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Structure-based optimization and synthesis of antiviral drug Arbidol analogues with significantly improved affinity to influenza hemagglutinin

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ABSTRACT

Influenza is a highly contagious respiratory viral infection responsible for up to 50,000 deaths per annum in the US alone. The need for new therapeutics with novel modes of action is of paramount importance. We determined the X-ray structure of Arbidol with influenza hemagglutinin and found it was located in a distinct binding pocket. Herein, we report a structure-activity relationship study based on the co-complex combined with bio-layer interferometry to assess the binding of our compounds. Addition of a *meta*-hydroxy group to the thiophenol moiety of Arbidol to replace a structured water molecule in the binding pocket resulted in a dramatic increase in affinity against both H3 (1150-fold) and H1 (98-fold) hemagglutinin subtypes. Our analogues represent novel leads to yield more potent compounds against hemagglutinin that block viral entry.

Influenza annually affects ~5–20% of the US population leading to nearly 200,000 influenza-related hospitalizations per year.[1] While vaccines have produced some measure of control over the risk of infection, rapid antigenic drift makes the selection of which strains to include in the seasonal vaccine an annual challenge. There are currently four licensed influenza drugs available for treatment use in the US; the M2 ion channel inhibitors Amantadine (Symmetrel®) and Rimantadine (Flumadine®),[2] and the neuraminidase (NA) inhibitors Osetlamivir (Tamiflu®) and Zanamivir (Relenza®).[3] However, as resistance to these drugs emerges in current strains, the quest for small molecule therapeutics with novel modes of action becomes more urgent.[4–6]

Arbidol (Umifenovir) is a broad-spectrum antiviral against a number of viruses, including influenza, Ebola, hepatitis B, and hepatitis C.[7,8] Despite initial lack of a known mechanism-of-action against any target virus, Arbidol is clinically used in Russia and China and is currently in phase IV US clinical trials (clinicaltrials.gov/ct2/show/NCT01651663). One major drawback to the use of Arbidol is the large dose required to achieve therapeutic efficacy.[9] Several groups have tried to improve the therapeutic potential by changing the substituents decorating the indole core (Fig. 1A). These have included changes to the nitrogen substituent, the hydroxy group in position 5, the bromo group in position 6, and the thiophenol in position 2.[10–15] However, despite the large number of structure-activity relationship (SAR) studies carried out to date, none of the compounds have shown a vast improvement in binding affinity to both group 1 and group 2 viruses over the parent compound Arbidol. This is perhaps not surprising as the exact binding site was unknown, although the mechanism of action was reported to involve increasing hemagglutinin (HA) stability through preventing the low pH-induced HA transition to the fusogenic state.[9,16,17]

To determine its binding site and mechanism of action, we recently determined crystal structures of Arbidol in complex with the influenza HA viral fusion glycoprotein from the pandemic 1968 H3N2 and recent 2013 H7N9 viruses.[18] Arbidol binds in a conserved hydrophobic cavity at the interface of the HA protomers within the upper stem of the fusion region (Fig. 1B and C). The structures demonstrate the molecular mechanism where Arbidol stabilizes the pre-fusion conformation of HA and prevents the conformational rearrangement required for membrane fusion and subsequent infection.

Using this structural data, we observed that Arbidol binds tightly to the pocket along the edge of the indole core at positions

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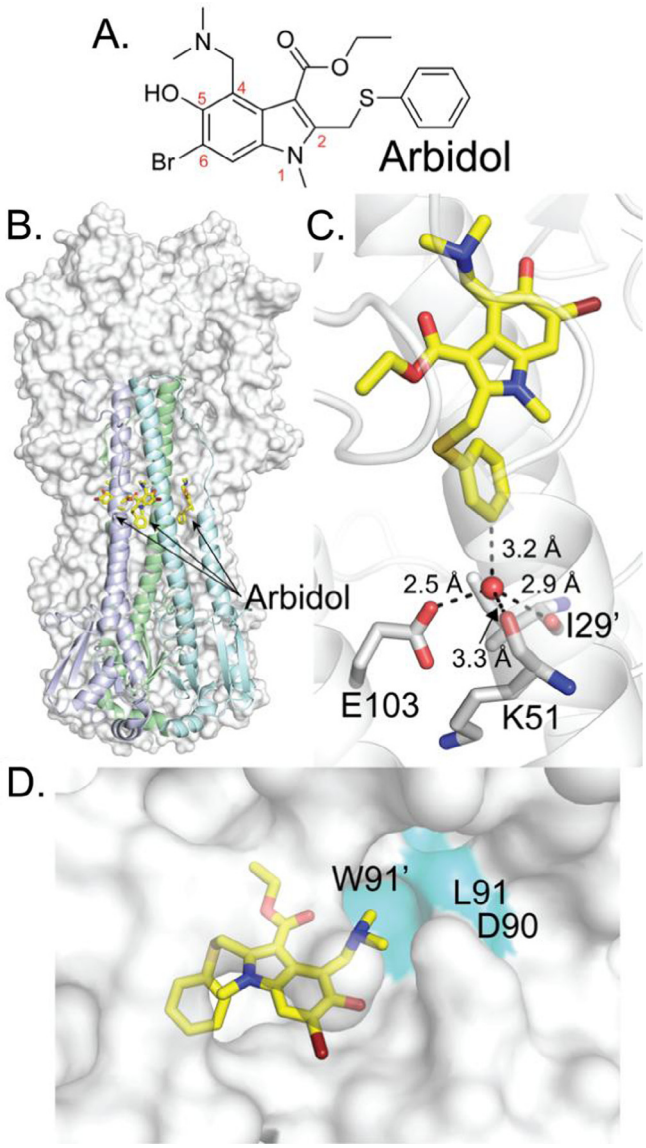

Fig. 1. A. Structure of Arbidol. B. Hemagglutinin trimer (H3 – HK68: A/Hong Kong/1/1968, PDB ID: 5T6N) with the location of the Arbidol binding site within the stem region.[18] C. A highly ordered water molecule adjacent to the Arbidol (yellow carbon, red oxygen, blue nitrogen, mustard sulfur, and maroon bromine) was exploited for structure-based design of Arbidol analogues. D. The HA pocket residues (cyan) near the Arbidol amino group at position 4 that could be used for additional interactions with Arbidol analogues.

1, 5 and 6 (Fig. 1A and C)[10–13] and explains why adding large chemical moieties at these positions can have a deleterious effect on the antiviral efficacy of those molecules.

Our structures also showed that there was additional space within the pocket not fully optimized for binding to the protein by the amino group in position 4 and the thiophenol group at position 2 (Fig. 1A and D). Using our structural data, we designed and synthesized several molecules to exploit these potential additional interactions and thus improve upon Arbidol binding. We herein report a new Arbidol analogue with significantly improved binding to HA in comparison to the parent compound. Our study provides new insights into how to manipulate compounds to bind to the influenza virus and presents exciting evidence for a possible new influenza therapeutic.

Results and discussion

Synthesis of Arbidol analogues

Our co-complex crystal structure of HA with Arbidol showed that there was underutilized space in the binding pocket to accommodate modifications to both the thiophenol group at position 2 and the dimethylamino group at position 4. To investigate the SAR, we optimized the original route to synthesize Arbidol to allow multiple analogues to be made from the common dibrominated intermediate (**2**) (Scheme 1).[19]

Crystallographic data highlighted the importance of a water molecule in the binding pocket in the *meta* position with respect to the thiophenol group. To exploit this potential interaction with HA, we added either an amino or hydroxy group at the *meta* position, as well as extending the size of the ring to investigate the effect of increased conjugation. To investigate the importance of the dimethylamino group, these analogues were synthesized with and without the presence of the amine in position 4 on the indole, as well as replacing the dimethylamino with a piperazine to investigate if any further interactions towards the back of the binding pocket could be beneficial.

The synthesis began with the orthogonal protection and double bromination of the commercially available indole core (**1**) to give **2**, with a yield of 90% over 3 steps. Here the synthesis diverged to provide two separate sets of analogues; in route 1, thiophenol is added to give intermediate **3** in 86% yield. This was followed by reaction with *N,N,N′,N′*-tetramethyldiaminomethane to give Arbidol (**4**) in reasonable yield (51%) (Supplementary Methods). Reaction with two piperazine analogues gave compounds **14** and **15** (77% and 51% respectively). In route 2, various thiols were reacted to give intermediates **8**–**10**.[20,21] These were again reacted with *N,N,N′,N′*-tetramethyldiaminomethane to generate analogues **11**–**13** (Fig. 2).

During the synthesis of compound **11**,[22] it was found that the sulfur was prone to oxidation in the presence of strong acid or base, leading to a range of side products and hindered purification of the final molecule. By decreasing the strength of the base from potassium hydroxide to sodium carbonate in the penultimate step (yield increased to 67%) and changing the solvent from 1,4-dioxane to dichloromethane (increasing the yield to 99%), it was possible to prevent this oxidation from occurring *in situ* during the course of the last two reactions. Further analysis showed that **11** could not be stored in water for long periods of time (>4 h) which meant that biophysical assessments needed to be carried out as soon as the product was dissolved in buffer.

Evaluation of kinetics using bio-layer interferometry

To investigate the binding affinity of the compounds to the HA, the ratio of k_{on} to k_{off} to give K_d was determined by bio-layer interferometry (BLI) using an Octet Red instrument (ForteBio).[23–25] HA was loaded onto streptavidin-coated biosensors (SSA) and incubated with varying concentrations of small molecule in solution. The experiments comprised five steps: (1) baseline acquisition (60 s); (2) HA loading onto sensor (1800 s); (3) second baseline acquisition (120 s); (4) association of small molecule for the measurement of k_{on} (180 s); and (5) dissociation of small molecule for the measurement of k_{off} (180 s).

Arbidol is a broad-spectrum antiviral that can be used to treat influenza infection from both influenza A group 1 and group 2, and influenza B viruses. To test the affinity of our Arbidol analogues, each compound was assessed for binding against one HA from group 1 (H1 – PR8: A/Puerto Rico/8/1934) and one from group 2 (H3 – HK68: A/Hong Kong/1/1968) (Table 1).