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Identification of small molecules acting against H1N1 influenza A virus

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ABSTRACT

Influenza virus represents a serious threat to public health. The lack of effective drugs against flu prompted researchers to identify more promising viral target. In this respect hemagglutinin (HA) can represent an interesting option because of its pivotal role in the infection process.

With this aim we collected a small library of commercially available compounds starting from a large database and performing a diversity-based selection to reduce the number of screened compounds avoiding structural redundancy of the library. Selected compounds were tested for their hemagglutination-inhibiting (HI) ability against two different A/H1N1 viral strains (one of which is oseltamivir sensitive), and 17 of them showed the ability to interact with HA. Five drug-like molecules, in particular, were able to impair hemagglutination of both A/H1N1 viral strains under study and to inhibit cytopathic effect and hemolysis at sub-micromolar level.

Introduction

Influenza A viruses cause acute respiratory infection and can represent a severe threat for aged and high-risk people. Moreover, the recent emergence of highly pathogenic avian and swine flu has become a global issue to humans.

To date, the treatment of influenza A infection relies on vaccination and four approved anti-influenza drugs that hit two viral targets: M2 ion channel (amantadine and rimantadine) and neuraminidase (oseltamivir and zanamivir). Unfortunately, side effects and the emergence of drug-resistant strains have been reported for the above approved drug classes, limiting their effectiveness (Cheng et al., 2010; Deyde et al., 2007; Hay et al., 2008; Gubareva et al., 2009). Potential novel targets are being explored for the development of new weapons against flu (Das et al., 2010). Among them, hemagglutinin (HA) deserves a special attention because of its pivotal role in the infection process.

HA is a glycoprotein expressed on the viral surface along with neuraminidase (NA). It is responsible for the attachment of the viral particle to the host cell through cell surface-sialic acid receptor (Hartshorn et al., 1995). At a later stage it is able to carry out a pH-dependent conformational rearrangement necessary to fuse the viral membrane with the endosome producing the infection (Skehel et al., 1982).

18 subtypes of influenza A HAs have been classified so far (Tong et al., 2012, 2013), that can be divided in two major groups: group 1 (with two clades including H3) and group 2 (with 3 clades including H1) (Russell et al., 2008).

From a structural point of view, HA is a homotrimer with a mushroom-like shape (Fig. 1).

During the maturation process each monomer is cleaved in two subunits: HA_1 and HA_2 connected through disulfide bridges. The HA_1 chain constitutes mainly the globular head of the HA and includes the receptor binding site (Wilson et al., 1981) that is responsible for sialic acid recognition (Skehel and Wiley, 2002) and undergoes frequent mutations to escape antibody protection (antigenic drift). The HA_2 chain occupies the stem region and contains the machinery able to produce a structural modification at low pH, thereby the hydrophobic fusion peptide is exposed and promotes the fusion with the endosome membrane, essential to virion internalization. Because of this functional role the HA_2 sequence is very conserved among the HA subtypes. Targeting this region of the HA may produce broad spectrum agents less subjected to the onset of resistances. Indeed, human monoclonal antibodies binding to the stalk region of HA have been recently reported with broad-spectrum activity (Sui et al., 2009; Ekiert et al., 2009).

Several small molecules binding the HA have been disclosed so far (Yang et al., 2013; Vanderlinden and Naesens, 2014; Li et al., 2015). Among the known ligands, a few have a widespread activity, as most of them are subtype-specific (Motohashi et al., 2013). Moreover, the binding site of these ligands on the HA

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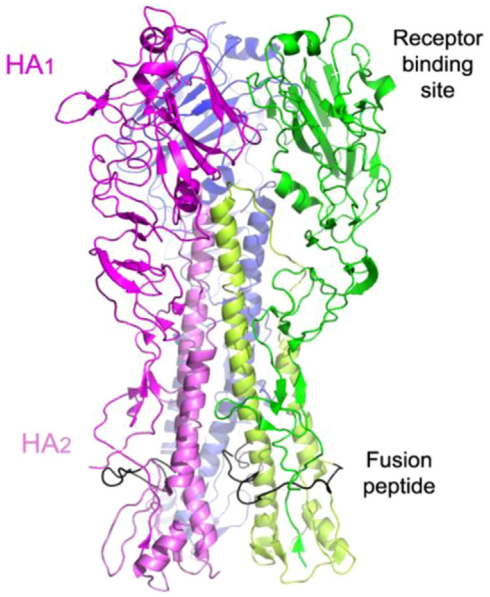


Fig. 1. Cartoon representation of the HA trimeric structure. Each monomer has a different color; the HA_2 subunit is represented in pale color, the fusion peptide is depicted in black, the receptor binding site on the upper part of HA is indicated.

surface has not been defined yet, except for TBHQ (Bodian et al., 1993), a small molecule active toward H3 subtypes that has been co-crystallized with the hemagglutinin (Russell et al., 2008). Its binding site may not be conserved in other HA subtypes therefore this information could not be exploited for the design of new ligands based on the structure of the receptor (structure-based drug design). On the other hand, ligand-based approaches, that uses the structural information of the known active molecules usually identifies compounds similar to the template molecules (Scior et al., 2012).

It is well known that to find new scaffold hits the random screening can be considered the best route even with low hit rate. To reach this objective a compound library ideally covering the whole chemical space (or better the "drug space") should be explored, submitting to experimental testing a huge amount of compounds, such in a HTS campaign, with high costs and long times. In this respect, chemoinformatics analysis of a virtual library can help to reduce the number of molecules to be tested, eliminating redundancy and selecting a small representative subset of the starting library (Rishton, 2008).

In the present work a small library of diverse compounds-selected from a large molecule dataset-was screened evaluating their ability to block the hemagglutination caused by the virus. Two different A/H1N1 viral strains were used for this study (A/RomaISS/02/08 H1N1 oseltamivir-sensitive virus, A/Parma/24/09 H1N1 oseltamivir-resistant virus). A good number of hits resulted from this test, some of them being able to block both A/H1N1 viral strains.

Results

Diverse library selection

The diversity-driven selection protocol was aimed to obtain a representative subset of the starting compound collection. The selection was performed *in silico* through chemoinformatics analysis of the virtual library.

For the screening purposes we downloaded almost 950,000 commercially available compounds from the Zinc database (http://zinc.docking.org/, Irwin and Shoichet, 2005).

The *in silico* exploration of the chemical space was confined to a smaller region occupied by "drug-like" compounds. This region is defined by physicochemical properties accounting for the drug-likeness of compounds, i.e. physical properties and chemical functionalities consistent with the majority of known drugs and correlated with an adequate bioavailability (Lipinski et al., 2001; Veber et al., 2002; Walters and Murcko, 2002). This methodology is aimed to apply ADME (absorption, distribution, metabolism, and excretion) considerations early in the drug discovery process to avoid costly late stage preclinical and clinical failures (Egan et al., 2000; Knox et al., 2005).

Moreover, undesired structures, containing reactive or toxic functional groups, were removed form the collection. The filtering procedure afforded a final collection of 495,000 molecules that were then used for the diversity-based selection.

The chemical similarity/diversity of molecules can be expressed as a number that quantifies the "distance" between two compounds. The number representing each compound can vary depending on the structural properties (descriptors) calculated to represent the molecules (Olah et al., 2004). In our approach we described compounds through their fingerprints: a widely used 1D representation of a chemical structure allowing for the chemoinformatics analysis of a large library (Willett, 2006). In our study the 2D fingerprints were calculated and used to cluster all structures, applying the widely used Tanimoto similarity metric. The selection of the most representative molecule from each group afforded a final non-redundant library of compounds. On the basis of our screening capabilities, a final subset of 105 readily available compounds have been purchased and submitted to HI assays. Structures of all tested compounds are reported in Electronic Supplementary material (Fig. S1).

Interaction of compounds with viral hemagglutinin and neutralization of Influenza virus

Selected compounds were tested in HI assay on two A/H1N1 viral subtypes (one sensitive and one resistant to oseltamivir) in order to check the influence of the NA mutation on the HA activity. As a matter of fact, both proteins are expressed on the virion surface and changes in NA sialidase conformation likely influence HA receptor-binding activity.

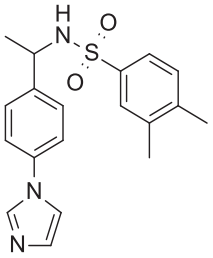
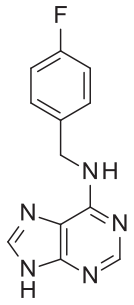
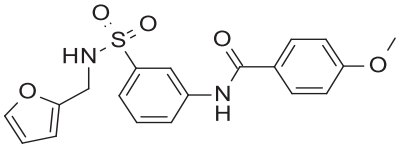
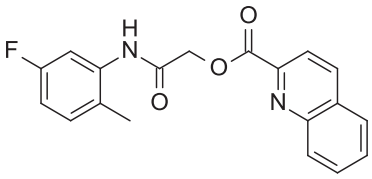
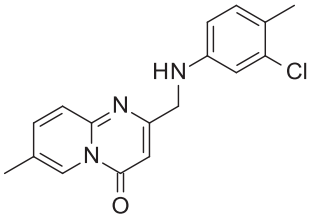
HI screening afforded 17 compounds with sub-micromolar activity over 105 assayed. The chemical structure of active compounds and results of HI activity are reported in Tables 1–3.

Among the compounds showing inhibiting effect, four were selective for A/Roma-ISS/2/08 (Table 2), eight were selective for A/Parma/24/09 (Table 3) and five were active on both H1N1 strains (Table 1). We also examined whether, and to what extent, these five compounds were able to neutralize virus infection. Cytotoxicity studies demonstrated that identified compounds did not induce significant cytotoxicity in MDCK cells until 64 μM. Thus, subsequent neutralization studies were performed with compound concentrations ranging from 0 to 64 μM. All compounds were able to prevent Influenza virus infection at concentration ranging from 250 to 500 nM confirming HI results (data not shown).

Pharmacophore analysis

Active compounds in HI assays share similar structural features: two or three aromatic or heterocyclic groups spaced by an amide, amine or sulfonamide function. However, the simple analysis of the chemical structures does not readily provide an

Table 1
HI titers of compounds interacting with both strains.

Compound ID	Compound structure	HI titer (μM)	
		A/Parma	A/Roma
3		0.48	0.24
4		0.48	0.48
5		0.48	0.48
6		0.48	0.48
7		0.48	0.48

indication about the structure–activity relationship. To this aim active compound common pharmacophore models were generated in order to elicit the main features responsible for the activity toward the two A/H1N1 strains.

Active compounds were aligned with Phase attempting to find a common pharmacophore hypothesis separately for compounds actives toward oseltamivir-resistant (A/Parma) and those active toward oseltamivir sensitive (A/Roma) strain.

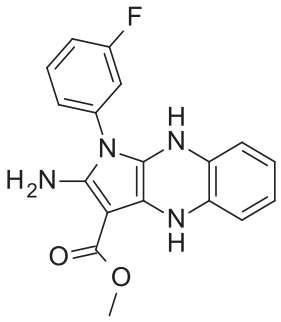
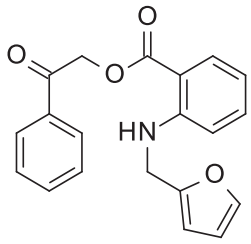
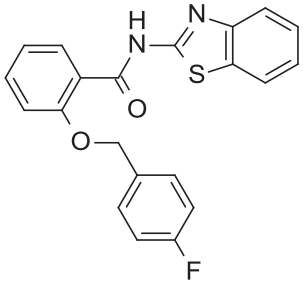
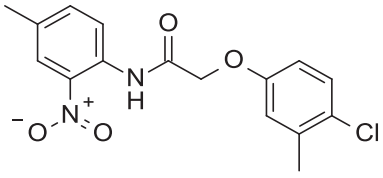
The two models were obtained using Phase (Schrödinger, 2012) and aligning all compounds active toward the same viral strain. Resulting hypotheses are composed by the same combination of features (ADHRR; Acceptor, Donor, Hydrophobic and two aromatic Rings), but are differently arranged in the 3D space (Fig. 2).

Anti-influenza activity

Inhibition of infection and hemolysis assays confirm the activity of broad-spectrum compounds toward both subtypes (Table 4).

The first assay assesses the ability of the compounds to protect cells against viral infection. Compounds **5** and **6** showed the best

Table 2
HI titers of compounds interacting with oseltamivir-sensitive strain (A/RomaISS/02/08 H1N1).

Compound ID	Compound structure	HI titer (μM)
8		0.48
9		0.48
10		0.48
11		0.48

activity profile with a nanomolar activity toward A/Parma and A/Roma, respectively, and a good selectivity profile.

Time course assay

The antiviral mechanism of selected five compounds was examined by a time course assay (time of drug addition, Fig. 3) in a single infectious cycle using both Influenza A H1N1 strains. Results from our experiments showed that all the compounds totally prevented viral infection when present throughout the infection and, in particular, their antiviral effect was exerted at the early stage of virus infection (0–2 h). Moreover, the temporal kinetic of the inhibitory activity of all compounds was quite similar. These results suggest that these molecules could affect Influenza virus infection by acting not only on viral binding to host cells but also on the fusion step required for the viral uncoating.

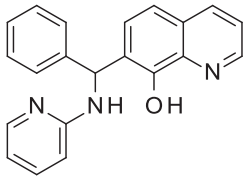
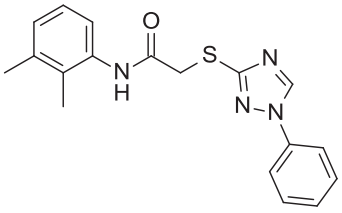
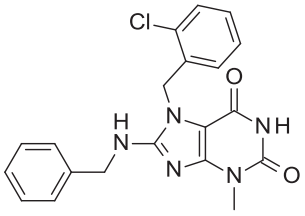
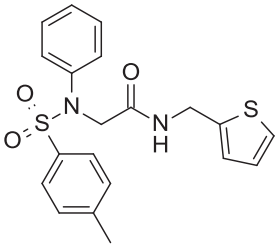
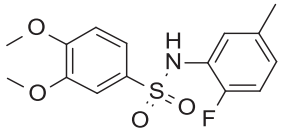
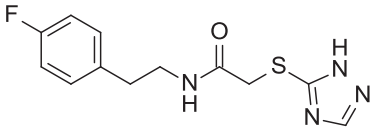
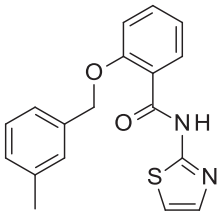
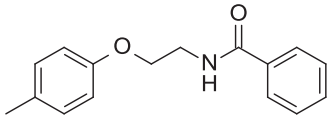
Combination treatment of selected compounds and oseltamivir

Finally, the potential additive or synergistic effect with oseltamivir of the five most promising molecules (compounds **3**–**7** active on both H1N1 strains) was performed to gain deeper insight into the mode of their antiviral action.

To this aim a combination treatment of these molecules and oseltamivir was carried out on the oseltamivir-sensitive viral strain (A/RomaISS/02/08 H1N1). Synergistic effect was observed only when virus-infected cells were incubated in the presence of compound **6** (from 0.97 nM to 500 nM) and oseltamivir (from 117 nM to 15 μM) (Fig. 4).

A quantitative measure of the total synergy of a drug combination can be expressed in terms of synergy volumes (Prichard et al., 1992). As synergy volume values $> 100\ \mu M^2\%$ are considered synergic, compound **6**, with a synergy volume of 305.23 $\mu M^2\%$ in combination with oseltamivir, displayed marked synergistic inhibition of Influenza virus infection. The synergy produced by the

Table 3
HI titers of compounds interacting with oseltamivir-resistant strain (A/Parma/24/09 H1N1).

Compound ID	Compound structure	HI titer (μM)
12		0.24
13		0.48
14		0.48
15		0.48
16		0.48
17		0.48
18		0.48
19		0.96

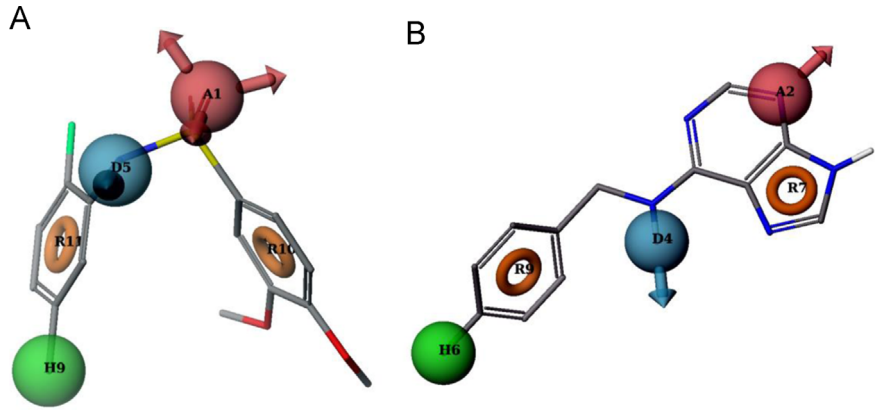


Fig. 2. Representation of the pharmacophore hypotheses obtained for compounds active towards A/Parma (A) and A/Roma (B). Pharmacophore features are represented: hydrophobic as a green sphere, aromatic as an orange ring, H-bond donor as a cyan vector and the acceptor as a red vector. The reference compound for each pharmacophore hypothesis is shown as well in stick (compound **16** in (A) and **4** in (B)). The program Phase, in fact, automatically selects the reference molecule to whom pharmacophore all other ligands are aligned.

Table 4
Anti-influenza activity evaluated using cell protection assay in MDCK cells infected with Influenza virus.

Compound	*CC_{50} (μM)	°EC_{50} (nM)		˙SI		#IC_{50} (nM)	
		A/Roma	A/Parma	A/Roma	A/Parma	A/Roma	A/Parma
3	300	500+/−0.01 $p<0.01$	125+/−0.01 $p<0.01$	6.10^2	$2.4.10^3$	254+/−0.001	220.7+/−0.002
4	200	200+/−0.09 $p<0.01$	250+/−0.07 $p<0.01$	1.10^3	8.10^2	241.5+/−0.003	229.9+/−0.001
5	450	150+/−0.07 $p<0.01$	62+/−0.02 $p<0.01$	3.10^3	$7.25.10^3$	244+/−0.002	230.7+/−0.001
6	250	62+/−0.07 $p<0.01$	125+/−0.03 $p<0.01$	4.10^3	2.10^3	242.4+/−0.002	240+/−0.003
7	100	800+/−0.06 $p<0.01$	250+/−0.01 $p<0.01$	$1.25.10^2$	4.10^2	277.7+/−0.002	333.3+/−0.001

*CC_{50} cytotoxic concentration 50%, °EC_{50} effective infection inhibiting concentration 50%, ˙SI (selectivity index)=CC_{50}/EC_{50}, #IC_{50} hemolysis inhibiting concentration 50%.

combination was statistically significant, as indicated by the values at the 95% confidence level (data not shown).

Discussion

HA represents an attractive target to find new anti-Influenza agents, because of its important role in host cell attachment and fusion process (Hartshorn et al., 1995; Skehel et al., 1982).

Several small molecules have been identified so far that are able to interfere with the HA functions. They can hinder one or both the functions played by HA: some of them bind the receptor binding site competing with sialic acid such as triterpenoids (Yu et al., 2014) and sialic acid mimetic peptides (Matsubara et al., 2010). The majority of identified compounds, however, block the fusion process promoted by HA preventing the conformational rearrangement of HA at low pH. Among these molecules we can mention arbidol that has been commercialized in Russia and China (Bodian et al., 1993; Boriskin et al., 2008), stachyflin derivatives (Yoshimoto et al., 2000; Motohashi et al., 2013), CL-61917 and its analogues (Plotch et al., 1999), polyphenols (Yang et al., 2014), BMY 27709 (Luo et al., 1997) and many others that have been recently reviewed (Yang et al., 2013; Vanderlinden and Naesens, 2014; Li et al., 2015).

The claimed mechanism of action concerns the interaction with the HA_2 chain or, better, the stem region that, because of its role in the fusion machinery, is more conserved among viral strains and should be less prone to mutations. However, most of them are subtype selective and the mechanism of action has not been exactly clarified, while experimental binding mode has been assessed just for TBHQ, a simple ligand blocking fusion of H3 Influenza strains.

The rational design has been applied to identify TBHQ (docking) (Hoffman et al., 1997) and ciclopentane derivatives through ligand-similarity search (Tang et al., 2010), while most of HA inhibitors have been selected through HTS: in a recent work Basu and co-workers reported the results of a HTS screening that identified 36 primary hits over 106,000 tested compounds with a hit rate of 0.035% (Basu et al., 2014). In this context, the present study represents an example of an effective approach to identify active compounds avoiding the screening of a large number of compounds.

In our work we exploited the chemoinformatics analysis of a larger library to select a small diverse library screened in HI assays to identify new potential antiviral compounds. Chemoinformatics is a well-established tool in the drug discovery process, able to easily manage large molecule collections (Zhou, 2011). In this case its use allowed to strongly reduce the number of assayed compounds with a much smaller effort, in terms of costs and time, for the biological screening. In fact, we identified 17 active compounds over 105 tested molecules and 5 of them were actives on both H1N1 strains with a final hit rate of 5%.

The activity showed in the HI assays relies on the direct competition of the small molecules with the sialic acid (SA) on the host cell. The haemagglutination, in fact, is due to the recognition between the SA and the receptor binding site on the upper part of the HA (HA_1 subunit, see Fig. 1). Therefore, our molecules should be able to bind this site of HA competing with the natural binder

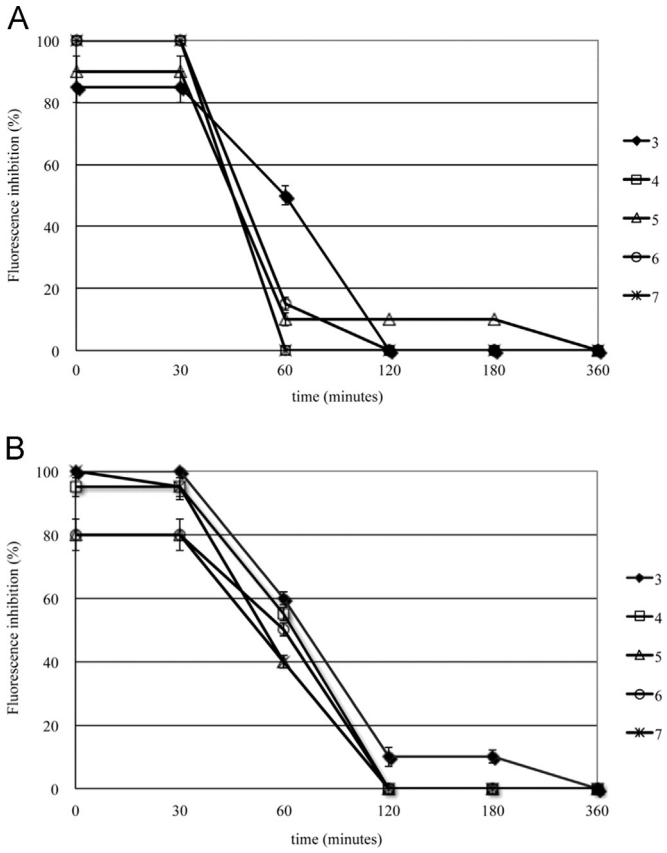


Fig. 3. Addition time effect of compounds on Influenza virus A/Roma ISS/02/08 H1N1 (A), and Influenza virus A/Parma/24/09 H1N1 (B) infection. After adsorption step (1 h at 4 °C; time zero), incubation temperature was shifted to 37 °C and EC50 concentrations of each compound were added to infected cells at different time intervals after time zero. After 6 h incubation at 37 °C, viral antigen synthesis was measured by immunofluorescence. Data represents the means of at least three independent experiments.

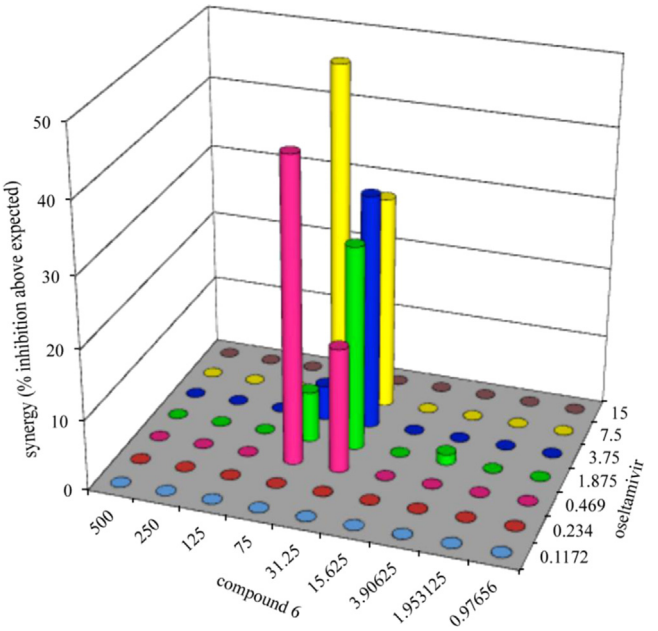


Fig. 4. Three-dimensional plots showing the interaction of compound **6** (nM) and oseltamivir (μM) on the replication of Influenza virus. Synergy was analyzed by the three-dimensional model of Prichard and Shipman, using the MacSynergy II software at 95% confidence limits.

even if the structural similarity with sialic acid is not evident. Actually, the displacement of sialic acid from its binding site can be carried out also by weak binders as it binds HA in the millimolar range (Takemoto et al., 1996).

Moreover, the selectivity observed in the HI assay for the two viral strains could be due to the mutated residues. The HA sequences of the two H1N1 strains considered in this work, are very conserved, with just four different residues surrounding the receptor binding site in the HA_1 chain (the alignment of the two HA sequences is reported in Supplementary material, Fig. S2). The obtained pharmacophore hypotheses highlighted that active compounds have the same features, differing for the 3D space arrangement, thus suggesting that these compounds may bind the same site with different conformations. We could hypothesize that compounds active toward both strains are able to satisfy the conformational requirements for both HAs.

Neutralization assay showed that the five compounds interacting with both strains were also able to prevent virus–cell interaction. As a matter of fact, pretreatment of virus samples with these molecules provided 100% protection against both viral strains *in vitro*. These results support the hypothesis that small identified molecules could be able to prevent infection by a direct interaction with HA_1 subunit.

The analysis of structural features of identified compounds (two aromatic portions linked by an amine/amide or sulfonamide function) put in evidence that they share common characteristics with already disclosed HA ligands such as 1e (Zhu et al., 2012) and MBX2546 (Basu et al., 2014), that have been demonstrated to block the HA mediated fusion. Indeed, the time course assay carried out for the five active compounds shows that they act in the early stage of the infection process.

Therefore, our results suggest that these molecules should be able to interact with the receptor binding site on the HA_1 subunit, competing with the sialic acid, and, at the same time, to interfere with the HA_2-mediated membrane fusion. In this respect, in is worth mentioning that the compound MBX2546 shares with our molecules a very similar activity profile: it is able to block both the hemagglutination and the fusion process and its binding the HA stem has been assessed by NMR studies. The already mentioned structural similarity with our ligands could suggest they interact similarly with HA.

Our results on double combination of small molecules and oseltamivir demonstrate that only compound **6** exhibits marked synergy with oseltamivir (synergy volume 305.23 μM^2%). This represents an interesting finding as values over 100 μM^2% indicate strong synergy and are considered probably important *in vivo* (Prichard and Shipman, 1990). The body of published work indicates that the synergy of double combinations varied and can be dependent on the drug combination, drug dose, experimental design and virus strain (Nguyen et al., 2009). For instance the combination of rimantadine and ribavirin was found synergistic against influenza H1N1 and H3N2 strains in one study (Hayden et al., 1980) but was found to be additive against different H1N1 and H3N2 strains in another investigation (Madren et al., 1995). Moreover, it has been reported that oseltamivir in combination with ribavirin was antagonistic, additive, or synergistic in mouse models, depending on the virus strain and/or the dose of the drugs (Govorkova et al., 2004; Smee et al., 2006). So, the variability in synergy for double compound combinations reported in the literature, due to the specific drugs in the combinations or to the virus strain, is consistent with the *in vitro* data we present here.

As Influenza virus productive infection requires a balance between the HA-mediated binding and entry process (early steps) and NA-mediated egress process (late step), the observed synergy is likely due to the simultaneous inhibition of HA-mediated activities by compound **6** (early steps) and NA-mediated egress

activities by oseltamivir (late step). Therefore, further optimization of this small molecule inhibitor as potential anti-Influenza therapeutic agent, either individually or in combination with current anti-viral treatments, appears promising.

In conclusion we have identified five novel H1N1 influenza A virus inhibitors. The activity profile along with their drug-like properties makes them an optimal starting point for the development of a therapeutic agent against flu.

In particular, one of these molecules showed attractive properties deserving further investigation about the mechanism leading to its high synergy with oseltamivir.

Materials and methods

Diverse library selection

2D structure data files (.sdf files) of commercially available compounds from Specs, Enamine and Asinex suppliers were downloaded from the ZINC database (Irwin and Shoichet, 2005; http://zinc.docking.org/). A total of almost 950,000 compounds were imported into CANVAS (Schrödinger, 2012; Sastry et al., 2010; Duan et al., 2010), an interface able to apply several chemoinformatics techniques. The following molecular properties were calculated for each compound: MW, number of rotatable bonds and H-bond donors and acceptors, PSA, AlogP, molecular polarizability and molecular refractivity. In order to obtain a collection of lead-like compounds the initial library was filtered applying the following parameter cut-off: MW <500 and $>=200$; HBD >0 and $<=5$; HBA $<=10$; AlogP $>=-2$ and $<=5$; RB $>=2$ and $<=8$; MR $>=40$ and $<=130$; PSA $<=120$. Resulting compounds were filtered also to eliminate all reactive and undesired functional group, applying a REOS filter available in Canvas (Walters et al., 1998).

Binary 2D fingerprints were generated for all resulting molecules ($\sim$495,000). The hashed linear fingerprints were calculated using the Daylight invariant atom types specification and distinguishing bonds by bond order. A 32-bit precision was applied, filtering bits by ON/OFF frequencies. Generated fingerprints were used to perform a clustering of all structures in order to collect most similar molecules in the same group. To this aim the Leader-follower clustering method was used, applying the Tanimoto similarity metric and a cluster radius of 0.5. Most representative compounds of each cluster were selected. The list of purchased compounds is reported as Supplementary material.

Pharmacophore model generation

Non-racemic compounds showing activity in the HI assays were submitted to the Develop Common Pharmacophore module in Phase (Schrödinger, 2012), Active compounds protonation state at physiological pH was calculated using Epik (Schrödinger, 2012). The conformational model for each compound has been generated using ConfGen and performing a thorough research. Five-sites hypotheses were generated applying the default parameters.

Virus strains

The following Brisbane-like Influenza A virus strains were used: A/RomaISS/02/08 H1N1 oseltamivir-sensitive virus and A/Parma/24/09 H1N1 oseltamivir-resistant virus. Virus titers were determined by a hemagglutination and/or plaque assays according to the standard procedures (Gaush and Smith, 1968; Rimmelzwaan et al., 1998).

Cells

Madin-Darby canine kidney (MDCK, ATCC, CRL-2936) cells were grown at 37 °C in minimal essential medium (MEM, Invitrogen, Paisley, UK) containing 1.2 g/l $NaHCO_3$ and supplemented with 10% inactivated fetal calf serum (FCS, Invitrogen, Paisley, UK), 2 mM glutamine, nonessential amino acids, penicillin (100 IU/ml) and streptomycin (100 μg/ml).

Compounds

Small molecules identified in the computational screening of ZINC database were purchased from Enamine Ltd. as dry powders. Compound purity, assessed by NMR and LC-MS, is higher than 92%. Chiral compounds are provided as racemic mixture. Compounds were dissolved in dimethylsulfoxide (DMSO, Sigma, Chemical Company, St.Louis, MO, USA) at 2 mmol/L and stored at −20 °C in amber glass vials.

Oseltamivir carboxylic acid, the active form of oseltamivir ((3R,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylicacid) (Roche) was dissolved in H_2O at 2.4 mmol/L and stored at −20 °C.

Cytotoxicity assay

Two-fold serial dilutions of each compound and DMSO or H_2O in culture medium were incubated at 37 °C with confluent MDCK cells grown in 96-well tissue culture microplates (Nalge Nunc Europe Ltd, Neerijse, Belgium). After 24 and 48 h, the cytoxicity was measured by the neutral red (NR) uptake assay. Briefly, the cells were rinsed in PBS, and NR incorporated in viable cells was extracted with a solution of 50% (v/v) ethanol and 1% (v/v) acetic acid for 10 min before measuring optical density at 550 nm using an ELISA plate reader (PerkinElmer Italia, Monza).

The ODs from identical wells were averaged, and compound cytotoxicity was expressed as the minimum cytotoxic concentration (MCC), i.e. the concentration producing minimal changes in cell morphology, or the CC_{50} value, i.e. the concentration causing 50% reduction in cell viability by NR assay. The cytotoxicity was computed by comparison to the average OD of wells containing compounds with the average OD of wells containing DMSO, H_2O and culture medium alone.

Hemagglutination inhibition assay (HI)

Virus in PBS was incubated for 1 h at 4 °C with serial dilutions of compounds in PBS. An equal volume of 0.5% turkey erythrocytes was then added and allowed to agglutinate. Titers were expressed as the reciprocal of the compound dilutions giving 50% hemagglutination of erythrocytes by four virus agglutinating units.

Neutralization assay

Neutralization was carried out by incubating serial twofold compound dilutions, starting from 1 μM, in culture medium with equal volumes of virus suspension containing 1.10^6 p.f.u. for 1 h at 4 °C. MDCK cells were seeded on 96-well tissue culture microplates (Nalge Nunc Europe Ltd., Neerijse, Belgium) at the concentration of 20,000 cells/well and allowed to grow for 24 h at 37 °C in 5% CO_2. After this time, cells were incubated for 1 h at 37 °C with 100 μl/well of the virus-protein mixtures. Infection was carried out in quadruplicate at a multiplicity of infection of 10 plaque forming units (p.f.u.)/cell. After viral adsorption, cells were rinsed thoroughly and incubated at 37 °C for 48 h. The cytopathic effect (c.p.e.) induced by infection was measured by the neutral red uptake assay (Marchetti et al., 2004), and results were

expressed as percentage of c.p.e. inhibition by comparison with untreated control cultures. EC_{50} was defined as the compound concentration that generates 50% of the maximal protection. Percentage of protection was calculated by the equation (A_{550} compound-virus-A_{550} virus)/(A_{550} mock infected cells-A_{550} virus) x100%. The data were expressed as the EC_{50} relative to the value observed in the absence of compound.

Hemolysis inhibition assay

Hemolysis inhibition assays were modified from those described previously (Bodian et al., 1993). Briefly, 100 µl (about 6 µg of protein) of purified virus were incubated at 37 °C for 1 h with an equal volume of PBS containing various concentrations of compounds. Then, 200 µl of a 2.0% solution of turkey red blood cells (RBCs) in PBS were added to the reaction and further incubated at 37 °C for 10 min. The virus-bound turkey RBCs were pelleted by centrifugation at 1600 rpm for 8 min and the RBC pellet was suspended in 450 µl of low pH PBS buffer (pH 5.0 +/−0.05) containing the corresponding concentration of compound. After 25 min incubation at 37 °C, the reaction was neutralized to pH 7.0 +/−0.2 by the addition of 1 N NaOH. Cell debris and unlysed cells were pelleted down through centrifugation at 2000 rpm for 8 min. Then 300 µl of supernatant were transferred to 96-well tissue culture plate (Corning Incorporated, Corning, New York) for measurement of optical density at a wavelength of 540 nm using a multilabel plate reader (PerkinElmer Italia, Monza). Percentage of protection was calculated by the equation (A_{540} compound-virus-A_{540} virus)/(A_{540} PBS-A_{540} virus)x100%. The data were expressed as the IC_{50} relative to the value observed in the absence of compound.

Time course assay (time-of-addition)

The effect of small molecules on Influenza virus infection was tested in a time-of-addition assay. For these experiments, infection was synchronized by incubating the virus (m.o.i. 10) with the cells for 1 h at 4 °C. After the attachment step, unbound virus was removed by washing twice with medium and cells were incubated for 6 h at 37 °C. The inhibiting activity of the five compounds was assessed by different experimental procedures: (i) the small molecules were added to the infected cells after the temperature shift and were present for the entire time of infection or, (ii) before the addition of chemicals, infected cells were incubated for well-defined times. Influenza virus antigen synthesis was measured by indirect immunofluorescence.

Immunofluorescence

MDCK infected cells were washed in PBS, fixed in acetone at −20 °C for 5 min, incubated with monoclonal anti-Influenza virus antibodies (Immunological Sciences, Rome, Italy) for 45 min at 37 °C. After washing in PBS, viral antigen synthesis was estimated by utilizing (FITC)-conjugated anti-mouse gammaglobulin antibodies (Sigma Chemical Company) and an UV Leitz microscope.

Combination treatment of selected compounds and oseltamivir

The combination treatment of selected compounds and oseltamivir was performed to demonstrate additive or synergistic effects on the replication of the oseltamivir sensitive Influenza virus strain A/RomaISS/2/08 H1N1. MDCK cells were infected at a multiplicity of infection of 10 p.f.u./cell. After 1 h incubation at 37 °C the medium was replaced with mock-medium, or medium containing oseltamivir (from 117 nM to 15 µM) compound 6 (from 0.97 nM to 500 nM) or combinations of both. The infected cells were further incubated for 48 h, and virus replication was measured by Neutral Red assay. Drug–drug interactions were analyzed by the three-dimensional model of Prichard and Shipman (1990), using the MacSynergy ™II software (Prichard et al., 1992) at 95% confidence limits. Theoretical additive interactions were calculated from the dose–response curve for each compound individually, and the calculated additive surface was subtracted from the experimentally determined dose–response surface to give regions of synergistic or antagonistic interactions. The resulting surface appears as horizontal plane at 0% of synergy if the interactions of two compounds are additive. Any peak above or below this plane indicates synergy or antagonism, respectively.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.11.024.

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