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Design of oseltamivir analogs inhibiting neuraminidase of avian influenza virus H5N1

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ABSTRACT

Neuraminidase is an important target for design of antiviral agents in the prophylaxis and treatment of avian influenza virus infections. We have shown the applicability of computer-assisted combinatorial techniques in the design, focusing and *in silico* screening of a virtual library of analogs of oseltamivir (Tamiflu) with the goal to find potent inhibitors of influenza A neuraminidase N1 that fill the cavity found adjacent to the active site. Crystal structure of oseltamivir–N1 complex was used in the structure-based focusing and virtual screening of the designed library. A target-specific Piecewise Linear Potential type 1 scoring function fitted for a training set of 14 carbocyclic inhibitors and validated for three other inhibitors was used to select virtual hits with predicted inhibitory activities in the subnanomolar range. The results of this computational study are useful as a rational guide for synthetic and medicinal chemists who are developing new drugs against the avian influenza virus H5N1.

1. Introduction

In the recent years, the emergence and worldwide spread of the avian influenza A virus subtype H5N1 has raised concerns of possible easy human-to-human transmission, which calls for the need to develop more potent antiviral drugs to be used for the prophylaxis and treatment of influenza infections. Neuraminidase (NA), a membrane glycoprotein of the influenza virus, which is required for the release of budding virions from the host cell, is one of the potential drug targets of antiviral agents. Several potent and specific inhibitors of the NA have been developed through structure-based rational design, however, only two of them, oseltamivir (Tamiflu) and zanamivir (Relenza), have been approved for human use (Fig. 1) (Kim et al., 1997; Gubareva et al., 2000). A third NA inhibitor, namely peramivir (BCX-1812), failed to show statistically significant viral inhibition due to the relatively low blood levels obtained after oral administration (Bantia et al., 2006). High mutation rate and emerging drug resistance to the commercially available drugs, especially to oseltamivir, have been widely reported (Gubareva et al., 2001; Moscona, 2005). Therefore, finding novel potent inhibitors of NA less affected by cross-resistance as well as identification of new drug targets is a vital goal.

Based on the recent X-ray crystallographic studies of influenza A virus NAs of the group-1, it was shown that their structures contain a larger cavity adjacent to the active site, formed by residues 147–152 (150-loop) (Russell et al., 2006). This cavity was not found in the group-2 NAs. The 150-loop switches from open to closed conformation upon the ligand binding, however, the group-1 NAs can bind ligands in both conformational states of the loop. The closed conformation of the loop is similar to that observed in the group-2 NAs (Russell et al., 2006). Residues of the flexible 150-loop are located in the vicinity and interact mainly with the amino and acetamide groups of the oseltamivir moiety (Fig. 2). The active sites of both NA groups retain a conserved structure of three arginine residues (Arg118, Arg292 and Arg371) that bind the carboxylate group, Arg152 that interacts with the acetamido group of the sialic acid substrate and Glu276 that forms hydrogen bonds with the substrate hydroxyl groups.

Shikimic acid is an important metabolic intermediate in the shikimate pathway found in microorganisms and plants, commonly known as a precursor for the biosynthesis of aromatic amino acids and aromatic secondary metabolites. Recently, this phytochemical

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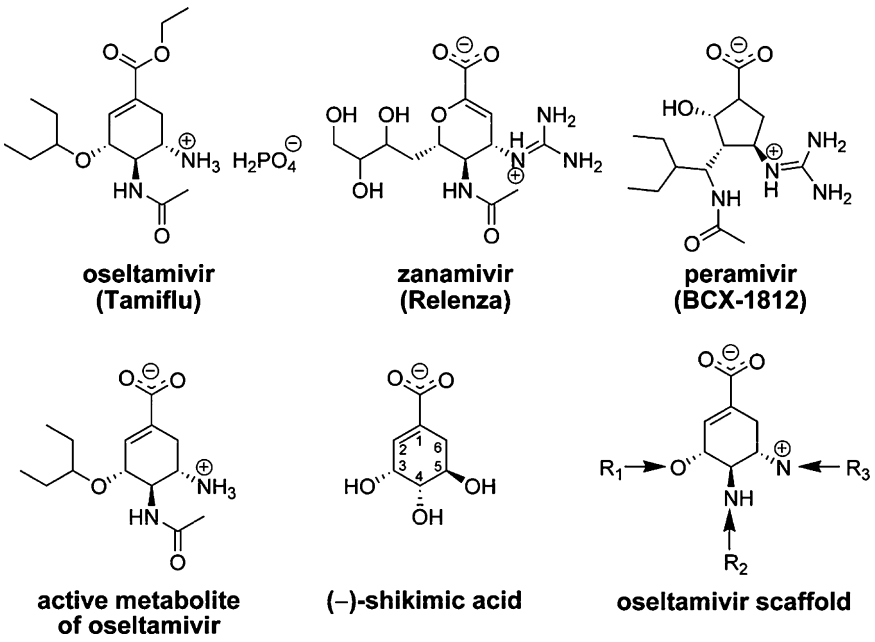

Fig. 1. Chemical structures of antiviral drugs inhibiting the neuraminidase of influenza A virus: oseltamivir, zanamivir and peramivir, the active metabolite of oseltamivir, (−)-shikimic acid (3*r*,4*s*,5*r*)-(−)3,4,5-trihydroxy-1-cyclohexenecarboxylic acid, and cyclic scaffold of oseltamivir analogs with R-groups (substitution points) notation.

has gained importance as the precursor used in the synthesis of oseltamivir (Fig. 1) (Federspiel et al., 1999; Karpf and Trussardi, 2001). The commercial synthesis of oseltamivir by Roche uses the (−)-shikimic acid and proceeds via 12 reaction intermediates. Derivatization of the shikimic acid at the position 3 (R_1) to obtain aliphatic and aromatic ethers proceeds via addition of ketones to an olefin intermediate (Federspiel et al., 1999). Amides at position 4 (R_2) can be obtained by acylation with anhydrides (Harrington et al., 2004). Introduction of amines or guanidines at position 5 (R_3) results from the addition of allyl amines to an epoxide intermediate (Federspiel et al., 1999; Harrington et al., 2004) or from modification of the resulting amines by thioureas (Poss et al., 1992).

Combinatorial chemistry approaches have been developed to generate a large quantity of compounds prepared via parallel

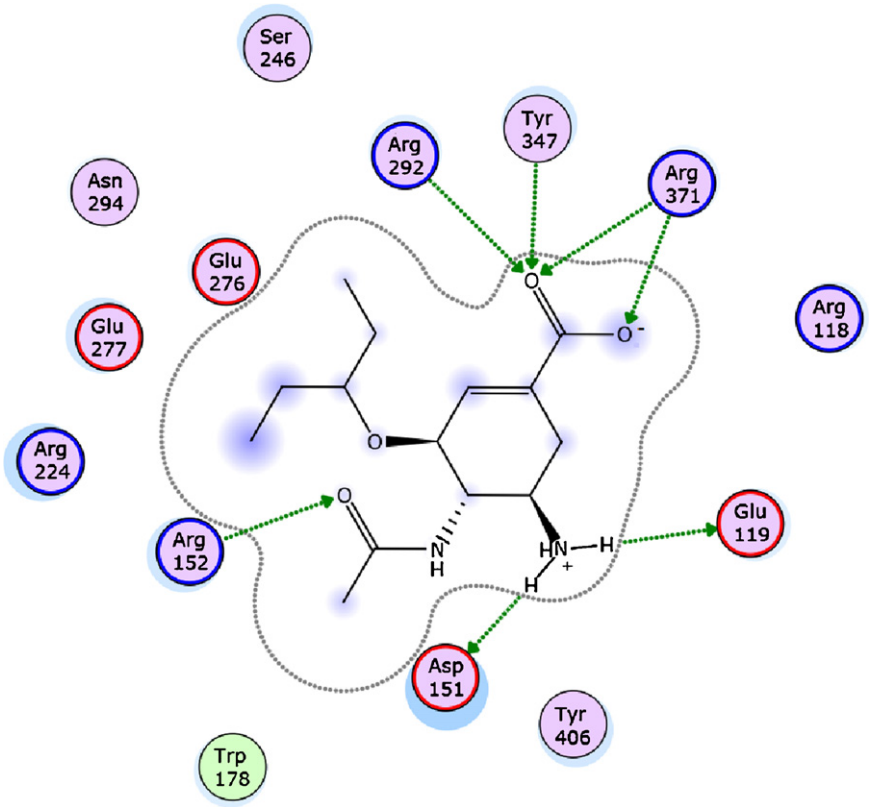

Fig. 2. Interactions of oseltamivir at the active site of N1 neuraminidase.

synthesis of libraries that are screened in high-throughput bioassays to identify hits, which may yield new lead compounds with desired bioactivity (Warr, 1997; Kubinyi, 1998). Computational methods are being increasingly used to assist the combinatorial library design, focusing and virtual screening by introducing selection criteria such as molecular diversity, drug-likeness, Lipinski criteria for bioavailability, and predicted receptor binding. Computational approaches can thus significantly decrease the size of libraries to be synthesized and screened to generate lead compounds. We have designed several classes of antiviral agents using combinatorial and computer-assisted approaches (Frecer et al., 2004, 2005) and obtained insight into inhibitor–N1 interactions from computer simulations (Malaisree et al., 2008).

In the present study, computer-assisted combinatorial chemistry techniques have been applied to design, focus and *in silico* screen a virtual library of oseltamivir analogs, which fill also the cavity adjacent to the active site of the N1, aiming at finding new potent NA inhibitors of the avian influenza virus H5N1. We have derived a small highly focused combinatorial library subset of analogs of oseltamivir (IC_{50} = 1 nM (Kim et al., 1997)), which contain compounds that are predicted to inhibit NA of the subtype N1 with IC_{50} values in the subnanomolar range.

2. Materials and methods

2.1. Virtual library generation

All calculations were performed with Cerius^2 program (Cerius^2, 2000). CFF91 force field (Maple et al., 1994) and the Rappé and Goddard equilibrated charges (Rappé and Goddard, 1991) were used. The library of oseltamivir analogs was enumerated by attaching of the R-group (fragments, building blocks, reagents) obtained from the directory of chemicals available from the commercial suppliers (ACD, 2002) onto the six-membered scaffold ring of the oseltamivir moiety using the CombiChem module. Each analog was built in a zwitterionic form and its molecular structure was refined by molecular mechanics optimization using smart minimizer with high convergence criteria (energy difference of 10^{-4} kcal mol^{-1}, *r.m.s.* displacement of 10^{-5} Å) and a dielectric constant of 2.

2.2. Fragment- and analog-based library focusing

Descriptors that characterize molecular surface, volume, mass, hydrogen bonding pattern (number of H-bond donors and acceptors), lipophilicity (log *P*), conformational flexibility (number of rotatable bonds), compositions and structural complexity (topological indices of Balaban, Wiener and Zagreb) were computed for the molecules of the training set of carbocyclic NA inhibitors (Williams et al., 1997; Lew et al., 1998; Kim et al., 1998) and their building blocks (substituents on the six-membered ring). Optimal ranges of the properties (descriptors) were defined in terms of upper and lower bounds and average values by analyzing eight most potent NA inhibitors and their building blocks. Penalty scores were assigned to fragments whose descriptor values lied outside the optimal ranges to filter out those with non-suitable properties. The diversity of fragments was evaluated via distance-based MaxMin function and the topological indices of Balaban, Wiener, and Zagreb (Willett, 1994).

2.3. Structure-based library focusing

Crystal structure of the closed form of NA subtype N1 complexed with oseltamivir (PDB code 2HU4 (Russell et al., 2006)), was used as the receptor target for docking of the analogs. The shape and size of the N1 ligand binding site was defined from the bound oseltamivir and was mapped onto a 3D energy grid of the size (73 Å × 73 Å × 68 Å) with a resolution of 0.25 Å per grid unit using the LigFit module of Cerius^2 (Cerius^2, 2000). The binding site model was enlarged by 3 layers to accommodate also bulkier R-groups. The analogs were docked into the binding site model as flexible molecules by generating conformers of each analog via randomizing the dihedral angles (10^4 Monte Carlo steps) and comparing the principal moments of inertia of the site and the analog after 50 rigid body minimization steps over four analog orientations (Peters et al., 1996). The docking score (ligand–receptor interaction energy) was computed as the nonbonding molecular mechanics term using CFF91 force field for each conformer by employing a grid representation of rigid N1 receptor, a cut-off distance of 20 Å applied to the non-bonded interactions and a dielectric constant of 2. Twenty best-fitting conformers were then energy minimized at the binding site and clustered into 10 conformational families according to

Table 1
Training and validation sets of carbocyclic NA inhibitors used in the QSAR model employed in definition of target-specific scoring function for the NA of subtype N1.

Training set	R_1	R_2	R_3	IC_{50}^{exp} (nM)[a]
TS1	–H	–(C=O)CH_3	–NH_3^+	6300
TS2	–CH_2CH_3	–(C=O)CH_3	–NH_3^+	2000
TS3	–$(CH_2)_2CH_3$	–(C=O)CH_3	–NH_3^+	130
TS4[b]	–$CH_2(CH_2CH_3)_2$	–(C=O)CH_3	–NH_3^+	1
TS5	–$CH_2(CH_2CH_3)CH_2CH{=}CH_2$	–(C=O)CH_3	–NH_3^+	1
TS6	–*cyc*Pen	–(C=O)CH_3	–NH_3^+	22
TS7	–Ph	–(C=O)CH_3	–NH_3^+	530
TS8	–CH_2*r*$(CH_2CH_3)(CH_2)_2$*cyc*Hex	–(C=O)CH_3	–NH_3^+	1
TS9	–CH_2*r*$(CH_2CH_3)(CH_2)_2$Ph	–(C=O)CH_3	–NH_3^+	0.3
TS10	–CH_2*s*$(CH_2CH_3)(CH_2)_2$Ph	–(C=O)CH_3	–NH_3^+	12
TS11	–$(CH2)_2CH3$	–(C=O)CH_3	–$NH^+{=}CHNH_2$	140
TS12	–H	–(C=O)CH_3	–$NH^+{=}C(NH_2)_2$	100
TS13	–$(CH2)_2CH3$	–(C=O)CH_3	–$NH^+{=}C(NH_2)_2$	1.8
TS14	–$CH_2(CH_2CH_3)_2$	–(C=O)CH_3	–$NH^+{=}C(NH_2)_2$	0.5
Validation set	**R_1**	**R_2**	**R_3**	**$IC_{50}^{exp}/IC_{50}^{pre}$[c]**
VS1	–*cyc*Hex	–(C=O)CH_3	–NH_3^+	1.40
VS2	–$(CH_2)_5CH_3$	–(C=O)CH_3	–NH_3^+	1.15
VS3	–CH_2*cyc*Hex	–(C=O)CH_3	–NH_3^+	1.08

[a] Experimental inhibitory activity of training and validation sets of carbocyclic inhibitors of influenza neuraminidase A/PR/8/34 (H1N1) were taken from references (Williams et al., 1997; Lew et al., 1998; Kim et al., 1998).

[b] Oseltamivir inhibitor.

[c] Ratio of experimental and predicted activities of the validation set. IC_{50}^{pre} was calculated from the PLP1 score using the QSAR regression equation (1).

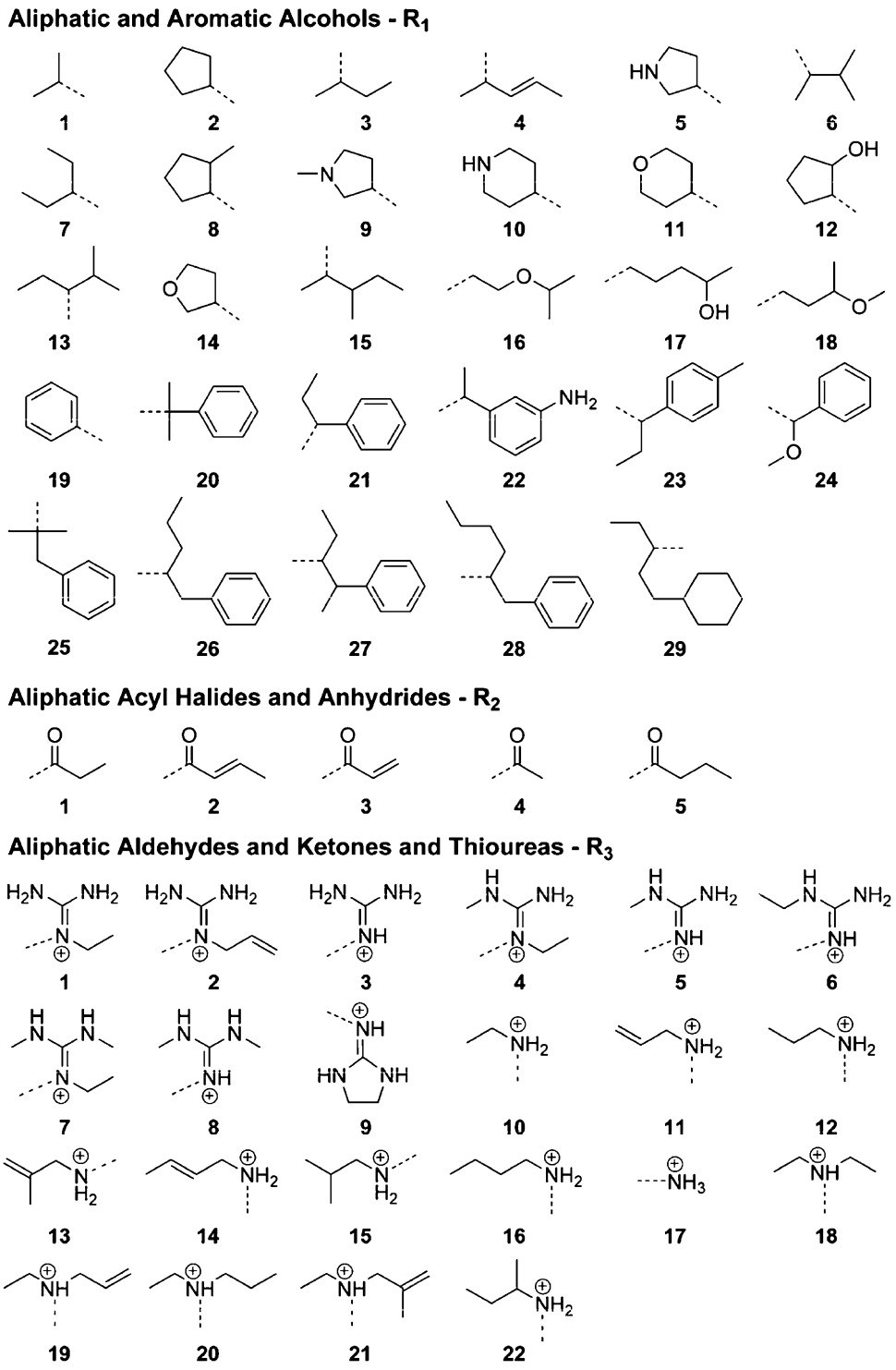

Fig. 3. R-groups selected by fragment-based library focusing.

their mutual *r.m.s.* deviations using the complete linkage clustering method of Jarvis–Patrick (Willett, 1994).

2.4. In silico screening

The conformer with the highest docking score in each cluster was selected for virtual screening by means of Piecewise Linear Potential scoring function type 1 (PLP1) (Verkhivker et al., 1995, 2000). The PLP1 score represents the sum of interaction energies of all heavy atom pairs of the docked analog and of the receptor. The PLP1 score was parameterized for prediction of the N1 inhibitory potencies of oseltamivir analogs in a QSAR model, which was derived for carbocyclic NA inhibitors with experimentally determined N1 inhibition potencies (IC_{50}^{exp}) (Williams et al., 1997; Lew et al., 1998; Kim et al., 1998).

2.5. QSAR model

The training set of 14 known carbocyclic NA inhibitors (Table 1) was docked to the binding site of the N1 receptor model using

the LigFit docking procedure (Cerius^2, 2000). Various scores implemented in the Cerius^2 (such as LigFit, LUDI1, LUDI2, PMF, PLP1 and PLP2 functions (Verkhivker et al., 1995, 2000; Böhm, 1994, 1998; Muegge and Martin, 1999; Muegge, 2001)) were computed and QSAR models, which related the IC_{50}^{exp} to the calculated scores were prepared by linear regression (least squares method) encoded in the QSAR module. Regression equation that correlates the PLP1 score with the IC_{50}^{exp} values against the influenza A subtype A/PR/8/34 (H1N1) (Williams et al., 1997; Lew et al., 1998; Kim et al., 1998), which displayed the highest statistical significance, was selected. The predictive power of this equation, which was used as the target-specific scoring function for *in silico* screening of the designed virtual library, was verified by applying it to a validation set of three carbocyclic NA inhibitors with known IC_{50}^{exp} values (Williams et al., 1997; Lew et al., 1998; Kim et al., 1998) (Table 1), which were not included into the training set.

3. Results and discussion

3.1. Library design and fragment-based focusing

Large diversity combinatorial library of oseltamivir analogs could be synthesized from shikimic acid and the ketone, anhydride, amine, and thiourea building blocks available in the commercial databases of suppliers of chemicals (Available Chemicals Directory (ACD), 2002). However, the size of such library would exceed the capacities of parallel synthesis and high-throughput screening equipment. Therefore, to design a small focused library we have introduced a set of filters and penalties, which can help to select the best building blocks (fragments) constituting the analogs. The fragment-based library focusing relied on the predetermined optimum ranges of nine structural and physicochemical properties (descriptors) that characterize molecular shape, size, polarity, hydrogen bonding, lipophilicity, conformational flexibility, and composition of the fragments. The optimum ranges (data not shown) were obtained by analyzing 3D models of the most potent compounds from a training set of 14 known carbocyclic inhibitors of the group-1 NAs (Table 1) (Williams et al., 1997; Lew et al., 1998; Kim et al., 1998), which were modeled in the bound conformation found in the crystal structure of the oseltamivir–N1 complex (Russell et al., 2006). Available fragments whose descriptor values lied outside the predetermined ranges and fragments with high similarity to other considered fragments were filtered out using combined penalty score and molecular diversity indices. The property upper limits for the R_2- and R_3-groups were augmented to permit the use of larger substituents due to somewhat limited diversity of the training set. Increased variation in the R_2- and R_3-groups is needed to design oseltamivir analogs inhibiting the NA subtype N1 as these groups directly interact with the 150-loop. Thus, 29 aliphatic and aromatic alcohols (R_1-groups), 5 aliphatic acyl halides and anhydrides (R_2-groups) and 22 aliphatic aldehydes, ketones and thioureas (R_3-groups) were selected as suitable and diverse building blocks (Fig. 3). Larger number of R_1- and R_3-groups was chosen in line with the elevated variability of the substituents filling the bulky R_1 cavity and the 150-loop cavity of the group-1 NAs (Fig. 2) (Russell et al., 2006). The size of the fragment-focused library of oseltamivir analogs was thus reduced to

$$29(R_1) \times 5(R_2) \times 22(R_3) = 3190 \text{ analogs}$$

The library of 3190 oseltamivir analogs was then generated by attaching of the R-groups from the fragment-focused sets (Fig. 3) onto the cyclic oseltamivir scaffold (Fig. 1).

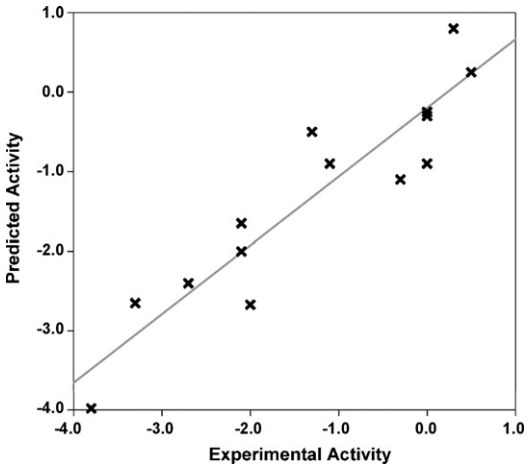

Fig. 4. Plot of regression equation of the QSAR model used for derivation of target-specific scoring function for the NA of subtype N1. Activity is expressed as $-\log_{10} IC_{50}$, IC_{50} is in nM.

3.2. Analog-based focusing

In the next step, analog-based focusing was applied to further reduce the size of the generated virtual library of oseltamivir analogs. The procedure was based on the molecular physicochemical descriptors and optimum molecular property ranges derived for the training set of carbocyclic NA inhibitors (Table 1). Two thousand most diverse analogs with molecular characteristics compliant with the predetermined property ranges were chosen from the fragment-focused library for a subsequent structure-based focusing step.

3.3. Structure-based focusing

In order to select a small highly focused combinatorial subset of oseltamivir analogs with good predicted binding affinities to the N1, structure-based focusing and *in silico* screening procedures have been applied to 2000 analogs. Each analog was docked into the binding pocket of the NA subtype N1 obtained from the crystal structure of NA-oseltamivir complex (Russell et al., 2006) by employing Monte Carlo ligand fitting algorithm of Cerius^2 (Cerius^2, 2000). The docking procedure yielded the best-fitting conformers, which were clustered into conformational families. In each cluster, the conformer with the highest docking score was then selected for subsequent virtual screening.

3.4. QSAR analysis of NA inhibitors and target-specific scoring function parameterization

To parameterize a scoring function specific for the NA subtype N1 target, we have correlated various scoring functions (Verkhivker et al., 1995, 2000; Böhm, 1994, 1998; Muegge and Martin, 1999; Muegge, 2001) implemented in the Cerius^2 program (Cerius^2, 2000) with the experimental activities (IC_{50}^{exp}) of the training set of carbocyclic inhibitors, Table 1 (Williams et al., 1997; Lew et al., 1998; Kim et al., 1998). The inhibitors of the training set were docked into the NA receptor model using the same LigFit procedure, which was used for the library of oseltamivir analogs. From the set of tested scoring function, the PLP1 score led to the best fit of the experimental activities to the predicted receptor binding energies against the N1. The following QSAR model was obtained by linear regression (Fig. 4):

$$pIC_{50} = -\log_{10} IC_{50} = -11.5254 + 0.1343\,\text{PLP1} \quad (1)$$

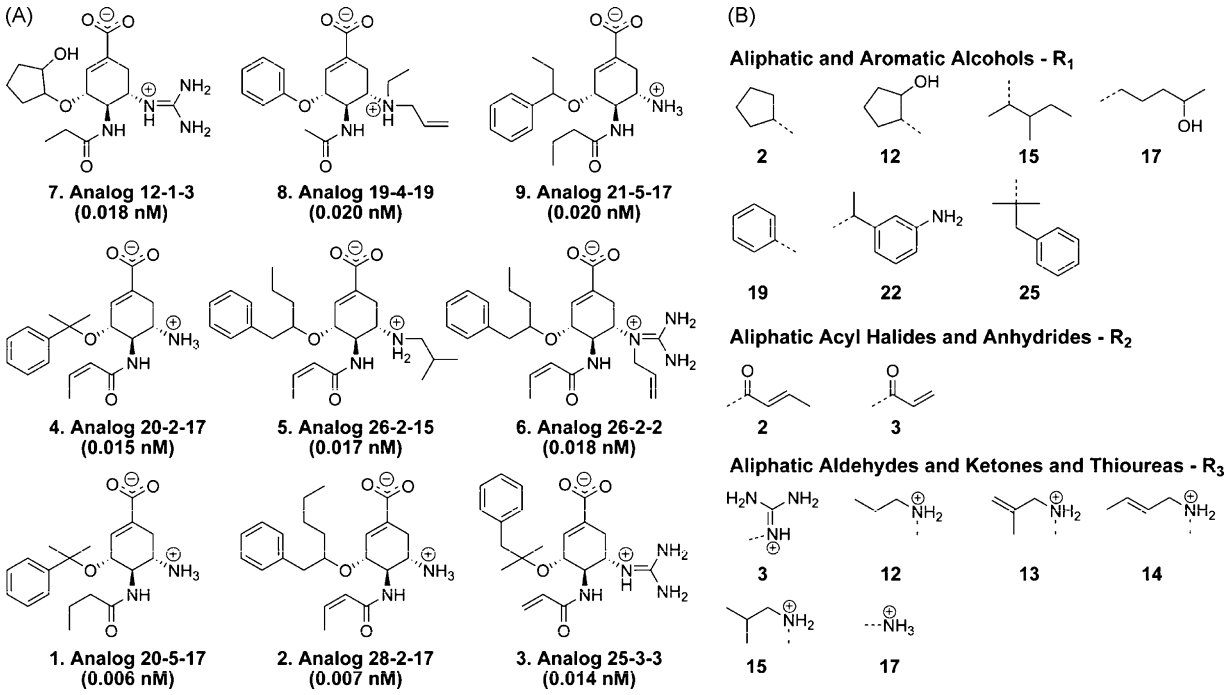

Fig. 5. (A) Chemical structures of the nine best oseltamivir analogs with the highest predicted activities against the NA subtype N1, Table 2. (B) List of aliphatic and aromatic alcohols (R_1-groups), aliphatic acyl halides and anhydrides (R_2-groups), and aliphatic aldehydes, ketones and thioureas (R_3-groups) selected in the final combinatorial subset.

(number of samples $n = 14$, correlation coefficient $R^2 = 0.88$, leave-one-out cross-validated correlation coefficient $R^2_{xv} = 0.84$, Fischer F-test = 85.1, statistical significance of the correlation $\alpha > 95\%$). The leave-one-out cross-validated correlation coefficient R^2_{xv} of 0.84 indicates that a major portion of the variance of the training set data was well described by this model. In addition, the quality of the model and the scoring function parameterization was confirmed by predicting the NA inhibitory activities for a validation set of three carbocyclic inhibitors (not included into the training set). Comparison of the observed and predicted activities, derived from Eq. (1) (ratio $IC_{50}^{exp}/IC_{50}^{pre}$ yielded values close to 1, Table 1), confirmed the predictive power of the model.

The training and validation sets used in the QSAR model (Williams et al., 1997; Lew et al., 1998; Kim et al., 1998) display only limited variation of the R_2- and R_3-groups space due to restricted availability of experimental activity data. Prediction of activities by the trained target-specific scoring function for analogs that somewhat exceed the boundaries of the training set diversity, is still possible, as QSAR models using ligand–receptor interaction energy of docked analogs (such as the PLP1 score) are less sensitive to training set molecular size and topology intervals than models using pure molecular descriptors (Frecer et al., 2008).

The derived scoring function specific for the NA of subtype N1 was subsequently used for *in silico* screening and scoring of the best-binding conformers of the library of oseltamivir analogs.

3.5. In silico screening

The best-binding conformers of 2000 oseltamivir analogs were screened *in silico* using the PLP1 score (Verkhivker et al., 1995, 2000). The predicted NA inhibitory activities of the designed analogs (IC_{50}^{pre}) were calculated from the target-specific scoring function (Table 2). The analogs were then rank-ordered according to the IC_{50}^{pre}. Absolute values of the predicted IC_{50}^{pre} in the low nanomolar range may be too optimistic, however, they served well for identification of the most potent analogs. Nine analogs with the highest predicted potencies (virtual hits) are listed in Table 2 and shown in Fig. 5A.

Table 2
List of the nine most active oseltamivir analogs (Fig. 5A) designed to inhibit the NA of avian influenza virus H5N1.

Oseltamivir analog[a]	PLP1[b]	IC_{50}^{pre} (nM)[c]
Analog 20-5-17	102.3	0.006
Analog 28-2-17	102.0	0.007
Analog 25-3-3	99.7	0.014
Analog 20-2-17	99.5	0.015
Analog 26-2-15	99.1	0.017
Analog 26-2-2	98.8	0.018
Analog 12-1-3	98.7	0.018
Analog 19-4-19	98.5	0.020
Analog 21-5-17	98.3	0.020

[a] Analog numbering contains the fragment numbers in positions R_1–R_2–R_3 shown in Fig. 3.

[b] Piecewise Linear Potential scoring function type 1 (PLP1) (Verkhivker et al., 1995, 2000) was used in the prediction of the binding affinity of analogs to the NA of subtype N1.

[c] Predicted inhibitory activity of oseltamivir analogs towards NA of subtype N1, calculated from the PLP1 score using the QSAR regression Eq. (1): $IC_{50}^{pre} = 10^{(11.5254-0.1343\,PLP1)}$.

An analog whose structure corresponds to oseltamivir was also included in the designed library. About 208 analogs scored better than oseltamivir based on the activities predicted from the PLP1 score.

The designed oseltamivir analog with the highest inhibitory activity, Analog 20-5-17 (Fig. 5A), contains an aromatic side chain filling the hydrophobic pocket of the N1 binding site occupied by the R_1-group. In this analog, the methyl of the acetamide R_2-group is replaced by a bulkier propyl chain, whereas the ammonium of the R_3-group remains identical to that found in oseltamivir.

3.6. Combinatorial subset selection

Considering only the 208 best scoring designed oseltamivir analogs with predicted anti-influenza NA inhibitory activities IC_{50}^{pre}

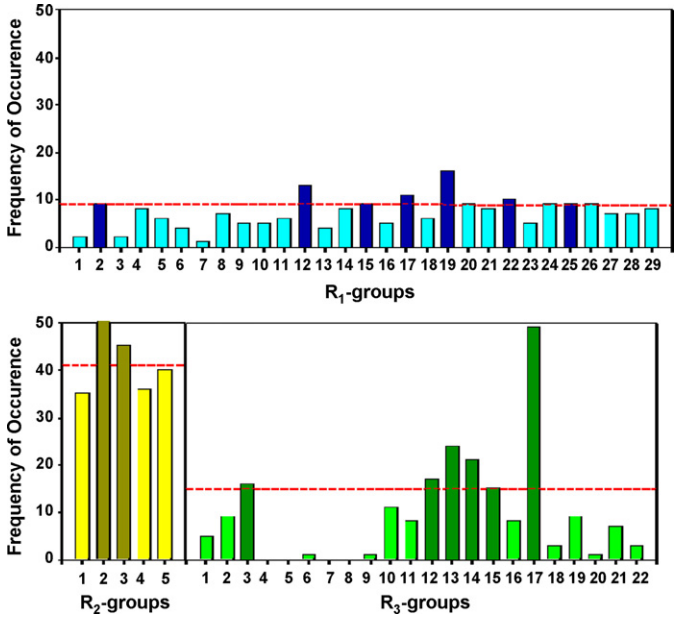

Fig. 6. Frequency of occurrence of individual R-groups (Fig. 3) in the 208 oseltamivir analogs with the highest predicted inhibitory activity ($IC_{50}^{pre} < 1$ nm). The fragments that display the highest frequency of occurrence (darker color bars above the threshold indicated by a red dashed line) were selected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

lower than 1 nM, the frequency of occurrence of the individual R-groups in the analogs was monitored (Fig. 6). The fragments that displayed the highest frequency of occurrence were selected to constitute a highly focused combinatorial subset. This subset displayed greatly increased probability to contain oseltamivir analogs with high predicted inhibitory activities toward the NA subtype N1, Fig. 5B. The size of the combinatorial subset was narrowed down to only:

$$7(R_1) \times 2(R_2) \times 6(R_3) = 84 \text{ analogs}$$

and permits rapid synthesis and testing for NA inhibitory activity.

4. Conclusions

The presented study demonstrated the applicability of computer-assisted combinatorial chemistry methods in the design and *in silico* screening of a virtual library of antiviral compounds. Our investigation yielded a small highly focused combinatorial subset of new oseltamivir analogs, which contains compounds with predicted inhibitory potencies against the wild type form of NA subtype N1 in the subnanomolar range. The predicted potencies of this combinatorial subset are lower than the IC_{50} values of oseltamivir and of the training set inhibitors. This study can thus help in drawing the attention of synthetic chemists working on the preparation of a next generation of agents against H5N1 avian influenza virus towards the explored subset of the chemical space, which is predicted to contain compounds with high N1 neuraminidase inhibition potencies.

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