes, Denaturation: 94°C for 1 minute, Annealing: 60°C for 1 minute 30 cycles, Extension: 72°C for 1 minute 30 sec, Final extension: 72°C for 5 minutes. PCR product was electrophoresed and the molecular weight was determined using DNA markers. DNA of unknown bacteria were sequenced by gene sequencer. The determined DNA sequence of unknown bacteria was then compared for homology to the BLAST database. CLUSTALW, ORF FINDER, BPROM, GENSCAN and FGENESH were also used for the sequence analysis.

**Media optimization**

Zobell Marine agar was used to isolate the bacteria; Modified Starch Agar, Modified
Carboxy cellulose agar, and Tryptic Soy Agar were used for the screening of primary metabolites. Zobell Marine Broth, Tryptic digest broth and Brain Heart infusion broth were used for the production of bioactive compounds. Maximum amount of secondary metabolite producing media was found out. Media compositions were slightly modified for the cultivation of marine bacteria.

**Strain improvement**

Primary and secondary metabolite producing ability was studied in both wild strain and UV treated strains. Rhodopseudomonas palustris MSB 55 was inoculated on Typtic soy agar and exposed to UV for 10 minutes. Plates were incubated at 37°C for 24 hours. Treated isolates were inoculated on Casein milk agar and incubated at 37°C for 24 hours. Zone of proteolysis on both the strains were compared with a wild strains proteolytic pattern.

**Purification of bioactive compounds**

Tryptic digest broth was prepared and optimum conditions were maintained to produce maximum yield and wide activity. Following inoculation with strains Rhodopseudomonas palustris MSB 55 and Rhodobacter sphaeroides MSB 57, it was incubated in a shaker at 25°C for 72 hours. It was centrifuged at 10,000rpm for 10 minutes, the supernatant was filter-sterilized (0.2μm pore-size filter), heated at 85°C for 10minutes, and stored at 4°C until use.

**Solvent extraction**

Cell free extract was acidified using 0.1N HCL and various solvents such as n-Butanol, Methanol, Hexane, Chloroform: Methanol (2:1) and Ethyl acetate were used to extract the bioactive compounds. Compound Purification & Characterization: (Fazuo et al., 2008) Ethyl acetate extract was purified by preparative TLC, followed by column chromatography was performed. The crude extract was subjected to chromatography over silica gel column using a stepwise gradient elution of petroleum ether/CHCl3/MeOH to yield 10 fractions (1–10). Fraction 3 was subjected to Sephadex LH-20 eluting with CHCl3/MeOH (1:1), followed by chromatographing on a silica gel column eluting with CHCl3/MeOH (60:1),to afford six sub fractions. Fraction 6 was subjected to repeated silica gel column chromatography using a stepwise gradient elution of petroleum ether/CHCl3/MeOH to yield three sub fractions . The procedure was repeated for three more times to get a pure compound. Purified compounds were analyzed by Nanoveu, Solubility was studied and other qualitative analysis was done.

**Gas chromatography and mass spectroscopy**

The GC/MS analyses were carried out on GC/MS [JEOL GCMATE II GC-MS] system equipped with a quantitative analysis by SIM mode detector (Indian Institute of Technology) (SAIF- Chennai) . A VF-5ms column of 30m length, 0.25mm diameter, and 0.25μm film thickness was used. The oven was programmed from an initial temperature 70 ºC (hold for 2 min) to the final temperature 300 ºC at the rate of 10(35.0minutes). The final temperature hold up time was 10 minutes. Helium at the rate of 1 ml/min was used as the carrier gas in constant flow mode. The inlet and interface temperatures were kept at 2800 ºC . The EI source was operated at 2300 ºC and the quadrupole temperature was 5000 ºC. The MS was scanned from 1-3,000 m/z. One micro litre of the sample was injected in split mode at a split ratio of 40. WILEY library search and NIST library search were used for compound identifications.

**Preparation of ligand**

The ligand compounds was drawn using ACD/ Chemsketch (12.0) (Alex, 2009) and saved in mol 2 format. The saved ligand compound was later imported and minimized in Argus Lab after adding hydrogen bonds. The molecule thus obtained was saved in PDB format.

**Argus lab**

Argus Lab is an electronic structure program that is based on the quantum mechanics; it predicts the potential energies, molecular structures, geometry optimization of structure, vibration frequencies of coordinates of atoms, bond length, bond angle
and reaction pathway (Cheng, 2003). The energy (E) of the molecule is calculated as E = E stretching + E bending + E torsion + E Vander Waals + E electrostatic + E hydrogen bond + cross term. These terms are of importance for the accurate calculation of geometric properties of molecules. The set of energy functions and the corresponding parameters are called a force field (Afshan, 2009).

**Genetic algorithm**

Genetic algorithm (GA) is a computer program that mimics the process of evolution by manipulating a collection of data structures called chromosomes. It is also stochastic optimization methods and provides a powerful means to perform directed random searches in drug designing (Shahper, 2008). It study properties of QSAR, utilizes the novel representation of the docking process, each chromosome encodes an internal conformation and protein active site and includes a mapping from hydrogen-bonding sites in the ligand and protein (Seiburg, 1990; Rajasekhar et al., 2010). On decoding a chromosome, fitness is evaluated by PLS (partial least squares) cross validation to position the ligand within the active site of the protein, in such a way that as many of the hydrogen bonds suggested by the mapping are formed (Kimura et al., 1998). Docking of flexible ligands to macromolecules is paramount in structure based drug design, few programs that work with GA also enable automated docking; another application of GA is the automated generation of small organic molecules using lipophilicity, electronic properties and shape related properties for calculation of the scoring function (Nissink et al., 2002).

Docking using gold

Genetic algorithm was implemented in GOLD 3.2 that was applied to calculate the possible conformations of the drug that binds to the protein (Selvaraj, 2008). The genetic algorithm parameters used are population size-100, number of islands-5, niche size-2, selection pressure-1.1, migrate-2, number of operators-100,000, mutate-95, cross over-95. During docking process, a maximum of 10 different conformations was considered for the drug. The conformer with highest binding score was used for further analysis (Girija et al., 2010)

**ADME/ toxicity testing**

ADME (absorption, distribution, metabolism and excretion) determines drug like activity of the ligand molecules based on Lipinski rule of 5 (Konstantin, 2005). Increasing clinical failures of new drugs call for a more effective use of ADME/TOX technologies, becoming more advanced and reliable in terms of accuracy and modeling show promise for increase in the accuracy of Insilco ADME-TOX prediction used for virtual screening in lead optimization (Gregory, 2004).

<table>
<thead>
<tr>
<th>S.No</th>
<th>Analysis</th>
<th>MSB 55</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ORF FINDER</td>
<td>FRAME 3 from reverse strand is the best ORF which potentially encodes a protein</td>
</tr>
<tr>
<td>2.</td>
<td>BPROM RESULT</td>
<td>Three Promoter regions and transcription factor binding sites were found at the 1094,227,772 position.</td>
</tr>
<tr>
<td>3.</td>
<td>GENSCAN</td>
<td>51.6% C+G, Two exonic region was found which is an initial exonic region at the positions 519,931</td>
</tr>
<tr>
<td>4.</td>
<td>FGENESH</td>
<td>One gene was found at the position from 3 to 170. GAPCYDFTPVMNPTLGSGLLTTRLPTSGETHSHGVTGGVYKTRERIHRDILIRDY</td>
</tr>
</tbody>
</table>
**Results and discussion**

Identification of sponge by scanning electron microscope

SEM examination helped to identify the sponge based on the shape of the spicules present inside the sponge. The diameter of the spicule was 50μm. The anterior end was sharper and tapered, where as the posterior end was broader. Maximum magnification power used was only 1000X because when the magnification power was increased inorder to study more morphological details, the naturally occurring metals collided with the beam of electron, which made the vision blurred.

16s rRNA sequencing for the identification of unknown bacteria

Universal primer was used for 16S rRNA sequencing. The determined DNA sequences of strains were then compared for homology to the BLAST database. CLUSTALW, ORF FINDER, BPRM, GENSCAN and FGENESH were also used for the sequence analysis. The result revealed that there were 97% homology of the sequence MSB 55 with Rhodopseudomonas palustris.(FASTA sequence)Fig.1. shows the phylogenetic analysis of unknown bacteria.

Sponges are a prolific source of biological compounds with diverse bioactivities. However,a structural similarities between the metabolites of the sponge and its associated bacteria, those found within its tissue indicates that these compounds are of bacterial origin. 16S rRNA gene sequence analyses have been widely used to identify and characterize both culturable and unculturable populations of marine bacteria. The purpose of this research was to isolate sponge associated bacteria and screen them for antibacterial activity (Luis et al., 2006).In this present study also the unknown bacteria were identified using 16S rRNA sequencing. In addition to that, other bioinformatics tools such as ORF FINDER, BPRM, GENSCAN and FGENESH were also used for the sequence analysis (Table. 1).

**Table 2.Effect of UV mutation on yield of the bioactive compounds**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Strain name</th>
<th>wild strain on DB(g/l)</th>
<th>wild strain on ZMB(g/l)</th>
<th>UV exposed strain on TDB(g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Rhodopseudomonas palustris</em> MSB 55</td>
<td>6.111</td>
<td>5.700</td>
<td>2.590</td>
</tr>
</tbody>
</table>

Media optimization

There were three different media used for the production of secondary metabolites. Their biomass production ability was studied. All the three media were slightly modified inorder to grow the sponge-associated bacteria. Rhodopseudomonas palustris MSB 55 had showed higher yield in Tryptic digest broth. It was 68.4% higher than Zobell marine broth and 81.6% higher than Brain heart infusion broth is predicted in Fig.2.

Strain improvement

UV mutation was accomplished to improve the product yield of the sponge associated bacteria. After mutation both their primary and secondary metabolite production ability was studied, which gave a characteristic result. There were no yield improvement in terms of secondary metabolites but their proteolytic activity was drastically increased. There was a 57.6% reduction in the bioactive compound yield by Rhodopseudomonas palustris MSB 55.
Liutskanova, et al., 2005, used conventional mutagenesis (UV irradiation and exposure to nitrosoguanidine) to produce and regenerate protoplasts, aiming at increasing the antibiotic activity of a Streptomyces fradiae strain producing tylosin. Variants exceeding the activity of the initial procedure strain by 0.5 – 28.3% were obtained. The most active variants were produced by a combined exposure to UV and nitrosoguanidine, as well as upon regeneration of protoplasts formed from the cells of clones produced by UV irradiation. Unstable inheritance of the trait of increased tylosin production was demonstrated. But in this present study we observed a novel result. There was no bioactive compound yield improvement after UV treatment. But proteolytic activity was improved.

Solvents such as n-Butanol, Methanol, Hexane, Chloroform: Methanol (2:1) and Ethyl acetate were used to extract bioactive compounds from the selected bacteria. Among the five solvents studied ethyl acetate was found to be a suitable solvent.

**GC/MS Analysis**

GC/MS analysis of the partially purified bioactive compound determined the presence of 3,6-diisopropyl-2,5-piperazinedione. (Fig. 3).

**Autodock & ADME/TOX analysis**

3,6-diisopropyl-2,5-piperazinedione had showed Maximum passive absorption 100%. Contribution from tranacellular route was 98% and paracellular route was 2%. Molecular Weight was 184.23, number of Hydrogen Bond Donors were 2, number of Hydrogen Bond Acceptors were 4, TPSA was 58.20 and number of Rotatable Bonds were 2. Log P was 0.55 and AMES test score was 0.073 represented in Fig.4 & 5.

The predicted target was Neuronal Nicotinic receptor. Docking energy was determined as -8.059kcal/mol and the source organism was human. The AChE-inhibitory compounds, isolated from the marine sponge R. sarai, are alkylpyridinium salts, recently isolated from several marine sponges. Natural AChE inhibitors are common, but only few have been isolated from marine organisms. Among them, we can only mention onchidal, an irreversible inhibitor from the mollusk Onchidella binneyi (Abramson et al., 1998) and a pseudozoanthoxanthin-like compound from the encrusting coral Parazoanthus axinellae. Synthetic alkylpyridinium compounds have recently been described as anti-cholinesterase agents (Barak et al., 1995). The anticholinesterase activity of the R. sarai extract is due to two compounds of this family, two large polymeric 3-alkylpyridinium compounds (poly-APS).

Fazuo et al, 2008, revealed that seven new prenylated indole diketopiperazine alkaloids, including compound 1,3 spirotryprostatins C-E (2-4), 2 derivatives of fumitremorgin B (5 and 6), and 13-oxoverruculogen (7), have been isolated from the holothurian-derived fungus Aspergillus fumigatus, along with 12 known ones (8-19). The structures of the new compounds were determined on the basis of extensive spectroscopic data and amino acid analysis. All new compounds were evaluated for their cytotoxic activities on MOLT-4, A549, HL-60, and BEL-7420 cell lines by the MTT and SRB methods.
Fig. 4. Docking - target neuronal nicotinic receptor
Fig. 5 ADME/TOX for 3,6-Diisopropyl-2,5-Piperazinedione

FASTA sequence

ATTAAGGCCTCTTTGGATTCATCGATCAGTACCCGTCCTACCCGTCATGAGGAAAGCCGCCTTACGTCTGAGGCTAC
CTACCTTTGCTGAAAACCCACACTCCATCTGTTGACGCGGGTGCTGTGAAAGCTAAGACCCTCCGGGAACTACG
ATCCGACATCCGAGGCTGTATCCTACCTGGCTGGGTTTGACATGACGGTGCTGTGAGGCTTCGAGCTATCAC
TGAGCCATGAGCTACACCGCTCTGAGGCTAGGTGACGATCTGAGGCTTACGAGCTACACCGCTCTGAGGCTA
GCATGAGGACTTGACGTCAT

Rani juenius et al.  "Production of novel antimicrobials" (Indian J. Med. Healthcare)
Sites | Residues and atoms
---|---
Site 1 | NH1 ARG A 187
Site 2 | NH2 ARG 30
Teresa et al. 2000, stated that the potentiation of central cholinergic activity has been proposed as a therapeutic approach for improving cognitive function in patients with Alzheimer’s disease. Increasing the acetylcholine concentration in brain by modulating acetylcholinesterase (AChE) activity is among the most promising strategies. We have used a combinatorial approach to identify different 2, 5-piperazinediones (DKP) with AChE inhibitory activity. Our goal was to find inhibitors exhibiting high AChE/BuChE (butryrylcholinesterase) selectivity, in order to reduce the undesirable side effects elicited by most of the inhibitors that have been developed to date. Screening of a DKP library constructed on solid-phase using the multiple parallel synthesis format, resulted in the identification of several compounds with moderate efficacy on AChE.

Conclusion

The exploitation of marine microbial life and the associated secondary metabolites, aided by genomic analyses, applying metabolic approach and employing combined biomedical and biotechnological efforts, which would lead to discovery of some novel, lead compounds of a varied degree of bioactivity. Rhodopseudomonas palustris MSB 55 had showed higher yield in Tryptic digest broth. It was 68.4% higher than Zobell marine broth and 81.6% higher than Brain heart infusion broth. There was no bioactive compound yield improvement after UV treatment. But proteolytic activity was improved.

Target for 3, 6-diisopropyl-2, 5-piperazinedione was found to be Neuronal Nicotinic receptor. 3,6-diisopropyl-2,5-piperazinedione had showed Maximum passive absorption 100%. Contribution from transcellular route was 98% and Paracellular route was 2%. Molecular Weight was 184.23, number of Hydrogen bond donars was 2, number of Hydrogen Bond Acceptors was 4, TPSA was 58.20 and number of rotatable bonds was 2. Log P was 0.55 and AMES test score was 0.073. Hence the compound 3,6-diisopropyl-2,5-piperazinedione produced from the natural source Rhodopseudomonas palustris MSB 55 may serve as a novel drug.

References

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http://www.iseeadyar.org/ijmhc.html

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