Screening and Identification of Potential iNOS Inhibitors to Curtail Cervical Cancer Progression: an In Silico Drug Repurposing Approach

Pavan Kumar Poleboyina1 · Shailima Rampogu2 · Ravinder Doneti1 · Akbar Pasha1 · Sneha Malleswari Poleboyina3 · Shivaaji Bhanothu1 · Deepthi Pasumarthi1 · Annapurna S.D.1 · DivyaVishambhar Kumbhakar1 · Keun Woo Lee2 · Smita C. Pawar1

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Abstract
Cervical cancer is the second most common cause of cancer deaths in women worldwide and remains the main reason of mortality among women of reproductive age in developing countries. Nitric oxide is involved in several physiological functions inclusive of inflammatory and immune responses. However, the function of NO in tumor biology is debatable. The inducible NOS (iNOS/NOS2) isoform is the one responsible to maintain the levels of NO, and it exhibits pleotropic effects in various cancers with concentration-dependent pro- and anti-tumor effects. iNOS triggers angiogenesis and endothelial cell migration in tumors by regulating the levels of vascular endothelial growth factor (VEGF). In drug discovery, drug repurposing involves investigations of approved drug candidates to treat various other diseases. In this study, we used anti-cancer drugs and small molecules to target iNOS and identify a potential selective iNOS inhibitor. The structures of ligands were geometrically optimized and energy minimized using Hyperchem software. Molecular docking was performed using Molegro virtual docker, and ligands were selected based on MolDock score, Rerank score, and H-bonding energy. In the study shown, venetoclax compound demonstrated excellent binding affinity to iNOS protein. This compound exhibited the lowest MolDock score and Rerank score with better H-bonding energy to iNOS. The binding efficacy of venetoclax was analyzed by performing molecular docking and molecular dynamic simulations. Multiple parameters were used to analyze the simulation trajectory, like root mean square deviation (RMSD), radius of gyration (Rg), and hydrogen bond interactions. Based on the results, venetoclax emerges to be a promising potential iNOS inhibitor to curtail cervical cancer progression.

Keywords Nitric oxide synthase · Cervical cancer · Drug repurposing · Anti-cancer drugs · Small molecules · Venetoclax
**Introduction**

Continual human papillomavirus (HPV) infection is the primary reason for cervical cancer, and it has led to the development of prophylactic vaccines to prevent HPV infection and HPV assays [1]. In the world, cervical cancer (CC) ranks second in causing cancer deaths in women. Due to the increased pap smear test for early detection of CC, the average death rate has declined in developed countries [2]. The global prevalence of cervical cancer is about 510,000 new cases annually, with about 288,000 deaths globally [3]. In India, 122,844 women get diagnosed and 67,477 succumb to death from the disease of cervical cancer annually. In the Indian population, 15 years and older-aged women are at high risk of developing cancer, and cervical cancer is the second most common cancer in women aged 15–44 years. By comparing the highest standardized incidence of cervical cancer in South Asia, India has the highest age at 22 than other countries, i.e., 19.2 in Bangladesh, 13 in Sri Lanka, and 2.8 in Iran.

Nitric oxide (NO) is one of the principal oxides of nitrogen that mediates a variety of actions such as neurotransmission, host defense, iron metabolism, and vasodilatation, but increased NO production contributes to a variety of disorders including cancer [4]. iNOS is a member of a nitric oxide synthase(NOS) family of enzymes and produces NO$^{-}$ [5]. There are three isofoms of NOS in nature: endothelial (eNOS), neural (nNOS), and inducible (iNOS). The inducible NOS exhibits increased expression in several types of cancer, but the prognostic role in cervical cancer still remains unclear [6]. High levels of NO produced by the NOS2 stimulate the angiogenesis process in tumors by controlling VEGF expression. NOS2 knockdown in SiHa and HeLa cells resulted in a reduction in cell proliferation due to a drop in VEGF levels, suggesting that NOS2 regulates cervical cancer cell growth in a VEGF-dependent manner. iNOS may influence the levels of vascular endothelial growth factor (VEGF), as VEGF is induced by iNOS during endothelial cell migration and angiogenesis, according to some experimental findings. Following the suppression of iNOS by a lentivirus, Dong et al. (2014) discovered that SiHa and HeLa cells have lower levels of proliferation [7, 8]. Hepatocellular carcinoma angiogenesis may be aided by the presence of iNOS and VEGF. Hepatocellular carcinoma tissues had higher levels of iNOS and VEGF expression, as well as a larger density of microvessels, than non-cancerous tissues [9].

In our previous study, we demonstrated that iNOS expression levels were reduced by using plant-derived diterpenoid andrographolide, in both in silico and in vitro [10]. In the present study, we adopt an in silico drug repurposing approach to identify a potential inhibitor for iNOS by using anti-cancer drugs and small molecules.

**Materials and Methods**

The study involves retrieval of target protein in PDB format from RCSB Protein Data Bank [11] and retrieval of ligands, anti-cancer drugs, and small molecules from Pubchem database [12], ZINC database [13], and DrugBank [14]. Due to the presence of cofactors and peptide bonds in ligands, some structures were drawn and converted into PDB files through Hyperchem8 software [15] and ChemDraw Pro 12.0 software (RRID:SCR_016768) [16]. Molecular docking was carried out by Molegro virtual
Retrieval of Target Protein

3d structure of NOS2 (iNOS) human inducible nitric oxide synthase with inhibitor protein (PDB ID: 4NOS) was retrieved from RCSB Protein Data Bank. Before docking, the unwanted hetero atoms, water molecules, and other ligand compounds were removed by MVD.

Retrieval of Anti-cancer Drugs and Small Molecules

After virtually screened various compounds, a total of 162 top hit lead optimized compounds were selected for further analysis. The 3d and 2d structures of ligands were retrieved from Pubchem database, ZINC database, and DrugBank, and some structures were drawn in ChemDraw pro 12.0 and Hyperchem8 package; they were pre-optimized using molecular mechanics force field procedure (MM+, AMBER) in Hyperchem8 [20]. To get the conformers of anti-cancer drugs and small molecules with the lowest energy, the semi-empirical method AM-1 was applied to the molecular structures. To avoid the local stability, each molecular structure was geometrically optimized several times with different starting points using the Polak-Rebiere algorithm, till the root mean square gradient becomes equal to 0.01 and 0.001 kcal Å−1 mol−1 [21]. Finally, the energy minimization of each ligand structure was performed. The first step and second step calculations, i.e., single point calculations that are used to determine the total molecular energy of structure and geometric optimization calculations (MM+, AMBER force field) to determine the energy minimization algorithms that locate the flexible structures, were done by using Hyperchem8 [22].

Prediction of Ligand-Binding Active Sites

The ligand-binding active sites in iNOS (iNOS, PDB ID: 4NOS) protein for anti-cancer drugs and small molecule ligands are not well characterized. The amino acid residues responsible for active site formation in iNOS protein were identified through the MVD cavity detection algorithm. This MVD detected 5 different active sites with high volume surface in iNOS protein.

Protein Preparation

iNOS human inducible nitric oxide synthase with inhibitor protein (PDB ID: 4NOS) was selected for the molecular docking studies using MVD. All necessary H atom addition, valency checks were performed. MVD provided utilities and protein preparation, i.e., protonation addition of mismatched amino acid residues was repaired, optimized, and rebuilt by MVD. For docking studies, it is important that the imported structures must be prepared accurately, the atom connectivity and bond orders are correct, and the partial atomic charges are assigned. PDB files have a poor or missing assignment of explicit hydrogens, and the PDB file format cannot accommodate bond order information. Then the repair, rebuilt, and optimized protein was saved in * .Mol format. The final structure was visualized and analyzed with
Ligand Preparation

The selected anti-cancer drugs and small molecules were downloaded from Pubchem, ZINC database, and DrugBank. Some torsion and peptide bond containing candidates were drawn and minimized its energy by using Hyperchem8 software and imported to MVD workspace in *.Mol format. Before import, the small molecules and anti-cancer drugs are subjected to series of steps to generate optimization and variation of the structure, as the energy minimized ligands have the capability to form better bonding with protein.

Docking Studies

In the current study, a docking algorithm called MolDock has been used. It is based on a new hybrid search algorithm, called guided differential evolution. It combines differential evolution optimization technique with an active site prediction algorithm [25]. For the docking process, GRID resolution was set to 0.30Å, and the cavities were set on the coordinate values $X=16.90$, $Y=61.22$, and $Z=17.64$. Other embedded parameters were used which consist of a maximum iteration of 1500, simplex evolution of 300, neighbor distance factor 1.00, and the highest population size of 50. For each ligand, 10 independent runs were performed with the differential evolution algorithm, with every 10 independent runs of these docking returning 5 docking poses and selecting the top most pose for consideration. MVD shows higher docking accuracy than the other docking software (MVD: 87%, Glide: 82%, Surflex: 75%, FlexX: 58%) [26]. The scoring function used by MVD is called MolDock which is derived from the PLP scoring functions. The docking scoring function MolDock score energy, $E_{\text{score}}$, is defined by (1), where $E_{\text{inter}}$ is the ligand-receptor interaction energy and $E_{\text{intra}}$ is the internal energy of the ligand. According to Eq. (2), $E_{\text{inter}}$ is calculated.

$$E_{\text{score}} = E_{\text{inter}} + E_{\text{intra}}$$

$$E_{\text{inter}} = \sum_{i=\text{ligand}} \sum_{j=\text{protein}} \left[ E_{\text{PLP}}(r_{ij}) + \frac{332.0}{4r_{ij}^2}q_i q_j \right]$$

The $E_{\text{PLP}}$ is a piecewise linear potential by using two different sets of parameters, i.e., the steric and hydrogen bonds [27]. According to Eq. (3), $E_{\text{intra}}$ calculates the energy involved in the pair of atoms of the ligand and torsional energy where $h$ is the torsional angle of the bond. $E_{\text{clash}}$ assigns a penalty of 1000 kcal/mol if the distance between two heavy atoms is similar than 2.0Å.

$$E_{\text{intra}} = \sum_{i=\text{ligand}} \sum_{j=\text{protein}} [E_{\text{PLP}}(r_{ij})] + \sum_{\text{flexible bond}} A[1 - \cos(m\theta - \theta_s)] + E_{\text{clash}}.$$

Pharmacophore Modeling

By using the LigandScout 4.4.5 software package (RRID:SCR_014889), the structure-based pharmacophore model was generated [28]. Herein, the proposed algorithm studies
and interprets ligand-receptor interactions such as hydrogen bonds, hydrophobic regions, and charge transfer regions of the macromolecular environment from PDB files allowing the automatic binding of the pharmacophore model. The exclusion volume spheres were added to the generated pharmacophore model which represents the inaccessible areas along with any potential ligand [29].

**Drug-Likeness and ADMET Properties**

The drug scan and ADMET properties were performed to determine that the inhibitor has the fulfilled conditions as the drug candidate based on Lipinski’s Rule of Five [30]. The pharmacokinetic properties, toxicity, and bioavailability of selected ligands were carried out by using the Molinspiration cheminformatics tool [31], SwissADME tool [32], and pkCSM tool [33].

**Molecular Dynamic Simulations**

Molecular dynamics simulation (MDS) studies were conducted to delineate on the stabilities of the protein-ligand complex and further to understand their interaction at atomistic level, as reported earlier [34–36] using GROMACS v2016.16 [37], using CHARMM27 all atom force field. The ligand topologies were obtained from SwissParam [38]. The dodecahedron water box of TIP3P water model was used to solvate the system and further the counter ions were added. This was followed by the minimization of the system after coupling of the protein and the ligand. The coupling process was proceeded by double equilibration method by the conserved number of particles (N), system volume (V), and temperature (T) (NVT) and the constant number of particles (N), system pressure (P) and temperature (T) (NPT) for 1 ns each. The NVT ensembles were escalated to MDS for 50 ns. The Parrinello-Rahman barostat [39] was used to monitor the pressure of the system. During the simulation process, the protein backbone was restrained, while the solvent molecules and the counter ions were permitted to move. The geometry of the molecules was maintained by LINCS algorithm that constrains the bond length [40]. All the analysis were carried out using visual molecular dynamics (VMD) [41] and DS. The results were studied according to the root mean square deviation (RMSD), radius of gyration (Rg) [42], number of hydrogen bonds, and the key residue interactions.

**Results and Discussion**

**Target Protein Structural Information**

The NOS2 (iNOS) human inducible nitric oxide synthase protein (PDB ID: 4NOS) with a resolution of 2.25Å was retrieved from the RCSB protein data bank. The structural confirmations of protein were obtained by PDBsum [43], and attained PROCHECK [44], and ERRAT [45] validations by uploading its structure in structure validation server (SAVESv6.0) [46]. The obtained 3d cartoon, secondary structural analysis, and Ramachandran plot are shown in Fig. 1.

The iNOS protein active sites were obtained by the MVD cavity detection algorithm the five detected cavities with their volume are illustrated in Fig. 2.
Fig. 1 Structural information of protein iNOS. a 3d cartoon structure, b secondary structure of 3pty mapped obtained using PDBsum (c), and d Ramachandran plot.

Fig. 2 MVD generated cavities of protein iNOS.
Ligand Structure Information

Total 162 ligand structures of anti-cancer drugs and small molecules were used in docking to find out its inhibitory binding effect with the target protein. The drug compounds structural information and its flexibility confirmation with after single point and geometry optimization minimized energies are represented in Supplementary Table 1.

Docking Result Validation

Herein, we used MVD for docking studies to obtain MolDock score, Rerank score, interaction energy, torsions, and H-bonding energy. To find out the protein-ligand interaction, each compound undergoes 10 runs with threshold energy 100 and is finally given 5 best poses. Out of 5 poses, we have selected the top 1 pose for each ligand having the lower energy along with higher MolDock score, lower Rerank score, and best hydrogen bonding. The obtained energies of each ligand are shown in Supplementary Table 2.

Among all docked 162 ligands, venetoclax showed harmonious inhibitory effect with iNOS protein. Based on the average MolDock score $-150$ kcal/mol to $-160$ kcal/mol, we have selected above-average MolDock score obtained ligands for further studies. The top compound venetoclax revealed the lowest MolDock score on iNOS protein which is $-276.37$ kcal/mol.

Venetoclax binds into the active site of iNOS protein with MolDock score $-276.37$ kcal/mol, and its binding site consists of the following amino acid residues: Phe488, Tyr489, Trp194, Ala197, Pro198, Leu125, Tyr491, Met355, Phe369, Val352, Pro350, Arg266, Tyr373, Cys200, Arg199, Trp463, ile462, Val465, Pro466, Pro467, Arg381, Met374, Gln357, Asp382, and Arg388. The docked amino acid residues against the ligand were Arg388, Tyr373, Tyr491, Trp463, and Val465. Figure 3 illustrates the ligand hydrogen bonding interactions with amino acid residues of iNOS protein. Figure 4 represents the key amino acid residues of iNOS protein forming steric interactions with ligands. Figures 5 and 6 show the ligand docked against the crystal protein structures of human inducible nitric oxide synthase and the amino acid residues around active and docked against iNOS protein, respectively. Our validated results of pharmacophore models are illustrated in Fig. 7.

ADMET Properties and Drug-Likeness Results

A rapid dissociation rate will occur with the weaker binding, the more negative binding energy analogue to the strong binding of selected compounds to the target iNOS protein [47]. The lower binding energy exhibited compounds give the strong binding with iNOS protein, and the selected compounds have shown the lower binding energies. According to the Rule of Five, a molecule might be no longer orally active if it violates or is greater than the 4 rules [48]. To obtain the pharmacophore properties, ligand canonical smiles were uploaded to pkCSM, SwissADME, and Molinspiration cheminformatics tools, and the results are illustrated in Table 1.
Fig. 3  Hydrogen bond interactions of venetoclax with protein iNOS

Fig. 4  Steric interactions of venetoclax with iNOS protein
Fig. 5 Ligand docked against the crystal protein structure of iNOS with venetoclax

Fig. 6 The amino acid residues around active site and docked amino acids against iNOS with venetoclax
Molecular Dynamic Simulation Results

RMSD

The RMSD calculates the deviations in the protein backbone from the initial to the final conformation and thus measures the stability of the protein [49]. In the present study, the RMSD of the protein backbone (bb) and the protein-ligand complex (com) were measured. The results have shown that calculated values have demonstrated a stable RMSD with any deviations. The bb RMSD has relatively shown a lower RMSD than the com, while both displayed stability throughout the simulation run (Fig. 8A).

Rg

The Rg interprets the compactness of the protein [50]. The Rg was found to be stable, existing between 2.32 and 2.27 nm indicating that the protein bb is highly compact (Fig. 8B), with an average of 2.28 nm.
Furthermore, the number of hydrogen bonds was monitored throughout the simulation run that exists between the protein and the ligand. It was noticed that the hydrogen bonds have existed throughout the simulation run inferring that the compound has accommodated within the binding pocket. In a noteworthy observation, the number of hydrogen bonds was more in the last 10 ns (Fig. 8C).

### Table 1 Pharmacophore kinetic and drug-likeness and ADMET properties of top selected ligand

| Property                      | Model name              | Venetoclax | Unit                        |
|-------------------------------|-------------------------|------------|-----------------------------|
| Absorption                    | Water solubility        | −3.03      | log mol/L                   |
|                               | CaCO₂ permeability      | 0.84       | log Papp in 10–6 cm/s       |
|                               | Intestinal absorption (human) | 100     | %Absorbed                  |
|                               | Skin permeability       | −2.73      | log Kp                      |
| Distribution                  | VDss (human)            | 0.329      | log l/kg                    |
|                               | Fraction unbound (human) | 0.169     | Fu                          |
|                               | BBB permeability        | −1.74      | log BB                      |
|                               | CNS permeability        | −3.11      | log PS                      |
| Metabolism                    | CYP2D6 substrate        | No         | Yes/no                      |
|                               | CYP3A4 substrate        | Yes        |                             |
|                               | CYP1A2 inhibitor         | No         |                             |
|                               | CYP2C19 inhibitor        | No         |                             |
|                               | CYP2C9 inhibitor         | No         |                             |
|                               | CYP2D6 inhibitor         | No         |                             |
|                               | CYP3A4 inhibitor         | Yes        |                             |
| Excretion                     | Total clearance         | −0.09      | log mL/min/kg               |
|                               | Renal OCT2 substrate    | No         | Yes/no                      |
| Toxicity                      | AMES toxicity           | No         |                             |
|                               | Max. tolerated dose (human) | 0.278  | log mg/kg/day               |
|                               | hERG I inhibitor        | No         | Yes/no                      |
|                               | Oral rat acute toxicity (LD50) | 2.60   | Mol/kg                      |
|                               | Oral rat chronic toxicity (LOAEL) | 1.924 | log mg/kg_bw/day           |
|                               | Hepatotoxicity          | Yes        | Yes/no                      |
|                               | Skin sensitization      | No         |                             |
|                               | T. pyriformis toxicity   | 0.285      | log μg/L                    |
|                               | Minnow toxicity         | −0.48      | log μg/L                    |
| Drug-likeness                 | Lipinski                | No         | Yes/no                      |
| Bioactivity score             | GPCR ligand             | −2.36      |                             |
|                               | Ion channel modulator   | −3.42      |                             |
|                               | Kinase inhibitor        | −3.00      |                             |
|                               | Nuclear receptor ligand  | −3.33      |                             |
|                               | Protease inhibitor      | −1.92      |                             |
|                               | Enzyme inhibitor        | −2.87      |                             |

**Hydrogen Bonds**

Furthermore, the number of hydrogen bonds was monitored throughout the simulation run that exists between the protein and the ligand. It was noticed that the hydrogen bonds have existed throughout the simulation run inferring that the compound has accommodated within the binding pocket. In a noteworthy observation, the number of hydrogen bonds was more in the last 10 ns (Fig. 8C).
From the last 5 ns stable RMSD, the representative structure was extracted and the interactions were visually inspected. The ligand has formed hydrogen bond interactions with residues Ser118, Glu377, and Arg388 (Fig. 9A), respectively, stabilizing the ligand at the active site. The ring F of the ligand has interacted with Phe369 and Cys200 residue by $\pi-\pi$ stacked and $\pi$-sulfur interactions. The ring E has prompted alkyl and $\pi$-alkyl interactions with several residues holding the ligand firmly at the active site.

Interactions

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binding pocket. The ring A and ring B are held via Met120 residues by π-alkyl interactions. The ring G and F have formed π-cation interactions and ring D has interacted with Val352, respectively (Fig. 9B). The other residues have formed van der Waals and carbon hydrogen bonds positioning the ligand at the binding pocket of the protein (Fig. 9B and Table 2).

The enzyme iNOS also called inducible NOS or inflammatory NOS plays a critical role in inflammation and tissue damage in cancer cells [51]. Docking studies have proved to be an essential tool to discover and identify the potential target candidates for a particular protein [52]. In this study, anti-cancer drugs and small molecules were used to identify the potential inhibitor for human inducible nitric oxide synthase protein. Among 162 docked compounds, we have identified that venetoclax showed better inhibitory effect on iNOS.

**Conclusion**

In summary, anti-cancer drugs and small molecules from the PubChem, ZINC database, and DrugBank were docked against iNOS protein. Molecular docking and molecular dynamic simulation studies revealed that venetoclax interacted with iNOS protein and demonstrated that it has an excellent inhibitory binding effect on iNOS and is a potential candidate to be used as drug to reduce iNOS expression in cervical cancer tumor cells. Venetoclax is sold under the brand name of Venclexta, and it is an antineoplastic agent which blocks BCL-2 and promotes apoptosis in various cancer cells [53]. In docking, it has shown the lowest MolDock score −276.37 kcal/mol, Rerank score −157.09 kcal/mol, and H-bonding energy −7.4 kcal/mol with iNOS protein. All these drug candidates are widely using in the treatment of various cancers. Thus, this study identified venetoclax as the best inhibitory compound from anti-cancer drugs and small molecules to inhibit iNOS protein in cervical cancer. However, further in vitro and in vivo studies are warranted to validate the promising potential of this compound to be used as iNOS protein inhibitor to restrain the cervical cancer progression. These results open the way for further experimental confirmation as a drug repurposing approach for iNOS inhibitor to combat cervical cancer.

| Name of the bond         | Interacting residues                                      |
|--------------------------|-----------------------------------------------------------|
| Conventional hydrogen bond| SER118, GLU377, ARG388                                    |
| Carbon hydrogen bond     | TRP463, ARG 381, TYR 491, MET 355, CYS 200, VAL352, ARG 388, MET120 |
| Pi-cation                | ARG A:381                                                 |
| Pi-sulfur                | CYS A:200                                                 |
| Pi-Pi stacked            | PHE A:369                                                 |
| Vander walls             | ILE 462, ALA282, ASN 354, GLY371, TYR489, PRO198, LEU125, TYR373, ILE 119 |
Findings

1. The key amino acids of iNOS protein which are forming H-bonding interactions with ligands are Phe488, Tyr489, Trp194, Ala197, Pro198, Leu125, Tyr491, Met355, Phe369, Val352, Asn354, Gln263, Pro350, Arg266, Tyr373, Cys200, Arg199, Trp463, ile462, Val465, Pro466, Pro467, Arg381, Met374, Glu377, Asp382, and Arg388.

2. The common amino acid Arg388 is involved in all ligand interactions and forms H-bonding with ligands. So, from this we deduce that the amino acid ARG388 of iNOS protein has the highest binding efficiency to form H-bonding with ligands.

3. The key amino acids which are involved in steric interactions are Cys200, Met355, Tyr489, Pro198, Arg199, Glu377, Tyr491, and Asp382.

4. Based on our in silico analysis, we conclude that venetoclax exhibits the potential to effectively inhibit iNOS expression in cervical cancer.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12010-021-03718-2.

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Author Contribution SCP and PKP conceptualized, designed interpreted data, and edited manuscript; PKP, AP, DR, SDA, SB, SMP, DK, SR, DP and KWL designed and conducted the study. All authors have approved the manuscript in the current form.

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Data Availability Data supporting the productivity of this investigation are available from the corresponding author upon request.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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Authors and Affiliations

Pavan Kumar Poleboyina1 · Shailima Rampogu2 · Ravinder Doneti1 · Akbar Pasha1 · Sneha Malleswari Poleboyina3 · Shivaji Bhanothu1 · Deepthi Pasumarthi1 · Annapurna S.D.1 · DivyaVishambhar Kumbhakar1 · Keun Woo Lee2 · Smita C. Pawar1

Pavan Kumar Poleboyina
pavanbiotech2012@gmail.com

1 Department of Genetics & Biotechnology, University College of Science, Osmania University, Hyderabad, Telangana 500007, India

2 Division of Life Sciences, Division of Applied Life Science (BK21 Plus), Research Institute of Natural Science (RINS), Gyeongsang National University (GNU), 501 Jinju-daero, Jinju 52828, South Korea

3 Department of Pharmaceutical Biotechnology, AU College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh 530003, India