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Development and validation of pharmacophore and QSAR models for influenza PB2 inhibitors

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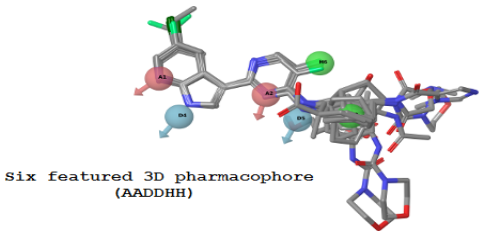
ABSTRACT



The Swine influenza is a highly contagious respiratory disease caused by one of several swine influenza A viruses. The viral replication complex of H1N1, formed by three proteins PA, PB1 and PB2, plays a major role in host adaptation and subsequent infection. The conserved interaction sites of these three proteins have been suggested as potential drug targets and several inhibitors are designed for the purpose. We report a six featured 3D pharmacophore model based on 38 reported inhibitors of PB2. It was used for development of a statistically significant 3D QSAR model with R^2 = 0.70 (internal training set), R^2 = 0.67 (internal test set) and Q^2 (0.64). The predictive power of concerned pharmacophore model was validated with an external test set R^2 (0.54) and used for screening of filtered PubChem database to fetch some important molecules. Both pharmacophore and QSAR models gave detailed structural insights as well as highlighted important features which can help in the design of potential PB2 inhibitors in further.

Keywords: H1N1, Influenza, Pharmacophore modeling, 3D QSAR, Virtual screening

INTRODUCTION

The H1N1 form of swine flu is one of the descendants of the strain that caused the 1918 flu pandemic.[1,2] The swine flu outbreaks were reported in India in late 2014 and early 2015.[3,4] At present swine flu is one of the major disease of concern. Both swine and avian hosts can serve as reservoirs of influenza. Occasionally, transmission of a new flu variant from other species to humans can lead to a global epidemic, such as the 2009 H1N1 swine flu pandemic. In 2009-10 there were two major outbreaks of the H1N1 flu. In India, the H1N1 virus outbreak killed ~2700 people[5] and in Mexico it caused ~400 deaths out of total 50000 infection.[6-8] During 2014-15 winter, there was a spur in cases with 1895 deaths from 31974 reported cases in India.

The new influenza H1N1 viral stain has emerged by the genetic combination of genes from human, pig and bird's H1N1 virus. The H1N1 virus particle is about 80-120 nanometers (nm) in diameter, roughly spherical and is enveloped by a lipid membrane.[9,10] There are two glycoproteins in this lipid membrane viz. hemagglutinin (HA) which helps in attachment of the viral strain on the host cell surface and neuraminidase (NA), responsible for initiation of viral infection.[11,12] The

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appearnce of highly pathogenic H5N1 and H7N9 avian influenza strains are responsible for most human illness and deaths worldwide.

Current antivirals for treatment of influenza cases are the neuraminidase inhibitors (NAIs) Oseltamivir and Zanamivir. While these agents can be effective against a variety of type A and B influenza viruses by blocking the function of the viral neuraminidase protein, they suffer from two main limitations. First, the neuraminidase inhibitors have only a moderate impact on the severity of symptoms as well as duration of sickness and they must be administered within 24−48 hours of infection.[13] Second, resistance to this class of antivirals has generated significant concern at times,[14] especially H5N1 influenza virus has shown resistance to Oseltamivir,[15] reinforcing the serious need for new anti-influenza therapeutics with novel mechanisms of action.

The pharmacophore modeling has been an important tool in the discovery of new chemical entities (NCEs). The term pharmacophore that means "carrier of drug" is three dimensional arrangement of features or groups that are required for a compound to show a particular biological activity. The specific arrangement of the groups and features ensures complementarity of a molecule and its target and is the key feature of the molecular recognition process.[16] In recent years there has been a great interest in the study of pharmacophore and their development, particularly due to the availability of better software and hardware coupled with a lot of biological activity data. Another reason is the versatility of the technique for application in virtual screening. The pharmacophore search has been reported to lead the identification of many diverse compounds for the same target.

In the current study, we report development and validation of a pharmacophore model for influenza PB2 inhibitors. The generated model was used for virtual screening of PubChem compound database (http://www.PubChem.com) to identify some important molecules. These molecules were then clustered and diverse molecules are reported that can help in design of potential PB2 inhibitors in future.

MATERIALS AND METHODS

2.1 DATA SET PREPARATION

A total of thirty eight PB2 inhibitors were selected through a systematic search of the literature by considering a wide activity range (Supplementary figure1), which spans over 5 orders of magnitude (EC_{50} 0.001μM - 20μM), and structural diversity.[17] The inhibitors phenotypic cell protection (CPE) assay activity was reported as EC_{50}. The EC_{50} values were converted to pEC_{50} (i.e. -log of EC_{50} value) for the purpose of QSAR model development. The correct choice of the compounds of the training and test sets is a key issue in an automated pharmacophore generation process. The training set compounds play an important role in determining the quality of the generated pharmacophore models, whereas the test set compounds serve to evaluate the predictive ability of the pharmacophore. In addition, both sets of compounds must have large range of activities to obtain critical information on the pharmacophoric requirements. Thus, the dataset was divided rationally into training set and test set by considering 70% (approx.) of the total compounds in the training set and rest 30% (approx.) in the test set using the following criteria: (a) both training and test sets should have compounds with similar structural features to ensure structural diversity and (b) both training and test sets should cover range of bioactivities (EC_{50}) as wide as possible. Therefore twenty nine compounds with significant structural diversity and wide coverage of molecular bioactivities in terms of EC_{50} value were selected for training set. The most active and least active compounds were included in the training set. In the current study all training set compounds were classified into highly active, inactive, and moderately active on the basis of their pEC_{50} values. Out of total 38 inhibitors 29 were kept in training set and rest 9 were in external test set as shown in Table 1. In training set 14 were considered as highly active (pEC_{50} value > 1.34), 5 as inactive (pEC_{50} value < 0) and rest 10 as moderately active (pEC_{50} = 0-1.34). Similarly the external test set was classified on the basis of their pEC_{50} values as employed for traing set previously. Thus there were 6 highly active, 1 moderately active and 2 inactive inhibitors.

2.2 METHODS

2.2.1 PHASE METHODOLOGY

The 3D QSAR studies were carried out using PHArmacophore Search Engine (PHASE)[18-20] application of Maestro 9.3 molecular modeling package from Schrodinger. Phase is one of the most sophisticated and powerful algorithm for pharmacophore development, structure alignment, activity prediction, and 3D database searching. It utilizes fine-grained conformational sampling and a range of scoring techniques to identify common pharmacophore hypotheses, which convey characteristics of 3D chemical structures that are critical for molecular recognition. A given hypothesis may be combined with known activity data to create 3D-QSAR models that identify various aspects of molecular structure that govern activity. These models may be used in conjunction with the hypothesis for virtual screening of chemical databases.[21]

2.2.2 LIGAND PREPARATION

The compounds were sketched in Maestro and their 3D structures were saved in sdf format. They were further processed with LigPrep[22] application for structure optimization and energy minimization. The compounds were prepared in OPLS-2005 force field[23,24] using default settings. A maximum number of 250 conformations for each compound were generated using 'Confgen' advanced option by applying OPLS-2005 force field with a constraint of 100 kJ/mol energy thresholds above the global energy minimum to ensure maximum coverage of the conformational space with RMSD cut off of 1.0Å. All conformers were then minimized by truncated Newton Conjugated Gradient minimization up to 100 iterations. Default settings were kept for the other parameters. The prepared structures were imported to PHASE along with their activity values to develop to pharmacophore model.

Table 1: The experimental and phase predicted activity values for training and test set compounds.

Sl. No.	Compound No.	QSAR Set	Experimental Activity	Phase Predicted Activity	Pharmacophore Set	Fitness Score
1	47	Training	3.000	2.750	Active	2.630
2	2	Training	2.699	2.910	Active	2.610
3	31	Training	2.699	2.800	Active	2.560
4	33	Training	2.523	1.610	Active	1.770
5	45	Training	2.097	1.381	Active	3.000
6	42	Training	1.959	1.070	Active	2.530
7	44	Training	1.745	1.390	Active	2.370
8	36	Training	1.602	0.600	Active	2.420
9	20	Training	1.357	1.780	Active	2.600
10	23	Training	1.347	2.200	Active	2.570
11	11	Training	1.092	0.870	Moderate active	2.390
12	24	Training	1.066	1.850	Moderate active	2.630
13	25	Training	1.000	0.680	Moderate active	2.380
14	10	Training	0.569	0.320	Moderate active	1.930
15	3	Training	0.292	0.570	Moderate active	2.400
16	18	Training	0.208	0.350	Moderate active	1.640
17	39	Training	-0.100	-0.750	Inactive	2.400
18	4	Training	-0.215	0.170	Inactive	2.560
19	6	Training	-1.301	0.210	Inactive	2.680
20	19	Training	-1.301	-0.420	Inactive	2.220
21	26	Internal Test Set	2.699	2.240	Active	2.610
22	28	Internal Test Set	2.398	1.960	Active	2.500
23	9	Internal Test Set	1.886	0.760	Active	1.860
24	21	Internal Test Set	1.444	1.180	Active	2.560
25	29	Internal Test Set	1.222	1.670	Moderate active	2.500
26	38	Internal Test Set	1.009	0.890	Moderate active	2.400
27	7	Internal Test Set	0.398	1.110	Moderate active	2.220
28	8	Internal Test Set	0.180	0.510	Moderate active	2.060
29	5	Internal Test Set	-0.272	0.300	Moderate active	2.240
30	22	External Test Set	1.357	0.629	Active	-
31	27	External Test Set	2.699	1.343	Active	-
32	30	External Test Set	2.699	1.486	Active	-
33	3	External Test Set	-1.000	0.428	Inactive	-
34	35	External Test Set	2.000	1.155	Active	-
35	37	External Test Set	1.022	0.780	Moderately Active	-
36	41	External Test Set	0.873	1.177	Inactive	-
37	43	External Test Set	1.745	1.061	Active	-
38	46	External Test Set	2.699	0.868	Active	-

2.2.3 PHARMACOPHORE HYPOTHESIS GENERATION

The compounds were assigned as active and inactive by giving an activity threshold value of (pEC_{50} > 1.34) for active compounds and (pEC_{50} < 0) for inactive. The activity threshold value was selected on the basis of dataset activity distribution (-1.301-3.000). The chemical features of all compounds were defined by six pharmacophorics features: H-bond acceptor (A), H-bond donor (D), hydrophobic group (H), negatively charged group (N), positively charged group (P), and aromatic ring (R). An active analog approach was used to identify common pharmacophore hypotheses (CPHs), in which common pharmacophores were extracted from the conformations of the set of active compounds using a tree-based partitioning technique that groups together similar pharmacophore according to their intersite distances. Hypotheses were generated by a systematic variation in the number of sites and the matching active compounds. The numbers of sites were altered from 6 to 3 until at least one hypothesis was found and scored successfully.

Among these pharmacophores, the models which are showing superior alignment with active compounds were identified and survival score was calculated. The "survival score" is a combination of scores (volume overlap of the aligned ligands, a selectivity score, a contribution for the number of matches, a contribution for the relative energy of the reference ligand and a contribution for the reference ligand activity) with user-adjustable weights. The scoring is performed on the ligands in the active set, which are the ligands used to develop the hypotheses. However, these pharmacophore models should also discriminate between the active (most active) and inactive (less active) compounds and thus, they were mapped to inactive compounds and scored. If inactive compounds score well, the hypothesis could be invalid because it does not discriminate between active and inactive compounds. Therefore, models with

maximum adjusted survival score and lowest relative conformational energy were selected for generating QSAR models. The scoring algorithm included the contributions from the alignment of site points and vectors, volume overlap, selectivity, number of compounds matched, relative conformational energy and activity.[25]

2.2.4 BUILDING 3D-QSAR MODELS

PHASE presents two options for alignment of 3D structure of compounds: the pharmacophore based alignment and the atom based alignment.[26] In this study, we have used an atom based QSAR model, which is more useful in explaining the structure activity relationship. In atom based QSAR, a compound is treated as a set of overlapping vander Waal's spheres. Each atom is placed into one of six categories according to a simple set of rules: hydrogens attached to polar atoms are classified as hydrogen bond donors (D), carbons, halogens, and C-H hydrogens are classified as hydrophobic/non-polar (H), atoms with an explicit negative ionic charge are classified as negative ionic (N), atoms with an explicit positive ionic charge are classified as positive ionic (P), non-ionic nitrogen and oxygen are classified as electron-withdrawing (W), and all other types of atoms are classified as miscellaneous (X).

The aligned training set compounds, on their common pharmacphoric features, were placed into a regular cubic grids of 1Å. Each cube was allocated 0 or 1 "bits" to account for the different types of atomic features in the training set compounds that occupy the cube. Each occupied cube give rise to one or more volume bits, where a separate bit is allocated for each different category of atoms that occupy the cubes. The generated binary values were used as independent variables for the development of 3D QSAR models using PLS analysis. As the number of PLS factors should not exceed 1/5 th of the training set compounds, an increase in the number of PLS factors did not improve the model statistics or predictive ability.[27] Thus, atom based 3D QSAR models were generated for the hypotheses with maximum three PLS factors and a grid spacing of 1 Å.

2.2.5 VALIDATION OF PHARMACOPHORE MODELS

Validation is a crucial aspect of *in silico* model development, particularly when the model is built for the purpose of predicting activities of compounds in external test set.[28] The validation is of two types (i) internal validation: where some of the compounds used in the model development are used for its validation. (ii) external validation: where a set of compounds which is not used for model development is used to test the predictivity of the model. This is usually considered a good standard for model validation.

An internal test set of nine compounds from the training set was selected to validate pharmacophore model (internal validation). The predicted squared correlation coefficient (R^2) was obtained by plotting the actual experimental activity and phase predicted activities of the internal test set compounds. Among the generated set of QSAR models, the best one was selected on the basis of the highest value of Q^2 (coefficient of determination for test set compounds). The selected model was further judged by R^2 (coefficient of determination for training set compounds), F-value (fisher test value) and SD (standard deviation).

The QSAR model was validated by using external test set of nine compounds selected earlier. The correlation between the experimental and predicted activities of the test set compounds was determined to check for the robustness of the generated models. The test set compounds were built and energy minimized and conformational analysis was done similar to the training set. Activities of individual compounds in the external test set were predicted by generated QSAR models. The squared correlation coefficient (R^2) was obtained by plotting the actual versus estimated activities of the test set compounds.

In order to further validate the model a systematic virtual screening protocol was employed. We generated a test database having many active molecules and decoys. The goal was to create a working protocol to identify most of the actives early in the virtual screening. The methodology used for the purpose is as follows. First all thity eight compounds were subjected to molecular property calculation using Canvas module of Schrodinger. Molecular properties like physiochemical descriptors (molecular weight, ALogP, hydrogen bond accepter, hydrogen bond donor, rotatable bonds), topological descriptors (polariity) and ligfilter descriptors (no. of rings) were calculated for all the compounds. Then the PubChem database that contains 459926 drug like molecules was filtered by Property Filter utility of Canvas module, for removal of duplicates and non-drug like compounds using the following criteria: [(AlogP >= 1) and (AlogP <= 4) and (HBA >= 3) and (HBA <= 6) and (HBD >= 2) and (HBD <= 4) and (MW >= 330) and (MW <= 510) and (Num rings >= 3) and (Num rings <= 5) and (Polar >= 34) and (Polar <= 58) and (RB >= 3) and (RB <= 7)].[29] After filteration the resulting compounds were taken for advanced pharmacophore screening in Phase. Prior to this, a Phase database was created using Generate Phase Database utility of Phase for all these compounds by generating 100 confomers per compound. The generated database was then screened against the best pharmacophore model AADDHH.3246 by Advanced pharmacophore screening with the following criteria: a compound must match minimum four sites, and inter site distance matching 2.0 angstrom. The top 20000 hits obtained were sorted according to the fitness score.

The resulting top screened compounds were clustered using hierarchical clustering to report their diversity. Hierarchical clustering is a method of cluster analysis which seeks to build a hierarchy of clusters. It has a distinct advantage that any valid measure of distance can be used. In fact, the observations themselves are not required: all that is used is a matrix of distances. Tanimoto matrix is used, and it is defined as $c/(a + b - c)$, where a is the number of bits set by structure A, b is the number of bits set by structure B, and c is the number of bits set jointly by A and B.

RESULT AND DISCUSSIONS

A total of eight hundred seventy nine pharmacophore models were generated with different combination of pharmacophoric

features. One of them was selected based on adjusted survival score for atom based QSAR model generation. Statistical details of this six featured hypothesis (AADDHH.3246) is presented in Table 2 with survival active score of 8.052.

It is recommended that for a reliable model, the R^2 value must exceed 0.50 and the squared predictive correlation coefficient (Q^2) should exceed 0.60.[30,31] For the above selected hypothesis the generated QSAR model also revealed good statistical significance as mentioned in Table 3.

However, when all generated models for corresponding hypothesis were evaluated against the training set and external test set, the model for hypothesis AADDHH.3246 showed predicted R^2 = 0.70 (internal training set), R^2 = 0.67 (internal test set) and R^2 = 0.54 (external test set). The plot of experimental vs. predicted activity using model AADDHH.3246 for training and external test set compounds is shown in (Fig. 1) and (Fig. 2) respectively. Both experimental and predicted activity values for all compounds of AADDHH.3246 hypothesis are shown in Table 1. After consideing the suitbale predictive power of AADDHH.3246 hypothesis for training set and test set it was selected for further study.

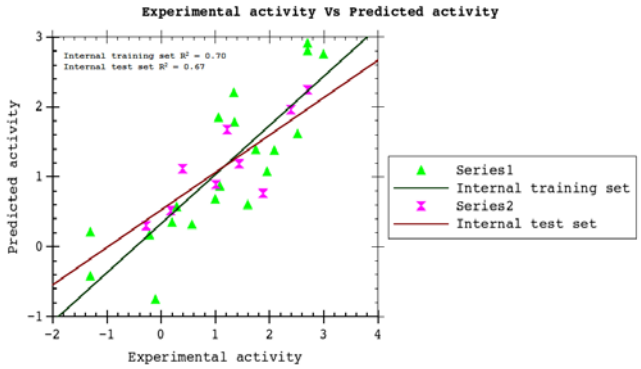

Figure 1 The plot of experimental vs. predicted activity for internal training set and test set.

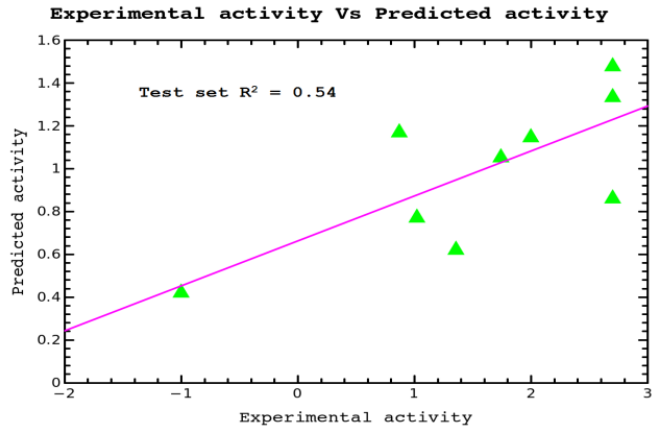

Figure 2 The plot of experimental vs. predicted activity for external test set.

The spatial arrangement of features along with their distances for the best model (AADDHH.3246) is shown in figure 3. The alignment of the best fit compound to this pharmacophore is shown in figure 4; whereas alignment of inactive compounds to the developed pharmacophore model is shown in figure 5. It is evident from figures 4, and 5 that the most active compound matches all six features very well while the least fit compounds miss one or more features.

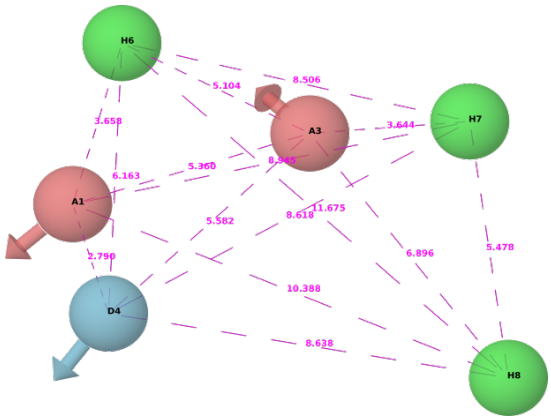

Figure 3 Interfeature distances for the best pharmacophore model. The green, blue and red spheres denote hydrophobic, donor and acceptor feature respectively

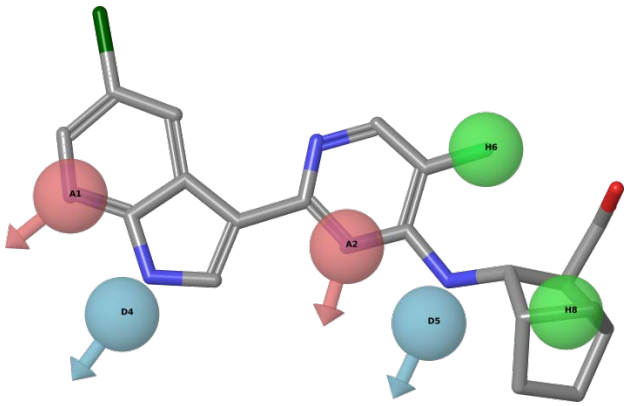

Figure 4 The most active compound superimposed on the best pharmacophore model.

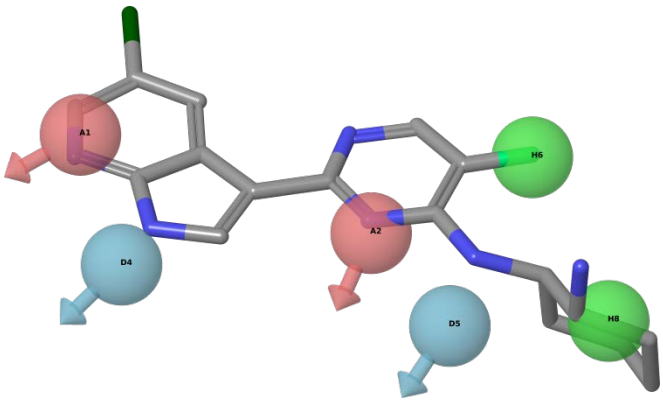

Figure 5 The least active compound superimposed on the best pharmacophore model.

Table 2: The statistical details of AADDHH.3246 hypothesis.

Hypothesis ID	Survival Score	Survival Inactive	Post-hoc	Site	Vector	Volume	Matches	Energy	Activity	Inactive
AADDHH.3246	8.052	5.631	4.059	0.76	0.995	0.675	14	4.575	2.097	2.421

Table.3: The statistical parameters of 3D QSAR Model

Hypothesis ID	PLS Factor	SD-Value	R^2	F-Value	P	RMSE	Q^2	Pearson-R
AADDHH.3246	1	0.7015	0.7018	42.4	4.042e-06	0.5691	0.6399	0.8176
	2	0.3887	0.9136	89.8	9.168e-10	0.7246	0.4163	0.7009
	3	0.192	0.9801	263.3	7.976e-14	0.7196	0.4243	0.6915

It is further clear from the alignments of all actives (Fig. 6) that they show a good alignment to the pharmacophore and among themselves. It is imperative as for molecular recognition they need the correct positioning of pharmacophoric features. Alignments of all compounds (actives and inactives) is shown in (Fig. 7) to indicate the differences in the superimpostion of these two groups of compounds (active and inactive) to the generated pharmacophore. The fitness score for all the compounds of training set is shown in Table 1.

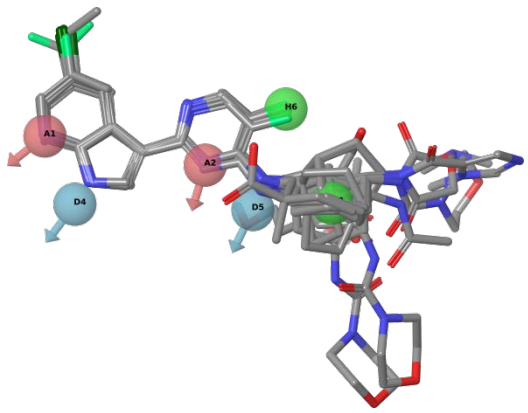

Figure 6 The alignment of active compounds on the best pharmacophore model.

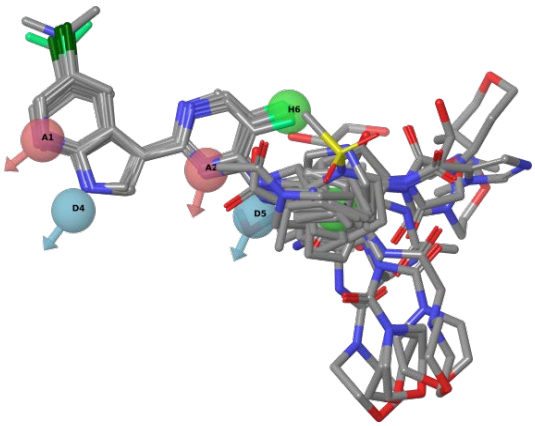

Figure 7 The alignment of all active & inactive compounds on the best pharmacophore model.

3.1 VIRTUAL SCREENING

Finally from 459926 compounds 24372 compounds were selected from property filtering assuming most of them will not be PB2 inhibitors and can be used as decoys. Only these are similar to the actives according to their low dimensional properties e.g. MW, number of HB atoms, LogP etc. All 38 previously selected PB2 inhibitors were mixed with 24372 decoys to result a total of 24410 compounds.

The performance of the virtual screening of these compounds with best pharmacophore model AADDHH.3246 was assessed and ranked according to the fitness score of the molecules and from these only top 50 compounds were considered as they were very close to the 38 PB2 inhibitors in terms of fitness score with the best pharmacophore model. The 2D structures and predicted activity value of top six compounds are shown in (Fig. 8) and remaining compounds are shown in supplementary figure2. When we considered predicted activity of the compounds it was found that out of top 50 compounds 2 were highly active and 48 were moderately active and non of them were in inactive range.

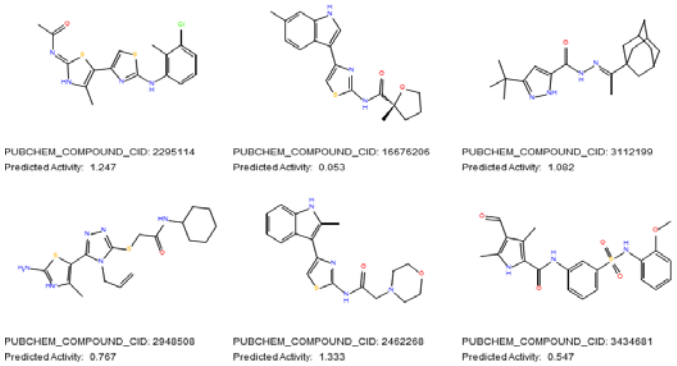

Figure 8 The 2D structures of top screened compounds by best pharmacophore model from PubChem compound database.

Then top 50 identified compounds were clustered using Molprint2D fingerprint to identify their diversity. Hierarchical clusreing was performed in Canvas which revealed 25 clusters as shown in figure 9.

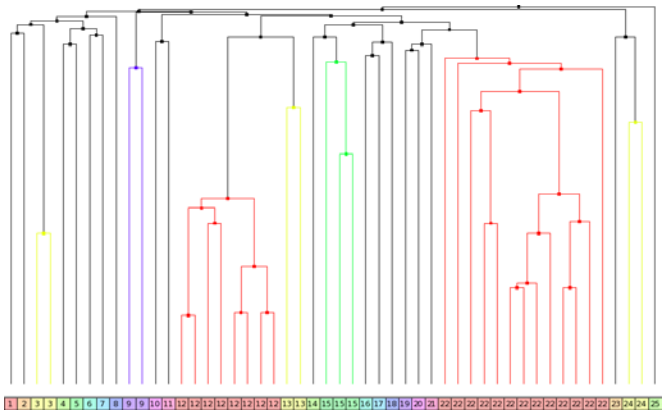

Figure 9 The clustering of top 50 compounds. The lines show connections to the nearest neighbours.

3.2 3D-QSAR VISUALIZATION

Additional insight into the PB2 inhibitor activity can be gained by visualizing the 3D QSAR model in the context of one or more compounds in the series with varying activity. This information can then be used to design new or more active analogs. 3D QSAR model based on the compounds of training and test set using various features, i.e. hydrogen bond acceptor (A), hydrogen bond donor (D), aromatic ring (R) and positively charged group (P) was studied.

Visual inspection of the QSAR model revealed that H-bond donor, hydrophobicity, and electron-withdrawing effects are crucial structural features for high activity. The most fit and least fit compound was mapped on the best model and are shown in (Fig. 10) and (Fig. 11) respectively. The blue cubes indicate positive coefficient (increase in activity), red cubes indicate negative coefficient (decrease in activity).

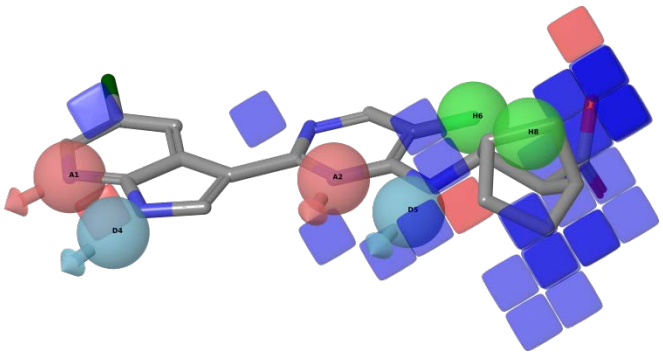

Figure 10 A visual representation of best QSAR model, in context of the most fit compound in the training set, where blue cubes indicate positive coefficient (increase in activity), red cubes indicate negative coefficient (decrease in activity).

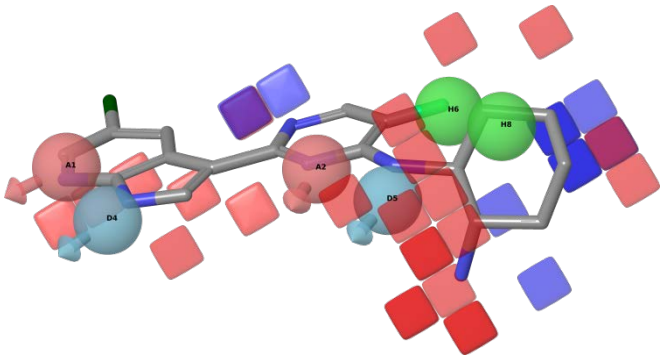

Figure 11 A visual representation of best QSAR model, in context of the most fit compound in the training set, where blue cubes indicate positive coefficient (increase in activity), red cubes indicate negative coefficient (decrease in activity).

CONCLUSION

A six featured pharmacophore with two hydrogen bond acceptor (A), two hydrogen bond donor (D), and two hydrophobic group (H) pharmacophore model was developed based on 29 training set compounds. The 3D QSAR model based on this pharmacophore revealed good statistical significance and predictive ability R^2 = 0.70 (internal training set), R^2 = 0.67 (internal test set), R^2 = 0.54 (external test set) and Q^2 = 0.64.

Visualization of the 3D-QSAR model provides details of relationship between structure and activity among these compounds and thus provide explicit indications for the design of better analogs. The obtained results suggested that the proposed 3D QSAR model AADDHH.3246 can be useful to rationally design new drug compounds as PB2 inhibitors and also to identify new promising compounds as potential influenza PB2 inhibitors in large chemical compound databases.

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Supplementary Material

Supplementary data (The thirty eight structure of the training test and test set compounds are presented in supplementary figure1 and PubChem screened top fifty compounds presented in supplementary figure2) associated with this article can be found in the supplementary material file.

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