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Urokinase Inhibitor Design Based on Pharmacophore Model Derived from Diverse Classes of Inhibitors

Liu Shui, Nagakumar Bharatham, Kavitha Bharatham, Keun Woo Lee*

Division of Applied Life Science, Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Korea

Abstract

A three-dimensional pharmacophore model was developed based on 24 currently available inhibitors, which were rationally selected from 472 compounds with diverse molecular structure and bioactivity, for generating pharmacophore of uPA (Urokinase Plasminogen Activator) inhibitors. The best hypothesis (Hypo1) comprised of five features, namely, one positive ionizable group, one hydrogen-bond acceptor group and three hydrophobic aromatic groups. The correlation coefficient, root mean square deviation and cost difference were 0.973, 0.695, and 94.291 respectively, suggesting that a highly predictive pharmacophore model was successfully obtained. The application of the model showed great success in predicting the activities of 251 known uPA inhibitors (test set) with a correlation coefficient of 0.837, and there was also none of the outcome hypotheses that had similar cost difference and RMS deviation (RMSD) with that of the initial hypothesis generated by *Cat-Scramble* validation test with 95% confidence level. Accordingly, our model should be reliable in identifying structurally diverse compounds with desired biological activity.

Introduction

It is widely documented that the progression of cancer cell invasion and metastasis is hinged on the ability of tumor cells to produce and recruit proteolytic enzymes. Among the diverse proteolytic enzyme systems produced by human cancers, the plasminogen activator-plasmin system is preferentially involved in cancer cell invasion and metastasis (Dano and Romer, 1999; Jan and Van der Pluijm, 1999). The plasminogen activators are a group of proteolytic enzymes existing mainly as urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (Dano and Andreasen, 1985).

uPA, a trypsin-like serine protease, is the key initiator of the extracellular proteolytic cascade driving cellular invasiveness (Saksela, 1985; Testa and Qiugley, 1990; Gunzler and Steffens, 1982). Proteolytically active uPA is a disulfide-linked two-chain protein generated from proteolytically inactive pro-uPA by the hydrolysis of the Lys158-Ile159 peptide bond (Mignatti and Robbins, 1986). Urokinase is secreted by tumor cells or adjacent stroma and exists either as the free enzyme or bound to its cell-surface receptor, uPAR. Binding to uPAR significantly increases the rate of cell surface-associated plasminogen activation by urokinase and can serve to spatially focus its activity. The uPA/uPAR complex plays a role in many normal physiological events, such as wound healing, but is also involved in tissue remodeling of various diseases, including arthritis (Cook and Braine, 2002; Busso and Pleclat, 1997), atherosclerosis (Noda-Heiny and Daughety, 1995; Carmeliet and Moons, 1997; Falkenberg and Giglio, 1998; Preissner and Kanse, 1999), vascular restenosis (Kanse and Benzakour, 1997), and cancer. In particular, urokinase is implicated in many tumor-associated processes, including extracellular matrix degradation, invasion, angiogenesis, and metastasis (Rabbani and Xing, 1997; Blasi, 1999)(Andreasen and Kjoller, 1997; Achbarou and Kaiser, 1994; Duffy, 2002).

Nowadays small-molecule inhibitors of urokinase have already been shown to inhibit tumor metastasis and slow cancer growth. Several compounds have been reported to inhibit uPA's activity at low micromolar or even nanomolar concentrations, and we found that all of the reported inhibitors of urokinase contain an amidine or guanidine group, and compounds designed from templates containing these positively charged moieties can be a liability in the search for oral therapeutic agents. Therefore, there is a need for alternative small-molecule inhibitors of urokinase which have favorable

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pharmacokinetic properties (Hajduk and Boyd, 2000).

With these recent experimental results, we decided to exploit this wealth of information to develop a small molecule-derived pharmacophore model. The approach we are using here is to develop a pharmacophore model using the *HypoGen* module in *Catalyst* (Accelrys, Inc., San Diego, CA, 2005) which can be used to correlate the observed biological activities for a series of compounds with their chemical structures. The 24 different kinds of compounds with biological activity data, covering six orders of magnitude were included in our study. These molecules were selected to span the range of activities from the most active compounds to almost completely inactive molecules that are publicly available.

As there is no report on developing pharmacophore models using newly published inhibitors of uPA until now, this present paper provides a hypothetical image of the primary pharmacophore features responsible for activity, and it is expected to provide useful knowledge for discovering novel potential inhibitors targeted to uPA.

Methods

A number of classes of uPA inhibitors have been identified during last few years. We have collected a set of 472 molecules of uPA inhibitors from the literature using the software *MDL ISIS/base 2.5*. The biological dataset was divided into a training set (Table 1) and a test set. The training set consists of 24 structures (Sturzebecher, 1999; Wilson, 2001; Subasinghe, 2001; Barber, 2002; Mackman, 2002; Bruncko, 2005; Wendt, 2004; Barber, 2004; Karanewsky, 1990; Renatus, 1998; Verner, 2001; Wendt, 2004;) and was selected by considering both structural diversity and wide coverage of the activity range (Ki ranging from 0.00062 uM to 72.4 uM (compound 1 and 24 respectively in Table 1). These molecules, whose activity span a range of 6 orders of magnitude, were selected based on the fact that each order of magnitude is represented by at least three compounds, including the most active and inactive ones. If two compounds had

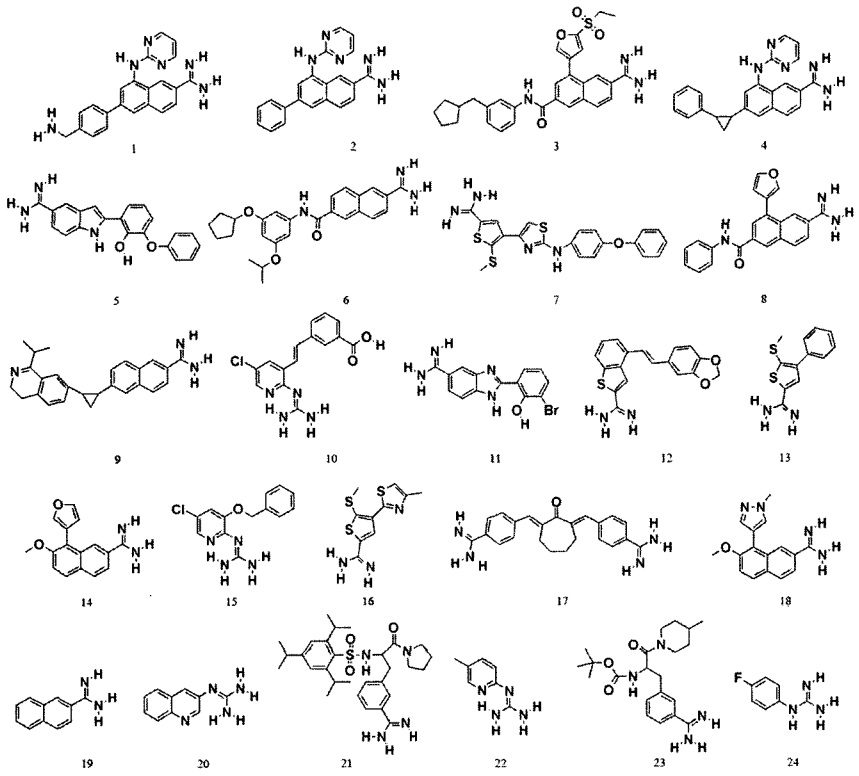

Figure 1. Chemical structures of the 24 training set molecules.

Table 1. Actual biological data and estimated Ki of training set molecules based on pharmacophore model Hypo1

Compound No.	True Ki(uM)	Estimated Ki(uM)	Error factora	Fit valueb	Activity scalec	Estimated activity scale
1	0.00062	0.00061	-1	9.27	+++	+++
2	0.002	0.0042	2.1	8.43	+++	+++
3	0.0021	0.0059	2.8	8.29	+++	+++
4	0.005	0.0026	-1.9	8.65	+++	+++
5	0.008	0.033	4.1	7.54	+++	+++
6	0.022	0.0085	-2.6	8.13	+++	+++
7	0.06	0.038	-1.6	7.48	+++	+++
8	0.091	0.041	-2.2	7.45	+++	+++
9	0.139	0.17	1.2	6.82	++	++
10	0.17	0.45	2.6	6.41	++	++
11	0.25	0.49	2	6.37	++	++
12	0.53	0.63	1.2	6.26	++	++
13	0.6	1.2	2	5.99	++	+
14	0.82	2.9	3.6	5.59	++	+
15	0.92	0.43	-2.2	6.43	++	++
16	1	0.97	-1	6.07	+	++
17	3.4	0.75	-4.5	6.18	+	++
18	4.6	5.5	1.2	5.32	+	+
19	5.91	4.2	-1.4	5.44	+	+
20	9.3	5.4	-1.7	5.33	+	+
21	19	10	-1.8	5.04	+	+
22	32.3	46	1.4	4.39	+	+
23	56	24	-2.4	4.68	+	+
24	72.4	61	-1.2	4.27	+	+

[a]The error factor is computed as the ratio of the measured activity to the activity estimated by the hypothesis or the inverse if estimated is greater than measured.
[b]Fit value indicates how well the features in the pharmacophore overlap the chemical features in the molecule.
[c]Activity scale: +++, Ki < 0.1 uM (highly active); ++, 0.1 uM < Ki < 1 uM (moderately active); +, Ki > 1 uM (inactive).

similar structures, they had to differ in activity by one order of magnitude to be included in our dataset. Otherwise, we selected only the most active one of the two. If two compounds were found to have similar activities, they had to be structurally distinct in order to be included. The structures of the 24 uPA inhibitors used in the training set are given in Figure 1.

Conformational analysis for each molecule was performed using the Poling algorithm (Smellie, 1994) to improve the broad coverage of the available conformational space. Poling explicitly promotes the conformational variation that forces similar conformers away from each other. All conformers are treated equally, each is considered as a possible configuration of functional groups or features. In this case, the setting in conformer generation was 250 as the maximum number of conformers for each molecule by using the 'best conformer generation' option with 20 kcal/mol energy cutoff.

Uncertainty influences the first step, called the constructive phase, in the hypothesis generating process (Guner, 2000). The default uncertainty value of 3 was used for the compound activity, representing the ratio of the uncertainty range of measured biological activity against the actual activity for each compound. Analysis of functional groups on each compound in the training set revealed that five chemical features, those are positive ionizable group (PI), hydrogen-bond acceptor (HA), hydrogen-bond donor (HD), hydrophobic aromatic (Z), and ring aromatic (RA), could effectively map all of the critical chemical features.

The 'Best Compare/Fit' procedure was then used in order to evaluate the geometry of the conformers of the compounds to test how well they fit the hypothesis. The fit value represents the quality of the mapping between the compound

and the hypothesis; the better the overall superimposition of functional groups of the molecule to the appropriate features of the pharmacophore model, the higher the fit score (Barnum and Greene, 1996).

Results and Discussion

Pharmacophore hypothesis generation

'Fixed cost' calculation carried out by the *HypoGen* in *Catalyst* represent the simple model that fits all data perfect, meanwhile, the 'null cost' calculation also persumes that there is no statistically significant structure in the data and the expertimental activities are distributed around their average value. Normally, when the difference between these two values should be in a certain range, the pharmacophore hypothesis will be meaningful. Here, we are now using a standard value of 70-100 bits for the pharmacophore hypothesis, and also, the total cost of any pharmacophore hypothesis should be close to the fixed cost to provide any useful models.

Catalyst produces 10 hypotheses, and Hypo1 is the best significant pharmacophore hypothesis shown the distance constraints between pharmacophore features in Figure 2. It is characterized by the highest cost difference, lowest error cost, and lowest RMSD as well as has the best correlation coefficient. The fixed cost, pharmacophore cost, null cost and configuration cost are 95.686, 102.126, 189.977, 13.835 bits respectively. The top ranked hypothesis Hypo1 showed the best result with a correlation of 0.973 and a RMSD of 0.695. The cost values, correlation coefficients (r), RMSD, and features for the top ten hypotheses are listed in Table 2.

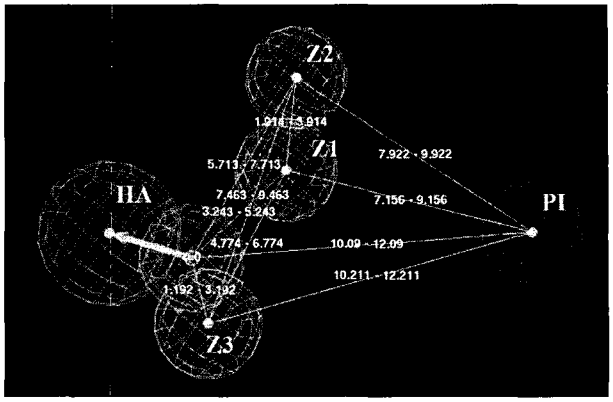

Figure 2. The best hypothesis model Hypo1 produced by HypoGen module in *Catalyst 4.10* software, pharmacophore features are color-coded with light blue for hydrophobic aromatic groups, red for positive ionizable group and green for hydrogen-bond acceptor. Distance range between pharmacophore features is reported in angstroms. HA, hydrogen-bond acceptor group; PI, positive ionizable group; Z1, hydrophobic aromatic 1; Z2, hydrophobic aromatic 2; Z3, hydrophobic aromatic 3.

From the table we can see 4 hypotheses including the best Hypo1 have the same 5 features: one positive ionizable, one hydrogen-bond acceptor and three hydrophobic aromatic groups, while another 5 hypotheses are slightly different with one ring aromatic replacing one hydrophobic aromatic group,

Table 2. Information of the cost values measured in bits RMSD, correlation values and features for top-ten hypotheses

Hypotheses No.	Training set				Feature[a]	Test set
	Total cost	□Cost	RMSD	Correlation(r)		Correlation(r)
1	102.126	87.851	0.695	0.973	AZZZP	0.837
2	111.702	78.275	1.153	0.924	AZZRP	0.68
3	113.052	76.925	1.203	0.917	AZZRP	0.703
4	113.514	76.463	1.219	0.915	AZZRP	0.628
5	114.118	75.859	1.239	0.912	AZZZP	0.808
6	118.877	71.1	1.368	0.891	AZZRP	0.71
7	121.799	68.178	1.475	0.872	AZZZP	0.652
8	123.124	66.853	1.511	0.866	AZZP	0.663
9	123.368	66.609	1.517	0.865	AZZZP	0.639
10	124.118	65.859	1.537	0.861	AZZRP	0.65

Null cost of ten hypotheses is 189.977 bits. Fixed cost is 95.686 bits. Configuration cost is 13.835 bits.
[a]Abbreviation used for features: A, hydrogen-bond acceptor; Z, hydrophobic aromatic; P, positive ionizable; R, ring aromatic.

and the last hypothesis had only 4 features: one positive ionizable, one hydrogen-bond acceptor and two hydrophobic aromatic group.

Hypo1, identified as the best hypothesis, has estimated the activity of the training set molecules accurately. In this study all compounds were classified by their activity as highly active (<0.1 uM, +++), moderately active (0.1-1 uM, ++) and inactive (>1 uM, +). Table 1 represents the actual and estimated uPA inhibitory activity of the 24 training set molecules based on the best hypothesis, Hypo1. Among 24 training set compounds, two moderately active compounds were predicted as inactive, and two inactive compounds were predicted to be moderately active by Hypo1. Consequently, for 20 of 24 training set compounds, the predicted Ki (uM) values were within the same activity scale as the experimental values in the training set.

Compound 1 shows a good fit with all features of the pharmacophore hypothesis Hypo1. In this case, the hydrogen-bond acceptor group seems to be mapped by a nitrogen atom in pyrimidine moiety, the positive ionizable sphere is also mapped by a nitrogen atom, and the three aromatic features are fitted by three phenyl rings, respectively. Figure 3 represents the top scoring hypothesis, Hypo1 aligned with the most active compound 1 (Ki = 0.00062 uM) and an inactive compound 18 (Ki = 4.6 uM) in the training set. The predicted activity for compound 1 and compound 18 are 0.00061 and 5.5 uM, and the fit values are 9.27 and 5.32 respectively.

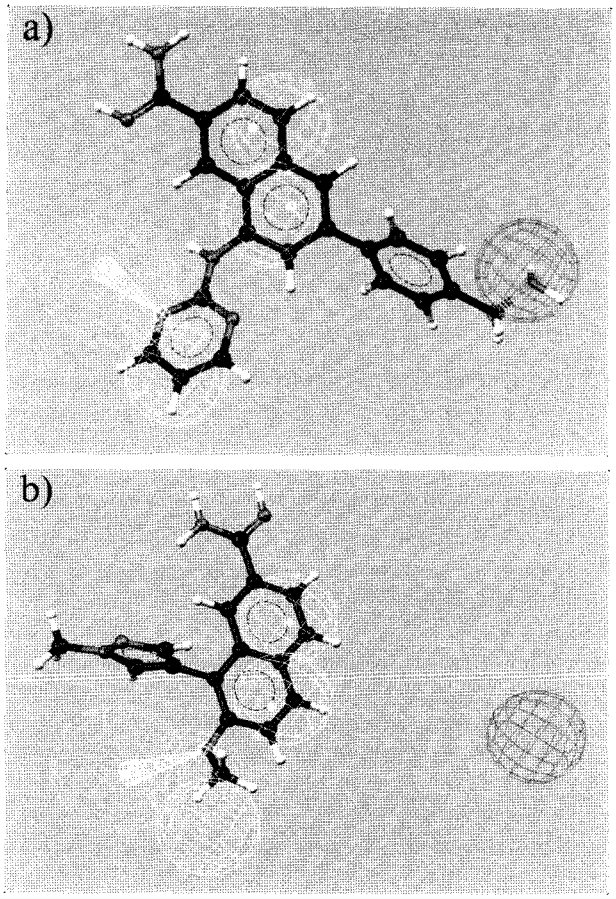

Figure 3. Mapping of the most active molecule, compound 1 (a) and inactive, compound 18(b) on the best hypothesis model, Hypo1.

Validation of the pharmacophore model generated from uPA inhibitors

In order to validate our pharmacophore hypothesis, we used a test set comprising of 251 molecules with uPA inhibiting activity from different activity classes and different structural information. Activities are reported as Ki values spanning from 0.001 uM to 100 uM ranging five orders of magnitude. All molecules in the test set were built, minimized as well as performed conformational analysis like the molecules in the training set. A correlation coefficient of 0.837 generated using the test set compounds shown in Figure 4 indicates a good correlation between the actual and estimated activities, which means the Hypo1 we selected before is convictive. Moreover, an error value less than 10 indicates that the activity predicted for all the molecules was within one magnitude range. One of the most active molecules, Test43, from the test set was selected to show its mapping on the selected pharmacophore hypothesis, Hypo1 (Figure 5). The true and estimated activities of Test43 are 0.018 and 0.049 uM respectively.

Another approach to assess the quality of *HypoGen* hypothesis is to apply cross validation using the *Cat-Scramble* program available in *Catalyst*. The validation procedure described here is based on Fischer randomization test. The goal of this type of validation is to check whether there is a strong correlation between the chemical structures and the biological activity. In this validation test, we chose 95% confidence level, and thus 19 spreadsheets were generated. These random spreadsheets were used to generate hypotheses employing exactly the same features as used in generating the initial hypothesis. The results of the *Cat-Scramble* runs are listed in Table 3. The data of cross validation clearly indicates that all values generated after randomization produced hypotheses with no significant value. Besides, out of 19 runs, only one, trial 18, had a correlation value of 0.81, but the

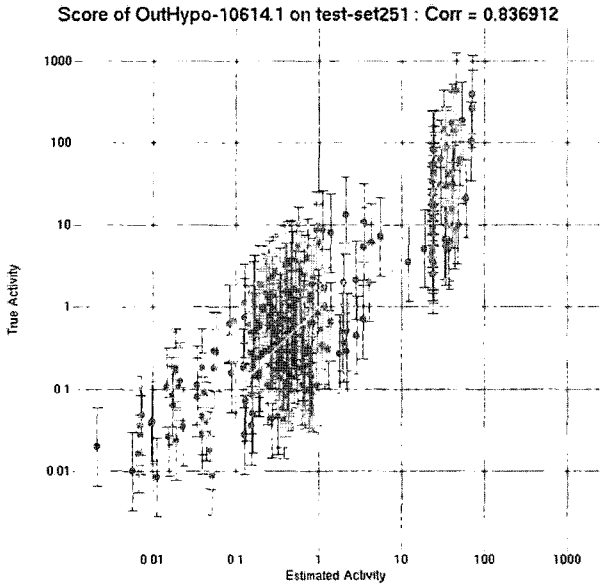

Figure 4. Correlation graph drawn between true and Hypo1 estimated activities for test set compounds.

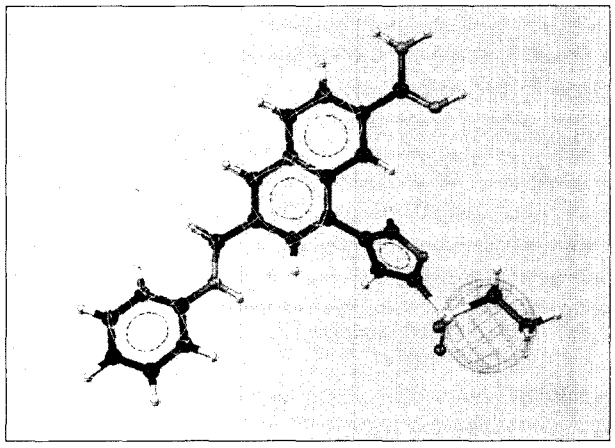

Figure 5. Mapping of one of the most active compound (Test43) from the test set. Pharmacophore features are color-coded with light blue for hydrophobic aromatic groups (Z), red for positive ionizable (PI) group and green for hydrogen-bond acceptor (HA).

RMS deviation and total cost were very high, which is not desirable for a good hypothesis. This cross validation also provided strong confidence on the best pharmacophore Hypo1.

It is interesting to note that the validated pharmacophore model has neglected the amidine or guanidine groups as they had no role in distinguishing active from inactive molecules. Our aim was to design new compounds lacking amidine or guanidine groups present in all existing inhibitors, as it is a liability in the search for oral therapeutic agents. Thus we have accomplished our goal and therefore propose that our pharmacophore model, Hypo1, would be very useful in discovering new lead molecules with good uPA inhibitory activity and also with physicochemical properties.

Table 3. Cat-Scramble Validation test of the best Hypo1

Trail No.	Total cost	Fixed cost	RMSD	Correlation(r)
	Results for unscrambled			
	102.126	95.6861	0.6951	0.973
	Result for scrambled			
1	187.714	86.7095	2.896	0.283
2	177.213	92.0463	2.653	0.479
3	167.962	92.6271	2.505	0.557
4	149.296	91.9782	2.184	0.69
5	171.178	88.2266	2.628	0.491
6	189.977	80.7265	3.017	0
7	148.049	92.1869	2.037	0.749
8	169.407	90.3984	2.563	0.528
9	158.624	93.1329	2.314	0.643
10	161.692	92.1903	2.405	0.604
11	161.342	90.5658	2.428	0.594
12	172.01	92.0695	2.558	0.534
13	163.985	89.9911	2.467	0.578
14	144.771	90.5026	2.117	0.713
15	142.8	89.3109	2.089	0.723
16	168.271	88.2266	2.571	0.525
17	160.991	92.0314	2.318	0.652
18	134.833	95.7672	1.762	0.813
19	170.33	87.6844	2.601	0.513

The null cost is 189.977 bits.

Conclusion

In conclusion, a ligand-based computational approach was employed to identify molecular structural features required for an effective uPA inhibitor, in an aim to discover drugs to prevent and cure its related diseases. A highly predictive pharmacophore model, Hypo1 was generated based on 24 training set compounds, which consists of three hydrophobic aromatic groups, one positive ionizable group, and one hydrogen-bond acceptor group. The utility of our pharmacophore model on 251 test set compounds showed that the model is able to accurately differentiate various classes of

uPA inhibitors. Thus, our pharmacophore model should be helpful in identifying novel lead compounds with improved uPA inhibitory activity as well as desired physiological properties through 3D database searches.

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