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Development and Validation of a Multi-Level Computational Protocol for Drug Re-Purposing in the Treatment of Bacterial Infections †

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Abstract: Here we report the optimization of a methodology using docking and virtual screening to identify novel clinical uses for already approved drugs. The molecular targets selected were MvfR and PqsD due to their crucial role in quorum-sensing and biofilm formation and development. The FDA approved subset of the ZINC database was screened after careful validation of the Virtual Screening protocol and molecules obtained in the top 1% for each target were further analyzed. Presented here are the top 5 molecules selected for each target.

Keywords: *Pseudomonas aeruginosa*; biofilms; quorum-sensing; PQS system; virtual screening; molecular dynamics; MM/GBSA

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1. Introduction

Pseudomonas aeruginosa is an opportunistic gram-negative bacterium, responsible for acute and chronic infections. It is a highly adaptable pathogen, and it is becoming extremely difficult to eradicate due to acquired resistance and tolerance to drugs [1,2]. This bacterium can be found in planktonic state or in an association called biofilm, the ultimate way of protection in adverse conditions [3]. Biofilms are an association of microorganisms organized within a self-produced extracellular polymeric substance matrix. This matrix confers stability and works like a protective armor against antimicrobial compounds as well as providing increased virulence that often leads to chronic infections [4–7].

Like many other bacterial species, *P. aeruginosa* can control the expression of genes, population density and biofilm formation through a process called quorum-sensing (QS). Quorum sensing is a cell-cell communication mechanism controlled by the release, detection, and response to signaling molecules called autoinducers. It controls, among other aspects, biofilm formation and the transcription of several virulence genes [8].

Quorum-sensing in *P. aeruginosa* is rather complex and hierarchical. It uses four types of signaling systems, two of which are based on acyl homoserine lactones (LasR, RhlR), one that uses quinolone as signaling molecules (PQS) and one whose mechanism and targets are still unknown (IQS) [9,10]. The LasR system is at the top of the hierarchy, but integration with the RhIR and PQS systems is fundamental as a regulatory link to control the direct and indirect expression of several virulence genes [11] Targeting the QS system will not kill the bacteria, but it will hamper its pathogenicity and the possibility to resistance is diminished as there is less selective pressure on the bacteria [12].

The focus of this work is the PQS system, more specifically the proteins PqsR (also known as Multiple Virulence Factor Regulator—MvfR) and PqsD. PqsD is a Anthraniloyl-CoA anthraniloyltransferase required for the biosynthesis of several signalling molecules such as HHQ. Catalyzes the transfer of the anthraniloyl moiety from antraniloyl-CoA to malonyl-CoA to form 2-aminobenzoylacetyl-CoA (2-ABACoA). Involves the formation of a covalent bond between Cys112 and antraniloyl-CoA [13]. MvfR is a transcriptional regulator responsible for the transcription of virulence genes). It interacts with two native ligands: 2-Heptyl-3-hydroxy-4(1H)-quinolone (also called the Pseudomonas Quinolone Signal, PQS) and its precursor 2-heptyl-4- hydroxyquinoline (HHQ). It controls its own activity by upregulating the expression of genes in the pqsABCDE and phnAB operons which encode other enzymes [1]. Studies have shown that interfering with PqsR and PqsD leads to a more efficient attenuation of pathogenicity than single target approaches [14].

In this work, a docking and virtual screening (VS) protocol was applied, to discover new inhibitors for MvfR and PqsD proteins, using the ZINC FDA approved database as starting point. Drug repurposing is becoming an attractive approach to the drug discovery process since the repurposed drugs are, safe, already in use and well characterized, reducing the drug developing time and cost [15].

2. Materials and Methods

2.1. Docking Protocol Validation

The Protein Databank [16] and in the Biofilms Structural database [17] were explored to find molecular structures of MvfR and PqsD. A total of 12 strucutres for MvfR and 3 for PqsD were found. All the fifteen x-ray structures were prepared for docking using Pymol, with the extraction of water molecules and crystallographic ligands (these were saved in separate files to be used as reference in the following steps). For MvfR there is a variety of X-ray structures and X-ray ligands, however, that is not the case for PqsD, as there are only 3 protein structures and 2 ligands.

For this work, the docking software GOLD [18] was used (with all its scoring functions (SFs): CHEMPLP, GoldScore, ChemScore and ASP). The purpose of testing all the different scoring functions was to evaluate which one is the best for these specific hydrophobic targets, as it has been demonstrated that docking results can vary significantly depending on the type of protein target and ligand [19,20]. The docking conditions were the same for every SF and every target to ensure consistency and reproducibility. The optimized conditions consisted of binding site coordinates and radius, number of runs and search efficiency. The protocol described was applied separately for MvfR and PqsD.

As a first step in the protocol validation, re-docking was performed to evaluate the ability of the docking software to reproduce the geometry and orientation of the crystallographic pose. The root mean square deviation (RMSD) between the heavy atoms of the crystallographic and docked poses was calculated, and the resulting scores were evaluated. The docking conditions were optimized with the goal of obtaining the lowest RMSD possible. The next step toward protocol validation was to perform cross-docking. This strategy, as a measure of robustness of target structures and methodology, is quite simple to perform. All the crystallographic ligand structures isolated from the protein structures of both target were "docked" into the different X-ray structures. This test aimed to evaluate the ability of individual X-ray structures in enabling the correct docking of different X-ray ligands, co-crystallized in other X-ray structures. The RMSDs in both cases was calculated using DockRMSD [21]. A good result is the one that presents a high positive score and a RMSD below 2 Å.

2.2. Virtual Screening Protocol Validation

For this stage, all the structures that presented mutations were removed. Only the best structures obtained in the docking protocol validation stage were selected to move on to the VS protocol validation (4JVI and 6B8A for MvfR and 3H76 and 3H77 for PqsD).

The VS protocol was validated with a benchmark dataset to ensure that it provides reliable results. For MvfR and PqsD a specific virtual screening training library was prepared, to evaluate and optimize the ability of the protocol in discriminating between binders and non-binders. After an initial query in the ChEMBL [22] and BindingDB [23] databases and a brief literature review, 40 molecules with experimental activity against MvfR were found, the active pool of the test set. Using the DUD-E [24] database, a set of 50 decoys for each ligand was created. Decoys are molecules that resemble the ligands in their physical properties but are chemically and topologically different so that they are most likely non-binders. The total number of decoys generated was 2000. The final test set for MvfR was composed of 2040 compounds. The same protocol was followed for PqsD and the final dataset was composed of 59 active molecules and 2950 decoys.

The discriminatory ability of the five scoring functions were assessed and the evaluation metrics were calculated using a web-based application, Screening Explorer [25] as well as Excel. The metrics used for the evaluation of the VS results were the enrichment factor at 1% (EF 1%), Receiver operating characteristic (ROC) curves and respective area under the curve (AUC) and Total gain (TG). TG quantifies the discrimination of actives over decoys attributable to score variations. TG values over 0.25 combined with an AUC over 0.5 indicate a good performance and reproducibility from the VS protocol [25].

2.3. Virtual Screening of ZINC FDA Approved Compounds

At this stage, only the best SFs and the X-ray structures that yielded a better actives/decoys discrimination in the validation stage was selected.

FDA-approved drugs, which is a subset of ZINC [26] a free database of commercially available compounds for virtual screening. ZINC contains over 230 million purchasable compounds. At the time of the VS experiments, the FDA-approved drugs dataset had 3207 compounds that were all docked against the target. The top 5 compounds for each of the protein targets, were selected to move on to further studies.

3. Results and Discussion

The two x-ray structures, from each target, that provided the highest scores and lowest RMSD values (data not shown) in the re-docking and cross-docking stage were selected to move on to the VS stage.

Table 1 summarizes the results obtained for these two chosen structures for all the SFs tested, for MvfR. CHEMPLP, ChemScore and ASP provided good discriminatory ability between binders and non-binders for both structures with an EF1% of 10.40. However, CHEMPLP did not provide a good TG value for both structures. The TG value for ASP and 4JVI structure was also not satisfactory. Ultimately, the best TG value was obtained for structure 6B8A and ASP SF, and that was the combination that was used in VS of the FDA approved compounds.

Table 1. Evaluation metrics for Virtual Screening results for x-ray structures for MvfR (4JVI and 6B8A).

	4JVI			**6B8A**		
	EF 1%	AUC	TG	EF 1%	AUC	TG
CHEMPLP	10.40	55.11	0.08	5.20	53.18	0.07
GoldScore	0.00	50.43	0.01	0.00	46.28	0.005
ChemScore	5.20	48.95	0.005	2.60	51.73	0.02
ASP	10.40	66.42	0.21	10.39	65.81	0.25

The same analysis was performed for PqsD and the results are presented in Table 2. In this case, the SFs that provided the best results across all the metrics were CHEMPLP and GoldScore in structure 3H76. Because the AUC of CHEMPLP is slightly higher, that was the SF selected to move on to the next stage.

Table 2. Evaluation metrics for Virtual Screening results for x-ray structures for PqsD (3H76 and 3H77).

	3H76			3H77		
	EF 1%	AUC	TG	EF 1%	AUC	TG
CHEMPLP	1.73	67.89	0.25	1.73	59.19	0.03
GoldScore	1.70	65.99	0.25	1.73	53.16	0.06
ChemScore	0.00	70.46	0.02	1.73	59.95	0.03
ASP	0.00	70.65	0.02	1.73	62.72	0.01

After performing the VS protocol for the ZINC FDA approved database, only the molecules present in the top 1% were analyzed, corresponding to a total of 30 compounds for each protein target. Table 3 lists the top 5 results obtained for MvfR and Table 4, the top 5 compound for PqsD. A brief description of the pharmaceutical use of each compound is provided, along with the score that was obtained in the VS. Different SF use different metrics and scales, hence the difference between the ASP and CHEMPLP scores.

Table 3. Top 5 hits of the FDA approved drugs database for MvfR.

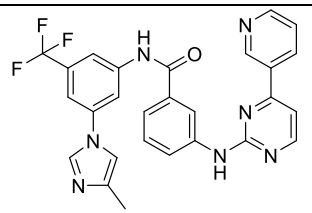
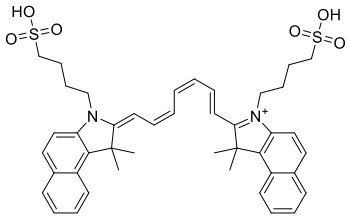
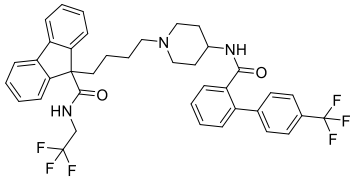
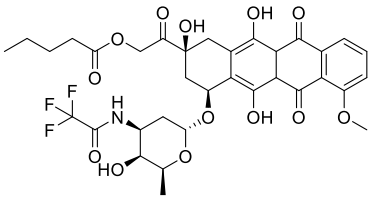
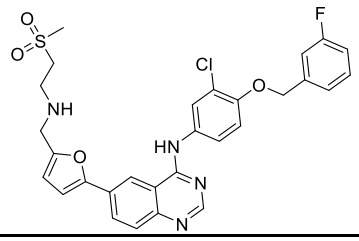
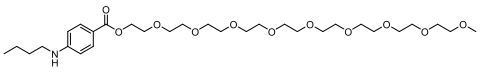
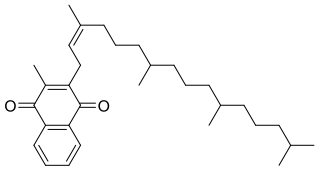
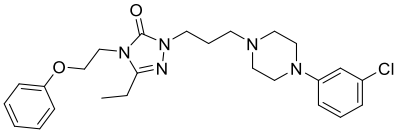
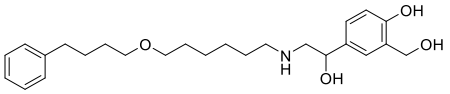
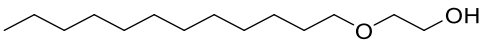
Drug Name	Description	Structure	ASP Score
Nilotinib	Bcr-Abl tyrosine kinase inhibitor (TKI) used in the treatment of chronic myelogenous leukemia (CML)		**54.96**
Indocyanine Green	Dye used in medical diagnosis. It has been used to measure cardiac output, liver function, and in ophthalmic angiography [27]		**50.55**
Lomitapide	Used to treat patients with Homozygous familial hypercholesterolaemia (HoFH). It is an inhibitor of MTP, an enzyme responsible for the synthesis of low-density lipoproteins in the liver [28]		**50.01**
Valrubicin	Chemotherapy drug used to treat carcinoma in situ bladder tumors		**49.86**
Lapatinib	Inhibitor of tyrosine kinase domains of epidermal growth factor receptor and human epidermal growth factor receptor (HER)-2. Used to treat metastatic HER-2+ breast cancer [29]		**49.89**

Table 4. Top 7 hits of the FDA approved drugs database for PqsD.

Drug Name	Description	Structure	CHEMPLP Score
Tessalon	**A** non-narcotic oral antitussive agent**.**		**93.53**
Vitamin K1	A lipid cofactor that is required for normal blood clotting**.**		**93.01**
Nefazodone	**A** phenylpiperazine antidepressant **that** potently and selectively blocks postsynaptic serotonin (5-hydroxytryptamine; 5-HT) 5-HT2A receptors [30]		**85.81**
Salmeterol	**A** β2 adrenergic receptor agonist (LABA) used in the treatment of severe persistent asthma and chronic obstructive pulmonary disease [31]		**85.26**
Polidocanol	Local anesthetic and antipruritic component of lotions and has also been approved for the treatment of varicose veins [32]		**84.70**

The top 5 molecules selected for each target are different structurally, with the molecules for PqsD presenting, in general, a higher molecular weight and long aliphatic tails. This is to be expected as the characteristics of each binding pocket is different. However, two compounds also stood out. Lapatinib, one of the top 5 results for PqsR, was also present in the top 10 molecules for PqsD. The same occurred with Salmeterol (a top 5 result for PqsD) was also one of the top 20 molecules for PqsR, being two strong candidates for dual inhibition.

4. Conclusions

A docking protocol was optimized using the crystallographic ligand as validation tools in the reproducibility of the pose generated by the docking software. The virtual screening protocol was adjusted to obtain the best discriminatory ability between known binders and non-binders, and it was applied to a database of 3207 FDA approved compounds for both MvfR and PqsD targets.

The top 5 compounds of each database obtained using the optimized VS protocol are presented and described. Further computational studies are going to be performed for all these compounds, using molecular dynamics simulation and free energy calculations, to confirm the docking binding predictions and stability of protein-ligand complexes. Also, experimental testing must be performed to confirm the quality and predictability of this in silico protocol. This optimized protocol can also be used in the future to screen additional chemical libraries in the search for novel drug candidates targeting MvfR and PqsD.

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