Journal of Medicinal Chemistry

Perspective

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Antivirals Targeting Influenza A Virus

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Flu is caused by influenza viruses. The primary defense against influenza A and B is through vaccination. Seasonal flu viruses constantly undergo antigenic variations by changing their surface glycoprotein hemagglutinin (HA or H). The flu vaccines are reformulated each year to match antigenic variations. The formulations are recommended by public health organizations such as WHO, CDC, and FDA.[1] Each year, the predominant strains expected to dominate the coming flu season are predicted based on global surveillance and analysis of circulating strains. For example, the strains selected for the 2011−2012 flu season are A/California/7/09 (H1N1-pandemic), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008 (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm263319.htm). Influenza A is responsible for pandemic flu. Influenza A subtypes are characterized by two different surface glycoproteins: hemagglutinin (HA or H) and neuraminidase (NA or N). Wild birds are the reservoir of influenza A viruses. Avian strains are periodically migrated into the pool of virus circulating in mammals including humans, and reassortments of genomic RNA segments from human and avian influenza A viruses can lead to emergence of pandemic strains.[2] Also, an avian strain can adapt to the human host and attain human-to-human transmission capability through acquired mutations. An unexpected human adaptation of an influenza subtype or strain other than currently circulating influenza viruses causes pandemic flu. The pandemics of 1918 H1N1 (Spanish flu), 1957 H2N2 (Asian flu), 1968 H3N2 (Hong Kong flu), 2005 H5N1 (bird flu), and 2009 H1N1 (swine flu) symbolize the devastating public health and socioeconomic impacts of pandemic flu and keep us alert for any such outbreak. Adaptation of 2005 H5N1 virus for human-to-human transmission and emergence of Tamiflu-resistant influenza A viruses are among the imminent threats. Additionally, seasonal flu is responsible for about 50 000 deaths per year.

GENES AND PROTEINS OF INFLUENZA A

Influenza A virus is a member of the Orthomyxoviridae family[3] and has eight negative-stranded RNA genomic segments (Figure 1a) within a lipid bilayer envelope. The eight RNA segments are responsible for the synthesis of 12 known polypeptide chains. The envelope surface is spiked with multiple copies of HA and NA and fewer M2 ion-channel proteins. The three largest RNA segments encode the three viral RNA-dependent RNA polymerase (RdRP) proteins PA, PB1, and PB2. The PB1 gene also encodes a small ∼90-amino acid nonstructural protein PB1-F2 that has apoptotic functions[4] and is involved in inhibiting interferon production;[5] however, the effect of PB1-F2 appears to be influenza strain specific.[6] The PB1 gene also encodes a newly discovered third protein "N40", an N-terminal truncated product of PB1. N40 does not interfere with virus viability; viruses that do not express both N40 and PB1-F2 have normal replication. However, viruses that have only the PB1-F2 gene replicate slowly if the N40 protein is not produced.[7] There are two specific genomic segments for encoding the two surface proteins HA and NA. The sixth segment encodes the nucleoprotein (NP) molecules that wrap the viral RNAs (vRNAs) to form viral ribonucleoprotein particles (vRNPs). In fact, each vRNA segment exists as a vRNP (Figure 1b) that has a heterotrimeric polymerase with the NP-encapsidated vRNA.[8] The M1 matrix protein and the M2 ion-channel protein are both synthesized from a single genomic segment. The smallest genomic segment encodes the nonstructural proteins NS1 and NS2/NEP (nuclear export protein). Influenza A life cycle in a cell includes the sequential steps, namely, viral attachment to cell surface sialic acid receptors, entry of the virus as endosome followed by fusion, replication and transcription of viral genes, suppression of host immunity, assembly, budding and release of new viruses; see a schematic representation of influenza A life cycle in our earlier review.[9] Each viral protein plays critical and specific roles to accomplish these steps, and therefore, these proteins are either targeted by current anti-influenza drugs/vaccines or are potential new targets.

MOLECULAR TARGETS OF INFLUENZA A

Current vaccines raise an immune response against HA, which is also a prime target for antibody-based therapy initiatives. Recently discovered, broadly neutralizing monoclonal antibodies CR6261,[10] F10,[11] and FI6[12] that bind to a common conserved region of HA, although via different sets of molecular interactions, provide promising leads. The other two surface proteins NA and M2 are currently targeted by chemotherapeutic agents, and specific mutations on the targeted proteins confer drug resistance. The heterotrimeric polymerase complex exists as an independent entity or as a part of vRNP, and the polymerase is responsible for transcription and replication of viral genetic information. Because of its multidomain structure and multiple enzymatic activities, the polymerase can be targeted at different sites. NP molecules are involved in multiple functions.[13] NP molecules (i) oligomerize and wrap around a vRNA to form vRNP, (ii) bind to ssRNA, (iii) contain nuclear localization signal (NLS) sequences, and (iv) interact with polymerase complex and M1 molecules at various stages in the influenza A life cycle. Current research initiatives have discovered potential druggable sites and inhibitors of NP.[14] The NS1 protein of influenza A binds dsRNA,[15] CPSF30,[16] and phosphoinositide 3-kinase (PI3K)[17] and suppresses the host responses to influenza A infection primarily via these molecular interactions. The distinct sites on NS1 that bind the above cellular entities provide

Received: March 31, 2012
Published: May 21, 2012

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dx.doi.org/10.1021/jm300455c | J. Med. Chem. 2012, 55, 6263−6277

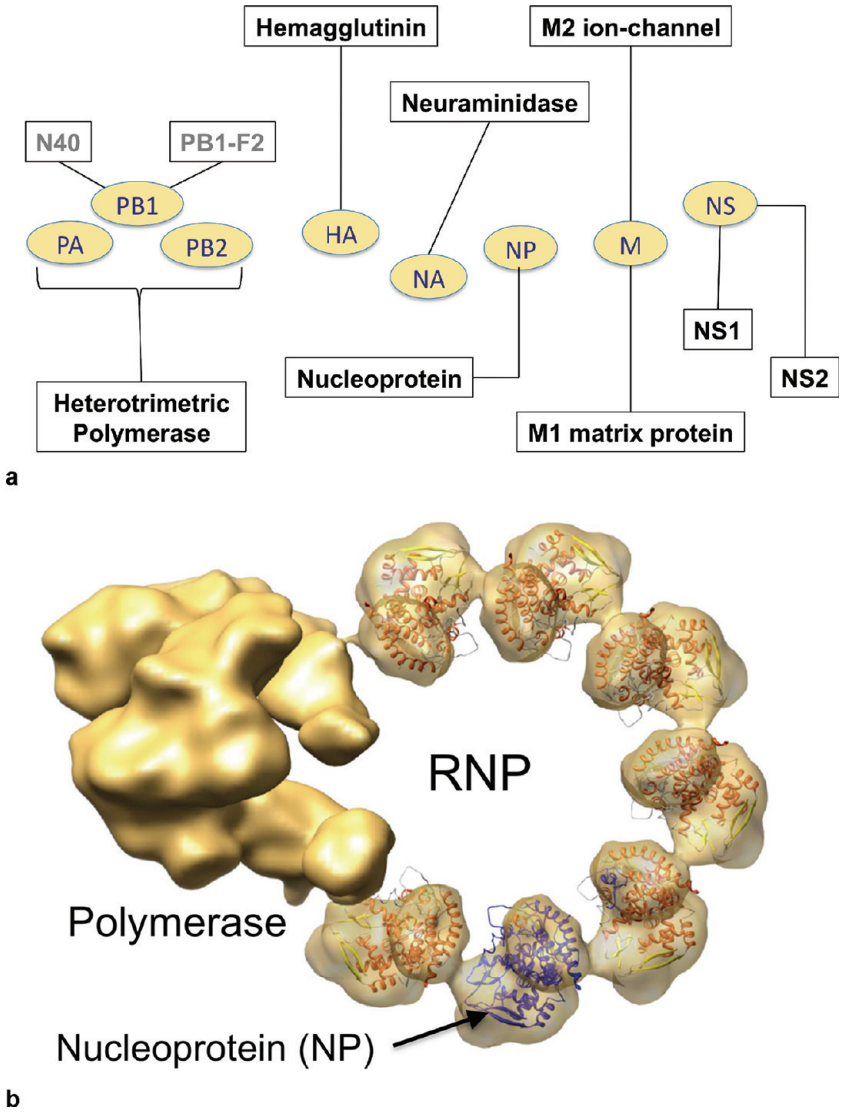


Figure 1. Segmented genes and proteins of influenza A. (a) Eight segmented viral genes (yellow) and 12 known polypeptides are shown. The PA, PB1, and PB2 chains assemble to form the viral polymerase. PB1 gene is also responsible for synthesis of two proteins PB1-F2 and N40 that are nonessential and influenza strain specific. The matrix protein M1 and ion-channel protein M2 are encoded from one genomic segment. Also, a single genomic RNA encodes both the nonstructural proteins NS1 and NS2/NEP (nuclear export protein). (b) An EM reconstruction image of RNP (ribonucleoprotein). Docked nucleoprotein (NP) molecules are shown as ribbons. Part b was reproduced from the open-access journal *PLoS Pathogens* with permission from authors Coloma et al.[75]

opportunities for drug discovery. Extensive ongoing research has discovered multiple potential targets to block critical steps of the viral life cycle by blocking functions of viral proteins and host–virus interactions.[18] This review analyzes the current understanding on the molecular mechanisms of resistance to existing drugs and provides a perspective into designs of new antivirals targeting resistant NA and M2 proteins and the multifunctional polymerase of influenza A.

GENETIC VARIATIONS AND DRUG RESISTANCE

Vaccines and drugs have been used to control many challenging pathogens. The pathogens that do not undergo high genetic variations or are less adaptive could be successfully irradiated or suppressed primarily through vaccination, whereas several viruses or bacteria such as HIV, tuberculosis bacterium, and malaria parasites, etc. have the ability to undergo genetic variations to overcome immune responses raised by a vaccine or reverse the interruption of pathogen functions by drugs. A viral infection, such as influenza, HIV, or hepatitis C (HCV), is transmitted as a pool of viral variants[19] in alliance with a predominant wild-type virus. A wild-type strain is predominant because it is the most fit for replication in a normal infection environment; however, the less-fit variants also replicate, although at a slower rate. A drug primarily targets the wild-type pathogen, and therefore, the use of drugs can considerably reduce the population of the wild-type variants. In contrast, a drug may have significantly less impact on some of the minor variants, and a most-fit variant gradually becomes the predominant strain under the selective pressure imposed by the drug. Often, a drug-resistant strain acquires compensatory mutations to enhance its fitness. The sequence of emergence of drug resistance and compensatory mutations is complex and appears to be resistance-path-specific; i.e., a drug resistant mutant may acquire compensatory mutations to enhance fitness or a resistance mutation may evolve from a preexisting variant containing compensatory mutations. Either of the two paths or a

dx.doi.org/10.1021/jm300455c | *J. Med. Chem.* 2012, 55, 6263–6277

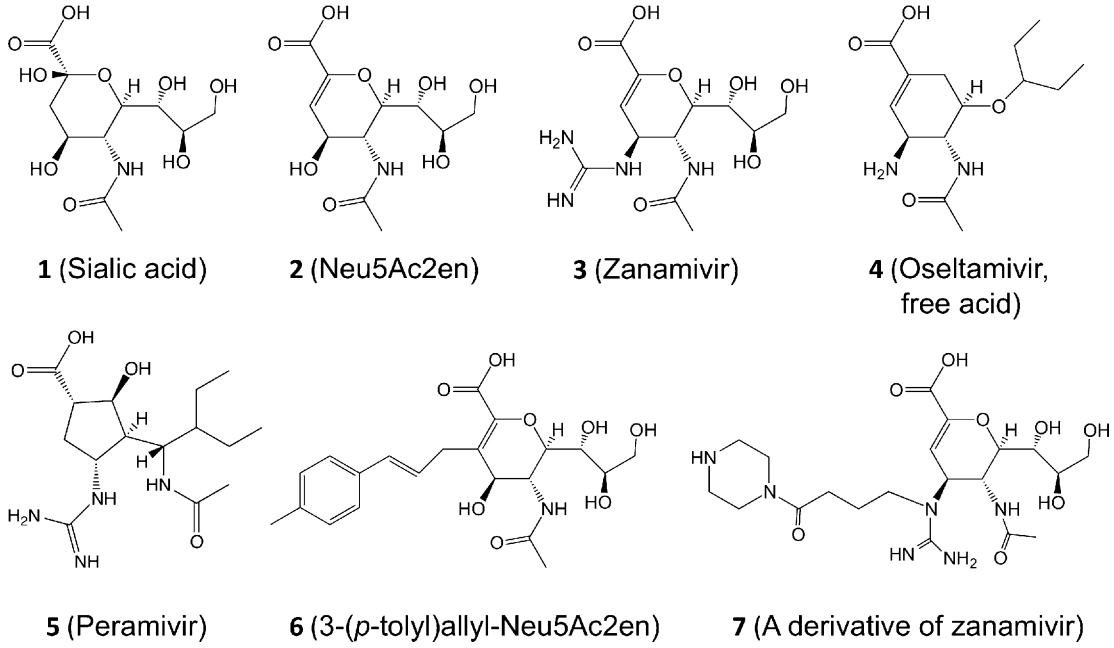


Figure 2. Sialic acid substrate and inhibitors of neuraminidase (NA): (**1**) sialic acid, (**2**) 2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en), (**3**) zanamivir, (**4**) oseltamivir (free acid), (**5**) peramivir, (**6**) 3-(*p*-tolyl)allyl-Neu5Ac2en,[41] and (**7**) 5-acetamido-2,6-anhydro-4-[*N*-(3-piperazinocarbonyl)propyl]guanidino-3,4,5-trideoxy-D-glycero-D-galactonon-2-enonic acid, a derivative of zanamivir.[42]

combination of them helps raise a population of a drug-resistant strain that then carries out new infections.

Antivirals are used for both prophylactic and therapeutic treatments during seasonal influenza epidemics,[20] and antivirals also provide a cost-effective stockpiling option for reducing the impact of a fast-spreading pandemic. Influenza antivirals are the inhibitors of M2 ion channel protein (adamantanes) and NA (zanamivir and oseltamivir). However, currently circulating influenza A virus strains[21] are mostly resistant to adamantanes. The NA-inhibiting drug zanamivir (Relenza) is inhaled, and the NA-inhibiting drug oseltamivir (Tamiflu) is a widely used oral drug. Emergence of oseltamivir-resistant seasonal and pandemic strains is on the rise.[22] The necessity of discovering new drugs to overcome resistance and looming threats of sporadic outbreaks of pandemic influenza A strains, particularly after the recent 2009 H1N1 outbreak, has renewed our interest in gaining a deeper understanding of the structures and functions of the viral protein components and their interactions. The array of information coming out of new research initiatives is providing a basis for designing new antivirals to block different steps in the virus life cycle.[9]

SIALIC ACID-MIMIC DRUGS TARGETING NEURAMINIDASE (NA)

Newly formed viruses are attached to the parent cell surface sialic acid (**1**, Figure 2). The cell surface enzyme NA releases the viruses by cleaving the glycosidic bond of the sialic acid residues.[23] NA has been the most successful influenza drug target. Compound **2** (2,3-didehydro-2-deoxy-*N*-acetylneuraminic acid or Neu5Ac2en)[24] was the first to be discovered as a potent NA inhibitor based on understanding the catalytic reaction-intermediate states of a sialic acid in NA. Studies of Neu5Ac derivatives by Glaxo Research Laboratories indicated that Neu5Ac2en had low therapeutic impact on infected mice, primarily because of rapid excretion.[25] Crystal structures of NA in the early 1980s revealed a conserved sialic acid binding pocket.[26,27] Subsequently, the structures and structure-based modeling[28] played a critical role in the development of NA substrate mimics, and these efforts[24] led to the discovery of **3** (zanamivir).[29] The compounds synthesized with a guanidine or amino group replacing the hydroxyl group of **2** at the C4-position showed higher retention in animal models. The 4-guanidine group improved the binding affinity by gaining several interactions with surrounding acidic side chains of Glu119, Asp151, and Glu227 and main-chain carbonyls of Asp151 and Trp178; these amino acid residues are highly conserved in NAs of influenza viruses. The resultant compound **3** demonstrated potency as an inhalant in influenza-infected mice and ferrets,[29] and **3** (zanamivir) was subsequently approved for human use as an inhalant drug.

The glycerol moiety of Neu5Ac compounds has O−H···O type interactions with Glu276 (Figure 3a). A systematic attempt to replace the glycerol moiety by a lipophilic group led the discovery of **4** (oseltamivir);[30] the chemical substitutions of (i) the glycerol moiety with a hydrophobic l-ethylpropoxy group and (ii) the 4-guanidine/4-hydroxyl group of zanamivir/Neu5Ac2en with an amine reduced the polarity and increased lipophilicity of the compound, leading to the formulation of oseltamivir as an oral prodrug. Both **3** (zanamivir) and **4** (oseltamivir) are highly effective in treating influenza A and B infections in humans and have favorable profiles for pre- and postexposure prophylaxis.[31] Peramivir (**5**),[32] the latest NA inhibitor developed by BioCryst, is approved as an intramuscular injection for patients with acute viral infections.[33] In contrast to the cyclohexene ring of oseltamivir, peramivir has a central cyclopentane ring. Despite differences in chemical compositions, all three NA drugs (**3**, **4**, **5**) share a common mode of binding (Figure 3a,b) within the conserved substrate-binding pocket of NA.[34] Peramivir binding preserves (i) the hydrophilic interactions of the guanidine group that it shares with **3** (zanamivir), (ii) hydrophobic interactions of the ethylpropoxy group that it shares with **4** (oseltamivir), (iii) the hydrogen bond of an OH group analogous to that of the 2-

dx.doi.org/10.1021/jm300455c | J. Med. Chem. 2012, 55, 6263−6277

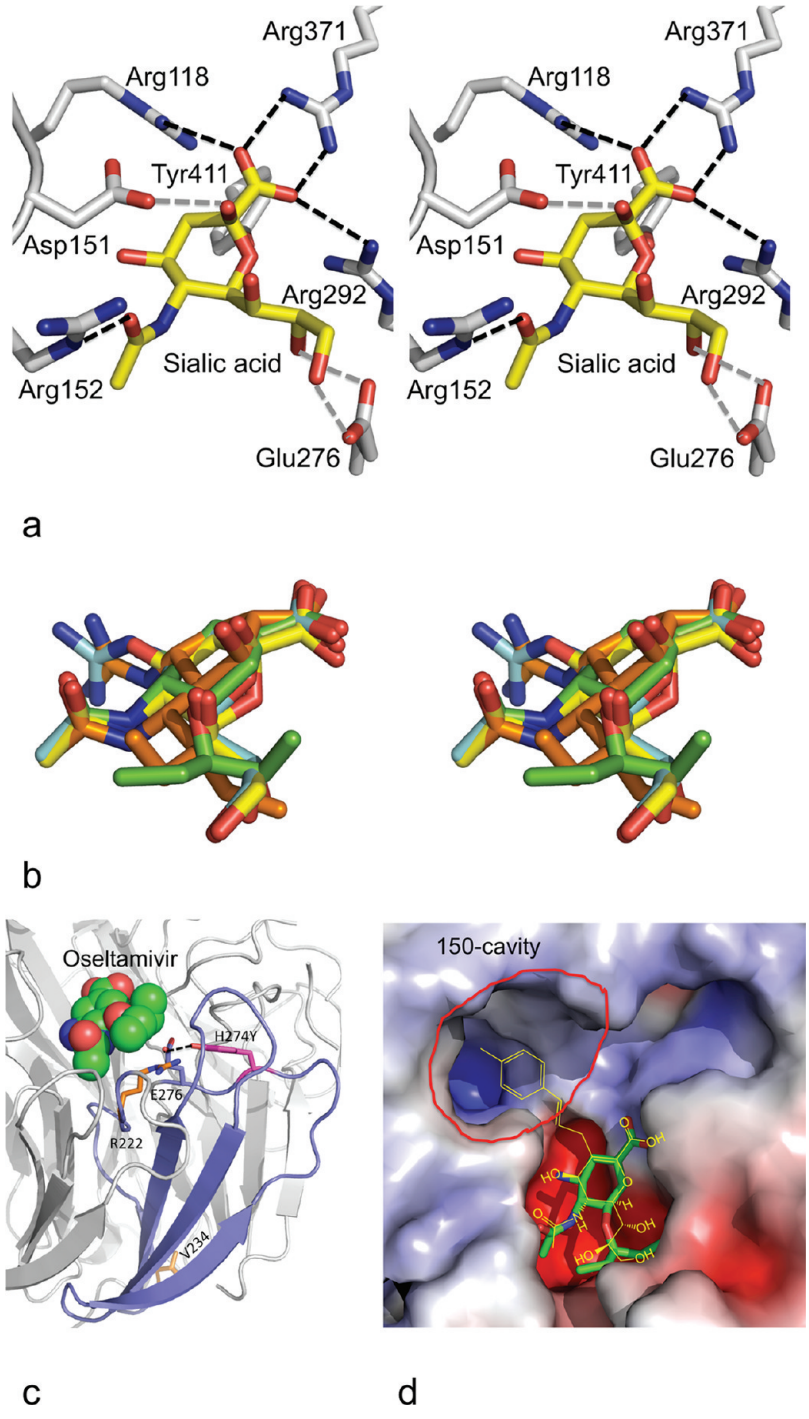


Figure 3. Structural basis for binding and resistance to drugs targeting neuraminidase (NA): (a) stereoview of binding of sialic acid to NA; (b) stereoview showing strikingly similar modes of binding of sialic acid (**1**, yellow), zanamivir (**3**, cyan), oseltamivir (**4**, green), and peramivir (**5**, orange). (c) Mutation H274Y causes resistance to oseltamivir (green space filling representation) by repositioning the pocket residue E276. The compensatory mutations R222Q and V234M restore the fitness of NA H274Y mutant virus[39] presumably by enhancing flexibility of E276 and the structural motif (in blue) that contains the residues H274 and E276. A crystal structure shows the 150-cavity[34] that was discovered upon a short soak of oseltamivir into an NA crystal. (d) Compound **6** is modeled as a 2D structure (yellow). The inhibitors (**6**[41] and **7**[42]) were designed to occupy the cavity.

OH group of **1** (sialic acid), and (iv) the conserved interactions of the carboxylate present in all the compounds.

OVERCOMING RESISTANCE TO NEURAMINIDASE INHIBITORS

The most widely used oral influenza drug **4** (oseltamivir) has a distinct chemical substitution of an ethylpropoxy group replacing the glycerol moiety of the substrate. This part has the largest structural and chemical differences when binding modes of NA substrate and inhibitors are compared (Figure 3b). The glycerol moiety has bidentate O−H···O interactions with the side chain of Glu276 (Figure 3a), whereas the ethylpropoxy group of oseltamivir has hydrophobic stacking with Glu276. Influenza viruses have acquired the ability to discriminate oseltamivir from the substrate by developing NA mutations. Several NA mutations[35] including E119V/I[36] and R292K cause resistance

dx.doi.org/10.1021/jm300455c | J. Med. Chem. 2012, 55, 6263−6277

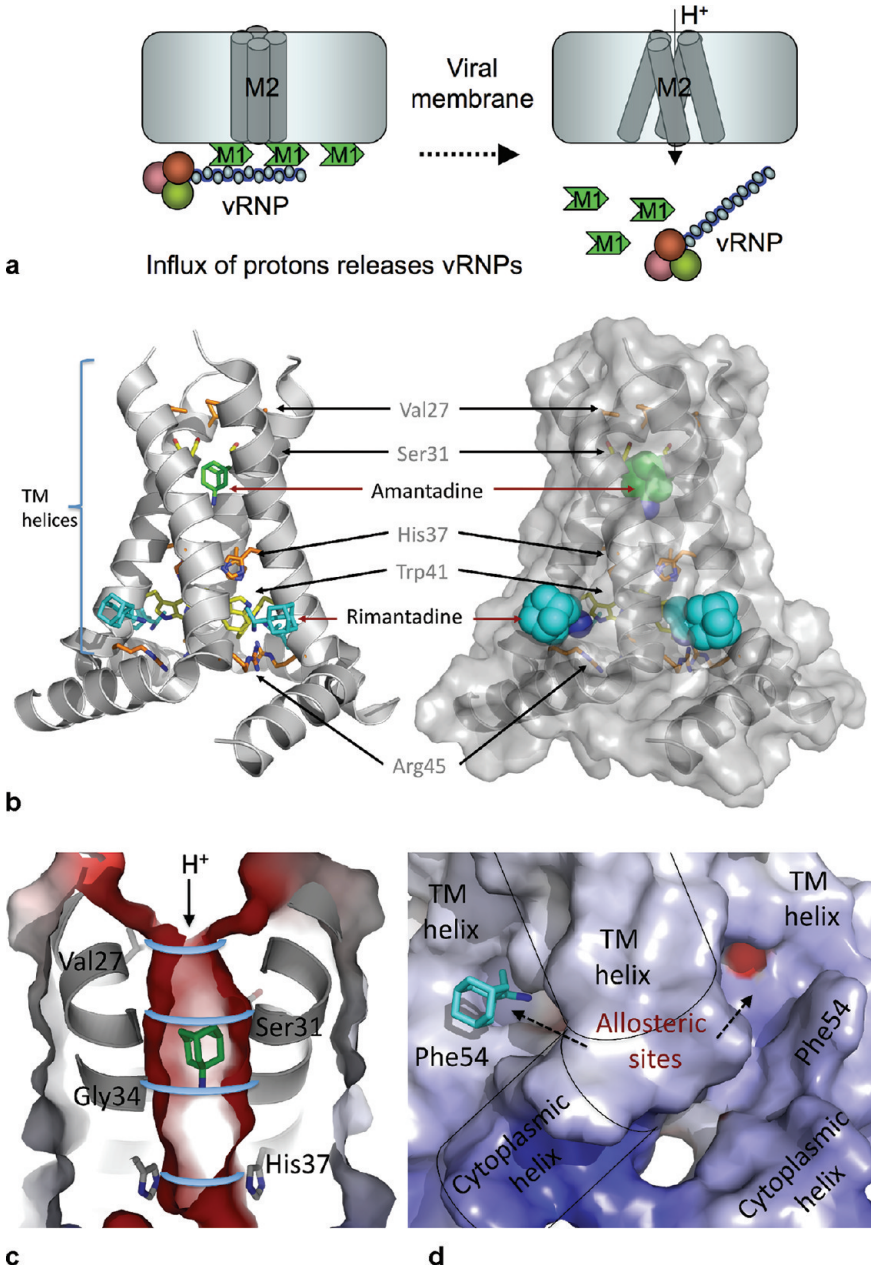
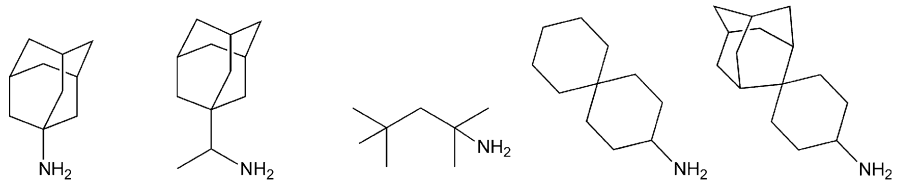


Figure 4. Structure, function, and inhibition of M2. (a) Schematic representation from Das et al. 2010[9] showing vRNPs attached to the inner surface of the lipid bilayer viral membrane and proton inflow into virus from endosome releases vRNPs. (b) Ribbon and surface representation of tetrameric M2 conductance domain (transmembrane (TM) and intracellular helices) structure (PDB code 2L0J).[62] The pore is blocked by an amantadine molecule (green), and the low-affinity allosteric binding sites are docked by rimantadine (blue) molecules based on the inhibitor positions in the NMR structure of the M2−rimantadine complex.[56] (c) Closer view of the amantadine in the pore. The tetrameric clusters V27, S31, G34, and H37 in the channel are involved in the positioning of adamantanes in the pore, and S31N is the primary clinical mutation associated with adamantane resistance. (d) A closer view of two of the four allosteric pocket regions in the solid-state NMR structure[62] shows the potential of the sites for binding of larger molecules than rimantadine that may have higher affinities.

to oseltamivir; however, H274Y mutation is predominantly observed in N1 oseltamivir-resistant strains.[22a,c] Crystal structures of H274Y mutant NA (N1 strain) complexes[37] provide a basis for understanding the molecular mechanism of the antiviral resistance by the mutation. The mutation site is located at a distance from the substrate-binding pocket (Figure 3c); however, Tyr274 in H274Y mutant NA repositions the side chain of Glu276, and the altered interaction between Glu276 and the ethylpropoxy group leads to discrimination against oseltamivir and the development of resistance. The structure of the mutant NA with **3** (zanamivir) revealed that the binding of **3** and interactions of its glycerol moiety with Glu276 are unaffected by H274Y mutation, which explains why H274Y develops significant resistance to **4** (oseltamivir) compared to **3** (zanamivir). Influenza strains that acquired NA H274Y mutation were found to replicate slowly, which reaffirmed the clinical benefit of oseltamivir even though the H274Y mutation causes oseltamivir resistance.[38] However, the emergence of compensatory mutations enhances the fitness of NA H274Y mutant viruses.[39] The H274Y mutant grows ∼100-fold more quickly when it contains two compensatory NA mutations R222Q and V234M compared to the variant containing only H274Y

dx.doi.org/10.1021/jm300455c | J. Med. Chem. 2012, 55, 6263−6277



8 9 10 11 12

Figure 5. Chemical structures of M2 inhibitors (**8**) amantadine, (**9**) rimantadine, (**10**) alkylamine, (**11**) spiropiperidine, and (**12**) spiroamantadine.

mutation; the triple mutant has a fitness comparable to that of the wild-type virus. Here is a possible structural explanation of the role of the compensatory mutations. In the NA enzyme, mutation sites R222 and V234 are parts of a structural motif that contains both His274 and Glu276 (Figure 3c). The repositioned Glu276 by H274Y mutation seemingly impairs the enzymatic activity of NA, even though the substrate binding is not significantly affected. E276Q/D mutant NA proteins were well folded, yet inactive[40] which suggests that right positioning and flexibility of the Glu276 side chain are essential for NA activity; however, a recombinant H3N2 virus containing E276D mutation replicates and confers resistance to both **3** (zanamivir) and **4** (oseltamivir).[35] The H274Y mutation restricts the flexibility and repositions the side chain of Glu276.[37] The R222Q mutation adjacent to Glu276 is likely to restore flexibility of the Glu276 side chain, and both R222Q and V234M mutations may allow a greater adaptability to the structural motif to help restore enzyme activity.

The drug **3** (zanamivir) that resembles the substrate more is less affected by resistance mutations compared to **4** (oseltamivir). By contrast, chemical modifications are essential to improve pharmacokinetic and bioavailability of a drug candidate. Upcoming NA inhibiting drugs should overcome the impact of existing resistance mutations. The recently identified transient "150-cavity"[34] (Figure 3d) provides opportunities for discovery of new NA inhibitors. Structures obtained from crystals of N1 and N8 NA[34] soaked with oseltamivir for a short period revealed the presence of the "150-cavity" adjacent to and accessible from the substrate-binding pocket.[34] These structural results indicate that initial binding of sialic acid or analogue inhibitors requires the opening of the 150-loop which creates the 150-cavity. The 150-cavity is prominent in group-1 NAs and not group-2 NAs.[34] Modified Neu5Ac2en derivative (**6**)[41] and zanamivir derivative (**7**)[42] containing C-3 and C-4 substitutions, respectively, could extend into the 150-cavity (Figure 3d). Both compounds demonstrated inhibition of wild-type and oseltamivir-resistant group-1 influenza A viruses and suggest the use of the 150-cavity for drug discovery. Because the pocket is transient, it is important to assess its conformational states in the process of substrate binding. Molecular dynamics studies suggest a wider open 150-cavity[43] in solution than that observed in crystal structures, existence of multiple conformational states of the cavity,[44] and possible existence of the cavity in group-2 NAs. Better assessment of the cavity and its evaluation for binding of small molecules may lead to new classes of NA-inhibiting influenza drugs.

M2 ION-CHANNEL ACTIVITY AND INHIBITION

The influenza A virus particle enters a host cell in an endosome. The surface of influenza A virus has few copies of M2 proteins[45] in addition to multiple copies of NA and HA. Low pH in the endosome causes conformational changes of HA that initiates the fusion of the viral and endosomal membranes.[46] Simultaneously, the ion channel protein M2[47] flows protons into the virus for releasing RNPs (ribonuclear proteins, Figure 1b) from the inner surface of the viral membrane into the cytoplasm (Figure 4a). The first line of flu drugs were adamantanes (amantadine (**8**,[48] Figure 5) and rimantadine (**9**)[49]) that target the M2 protein. A sharp rise in adamantane resistance in H3N2 and H1N1 influenza A viruses[50] undermined the utility of adamantanes; therefore, the treatment options with M2-inhibiting drugs were replaced with oseltamivir in the U.S. Recent surveillances have detected circulating seasonal H1N1 viruses containing resistance mutations to both adamantanes and oseltamivir.[51]

M2 protein has an N-terminal extracellular domain (residues 1−23), a transmembrane (TM) domain (residues 24−46), and a C-terminal intracellular domain (residues 47−97). The M2 molecules are functional as homotetramers; a bundle of four TM helices form the proton channel across the viral membrane. Extensive structural, biophysical, biochemical, and computational studies on the tetrameric arrangements of TM helices have been carried out in recent years that have increased significantly our understanding of the molecular mechanisms of proton flow, drug binding, and drug resistance. The conserved residues Ser31, His37, Trp41, Asp44, and Arg45 are critical for channel formation and proton transfer[47b] (Figure 4b,c). Tetrameric His37 cluster is important for pH activation of the channel,[52] and Trp41 cluster forms the "channel gate";[53] the Val27 cluster at the entrance to the channel also apparently forms a secondary gate.[54] Structures of influenza A M2 were determined in complexes with **8** (amantadine) and **9** (rimantadine) simultaneously by X-ray crystallography[55] and solution NMR,[56] respectively. Both structures agreed on the tetrameric arrangement of TM helices to form the ion channel; however, the structural studies uncovered two distinct binding sites and mechanisms of inhibition for adamantine drugs. The crystal structure determined by DeGrado and colleagues[55] revealed the binding of amantadine between the residues Ser31 and His37 in the pore, and the structure favored a "pore-blocking" model of inhibition by adamantanes (Figure 4b,c). Amantadine was positioned near the Ser31 cluster, and the structure supported a direct impact of the S31N mutation on the binding of adamantanes. The NMR structure of M2 protein (TM helix + cytoplasmic domain, residues 18−60) in dihexanoylphosphatidylcholine (DHPC, pH 7.5) micelles by Schnell and Chou[56] revealed a closed arrangement of TM helices compared to an open-pore conformation of TM helices in the crystal structure determined at pH 5.3.[55] The NMR structure revealed binding of four rimantadine molecules, each to a lipid-exposed allosteric site between adjacent TM helices near the TM−cytoplasm interface. The allosteric site is flanked by the conserved pore residues Tyr41, Asp44, and Arg45. Therefore, binding of an adamantine was proposed to stabilize the pore-closed conformation of the

dx.doi.org/10.1021/jm300455c | J. Med. Chem. 2012, 55, 6263−6277

TM helix bundle. This unexpected controversy on the binding of adamantanes triggered a series of follow-up studies.

A solid-state NMR study by Hong and colleagues[57] suggested existence of hydrogen bond interaction between Ser31 and amantadine. This study favored amantadine binding to the pore; however, the binding mode of amantadine was predicted to be flipped upside down compared to the crystal structure.[55] In a following solid-state NMR study of M2 TM domain in phospholipid bilayers and at varying concentration of deuterated amantadine by Hong, DeGrado, and colleagues,[58] the drug was found to bind in the pore and interact with Ser31 at 1:4 molar concentration of amantadine to protein. In the presence of excess amantadine, they observed additional interactions of the drug with Asp44, a residue at the allosteric side.[56] A surface plasmon resonance (SPR) study to assess binding of amantadine and rimantadine to M2 TM domain (residues 22−46) in 1,2-dimyristoyl-*sn*-glycero-phosphocholine (DMPC) liposomes[59] elicited two distinct binding sites for the adamantanes with binding affinity on the order of 0.1 mM and 0.1 μM, respectively. By use of M2 mutants (V27A, S31N, and D44A), the study confirmed "pore-blocking" (Figure 4c) as the high-affinity binding site and the "allosteric site" as the low-affinity binding site. Chou and colleagues have recently shown binding of rimantadine to an influenza A(M2)−B(M2) chimeric channel.[60] In the pore, the earlier ambiguity[55,57] on orientation of the amines of amantadine and rimantadine was ascertained by conducting solid-state NMR study of M2 TM in lipid bilayer and dodecylphosphocholine (DPC) micelles[61] rather than DHPC micelles that the authors used in their previous study;[57] the amines of both amantadine and rimantadine pointed toward His37, and the adjacent methyl group of rimantadine was positioned near Gly34. The study inferred significant impact of using different detergents in the experiment. In fact, a solid-state NMR study of M2 (residues 22−62)[62] in lipid bilayer found different tetrameric arrangements of TM helices and intracellular helices (Figure 4b) from that observed in DHPC micelles by solution NMR.[56] The arrangement of TM helices revealed by the solid-state NMR study was in agreement with that observed in a recent high-resolution crystal structure;[63] Cα superposition of TM helix bundle between the two structures was only ∼1.3 Å. The crystal structure[63] showed ordered water molecules between the tetrameric clusters Gly34-His37, His37-Trp41, and Trp41-Arg45 in the channel, and the study led to a hypothesis that water molecules along with tetrameric His37 cluster are involved in proton conductance through the channel. Aided by earlier biophysical studies on proton conductance[53,64] and UV resonance Raman scattering from Trp41,[65] the recent structure of M2 in lipid bilayer[62] suggested a mechanism of acid activation and proton conductance through His37-Trp41 coordinated rearrangements. The mechanism suggests that (i) a proton transfer to His37 from a hydronium atom in the channel initiates acid activation, (ii) in the activated state, the protonated Nε atom of His37 points down and forms a cation−π interaction with the indole ring of Trp41, whereas protonated Nδ atom interacts with a water molecule on its top, and (iii) the indole ring moves away to expose a water molecule at the bottom of the channel to receive the proton from the Nε atom of His37.

DESIGNING M2 INHIBITORS

Recent controversies on binding of adamantanes to M2 and extensive follow-up studies have (i) enhanced our understanding of proton conductance through the TM channel, (ii) defined binding of adamantanes to the channel ("pore-blocking" site), and (iii) discovered a new "allosteric" inhibitor target site; however, the druggability potential of the allosteric site needs to be validated. This information is likely to aid in the generation of new thoughts and ideas on how to effectively target M2 at its multiple sites for drug discovery. In the pore-blocking site, an adamantane binds to the channel near Ser31 with its amine group pointing down toward the His37 cluster. The space between the adamantine and His37 cluster is occupied by water molecules.[63] Earlier attempts at chemical addition to the hydrophobic adamantane core and designing larger molecules, however, did not substantially improve efficacy of the M2 inhibitors; see a recent review by Duque et al.[66] In fact a recent study on structural requirements of compounds to bind the pore identified a branched alkylamine (**10**)[67] of only nine non-hydrogen atoms exhibiting inhibitory activity similar to that of amantadine. The study also inferred that a hydrophobic head and an amine tail apparently are two important pharmacophoric requirements for potent M2 pore-blocking inhibitors. A systematic evaluation of pore-lining residues for their ion selectivity, amantadine sensitivity, specific activity, and pH-dependent conductance demonstrated that several mutations of the pore-lining residues enhance one or more functions; however, most of the residues are required to remain invariant for the pH-regulated proton flow through the channel.[68] This study correlates with the fact that only three mutations L26F, V27A, and S31N are predominantly observed in transmissible amantadine-resistant influenza A viruses.[50b,69] An interesting compound series synthesized as M2 channel inhibitors was spiropiperidine (**11**).[70] Importantly, a spiro-adamantane compound (**12**)[71] demonstrated inhibitory activity against L26F and V27A mutants.[72] The compound **12** inhibits V27A mutant channel at IC_{50} of ∼0.3 μM and the mutant virus in a plaque reduction assay at 1 μM, whereas 50 μM amantadine had no visible impact on the plaque reduction.[72] Admirably, recently gained knowledge on M2 structure and mechanism is starting to help design inhibitors to overcome drug resistance. Inhibitors that can additionally defeat the primary clinical resistance mutation S31N would bring new classes of M2 inhibiting influenza A drugs. S31N mutation appears to conflict directly with the binding of adamantanes. The V27A mutation is expected to open up the secondary gate; however, the mutation may cause resistance by increasing the off rate (k_{off}) for an inhibitor in the pore. Further understanding of the molecular mechanisms by which the mutations impact the binding of drugs is also essential to overcome resistance.

Although the M2 pore remains a valid target for small molecule drugs, future research focus on the allosteric site[56] may open up opportunities for the discovery of new classes of M2 inhibitors. Rimantadine binds to the allosteric site with an affinity that is about 3 orders of magnitude weaker than its binding affinity to the pore. Analysis of a recent M2 structure[62] (Figure 4b,d) reveals the existence of the allosteric pocket even in the absence of rimantadine. Docking of rimantadine into the site, as shown in Figure 3d, based on the binding of rimantadine to M2 in the solution NMR structure[56] suggests that the inhibitor binding to the site is not optimized, and significantly larger compounds may be accommodated at the site with enhanced binding affinity. The intracellular helices in the solid-state NMR structure[62] are positioned to form a wall for the allosteric site. The hydrophobic and aromatic side chains of the helix residues, in particular Phe54, may aid binding of M2 allosteric inhibitors. Rearrangement of TM helices and repositioning of the cytoplasmic helices with respect to TM helices are essential for proton transport. These rearrangements are also likely to provide multiple conformations

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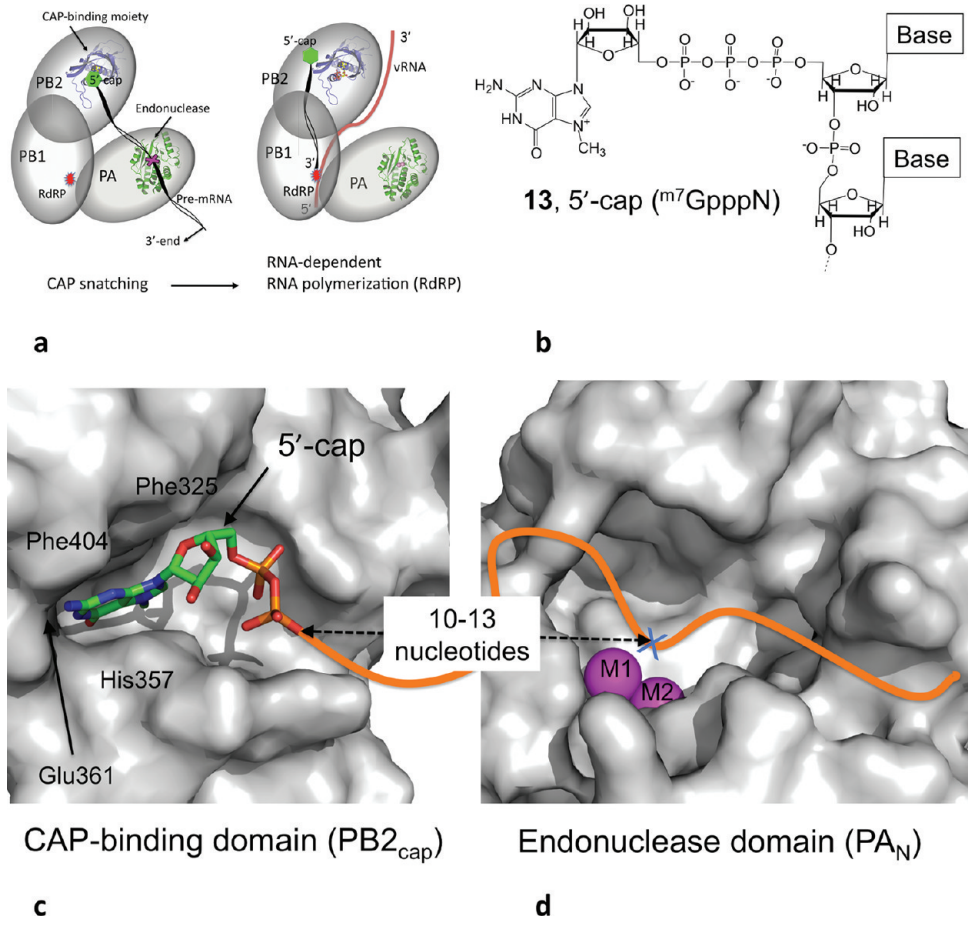

Figure 6. Cap snatching by influenza A polymerase. (a) Schematic representation of transcription of viral mRNAs. The PB2 subunit of influenza A heterotrimeric polymerase binds the 5′-cap of a host pre-mRNA, and the endonuclease active site in the PA subunit cleaves the pre-mRNA 10−13 nucleotides downstream from the cap. The viral polymerase initiates synthesis of its mRNAs from the cleaved end of the capped-RNA fragment by its RNA-dependent RNA polymerase activity (RdRP) and using a vRNA template. (b) Chemical structure of the ^{m7}GpppN cap (**13**) present at the 5′-end of mRNAs/pre-mRNAs. (c) Molecular surface representation of crystal structure of the cap-binding domain ($PB2_{cap}$) bound to an m7-GTP cap (green).[95] Only α and β phosphates are shown. (d) Molecular surface representation of the endonuclease active site present in the N-terminal domain of PA (PA_N)[97,98] that cleaves the RNA by a two cation dependent catalysis to complete the cap-snatching event.

of the allosteric pocket to trap chemically diverse compounds, analogous to the non-nucleoside binding site of HIV-1 reverse transcriptase that is currently targeted by five anti-AIDS drugs derived from four distinct chemical classes.[73] A potent allosteric M2 inhibitor, if discovered, would block proton flow by locking the TM helices in one conformation and by restricting the conformational mobility of Trp41 side chains.

■ VIRAL POLYMERASE

Three polypeptide chains PA, PB1, and PB2 assemble[74] to form a hetrotrimeric RNA-dependent RNA polymerase (RdRP) of mass ~250 kDa, the largest molecular machine of influenza virus. A trimetric viral polymerase is also associated with each vRNA segment, which is encapsidated with multiple copies of the nucleoprotein (NP) molecule, attached at an interval of approximately one NP per 24 nucleotides. Electron microscopic studies have revealed the overall conformations of influenza polymerase and vRNP (Figure 1b)[8,75] that, aided by biochemical studies, may help in understanding the molecular architecture and conformational states of the polymerase.

Fusion of virus into the host cell releases the eight vRNPs that were previously wrapped with the matrix protein M1 and attached to the inner wall of the virus (Figure 4a). The free vRNPs are then transported into the nucleus where the viral polymerase carries out catalytic reactions of RdRP to perform two distinct tasks on each vRNA: (i) transcription into mRNA (messenger RNA)[76] and (ii) replication to produce progeny genome copies (vRNA → cRNA (complementary RNA) → vRNA). Viral mRNAs are transported into the cytoplasm and translated to synthesize corresponding viral proteins. The polymerase uses different replication initiation approaches for synthesis of cRNA and vRNA;[77] however, replication of both requires NP molecules.[78] A vRNA produced at the end of a complete replication cycle is packaged as a vRNP. A new vRNP is associated with M1 molecules and exits to the cytoplasm via a M1:NS2/NEP mediated transport mechanism.[79] Trafficking of vRNPs, i.e., nuclear entry of vRNPs of an infecting virus or exit of newly formed vRNPs from the nucleus, is regulated by dissociation or association of M1 molecules, respectively.[80] Newly formed vRNPs and viral proteins are packaged into new virus particles that are budded out of the infected cell. For further details, see a schematic representation of the life cycle of influenza A in our previous review[9] and other recent publications on influenza polymerase.[81]

Atomic resolution structure(s) of the polymerase can play important roles in (i) understanding molecular mechanisms of

dx.doi.org/10.1021/jm300455c | J. Med. Chem. 2012, 55, 6263−6277

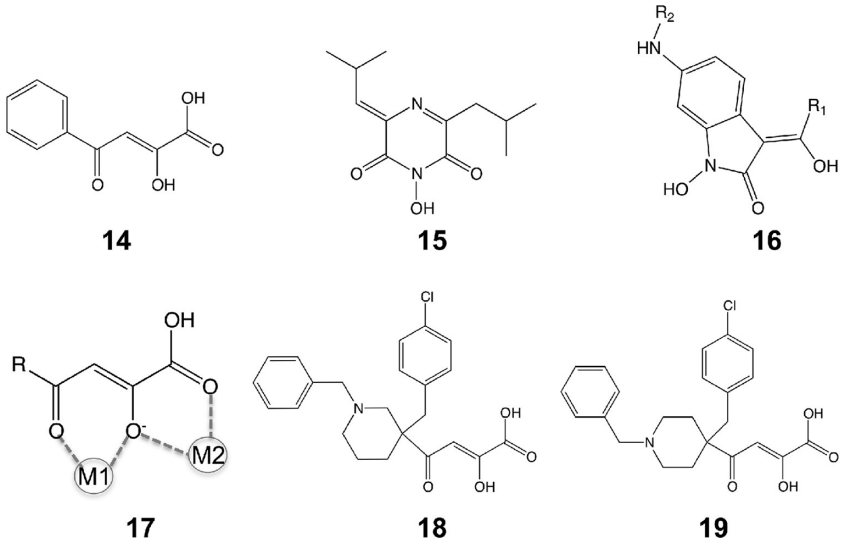

Figure 7. Chemical structures of cap-snatching inhibitors: (**14**) diketo acid (2,4-dioxobutanoic acid),[101] (**15**) flutimide,[102] (**16**) *N*-hydroxyimide,[103] (**17**) the endonuclease active cation sites that chelate the inhibitors in a precise geometry where the three chelating atoms are coplanar with the two metal ions. The chelation environment is also reminiscent of the inhibitor chelation at the retroviral integrase[127] and HIV RNase H active sites.[108] The diketo acid derivatives **18** and **19** were optimized by Merck as a highly specific influenza A endonuclease inhibitor[101] that inhibited influenza viruses.[104]

this multifunctional enzyme and (ii) visualizing structural rearrangements of functional domains that are responsible for the enzymatic activities and for switching between transcription and replication modes, and (iii) designing polymerase inhibitors by targeting specific function(s) or site(s). Although an atomic resolution structure of the full polymerase is not yet available, significant progress has been made toward understanding the domain structures of the polymerase and their functions and interactions; see our earlier review[9] for a description of structurally characterized domains of influenza A polymerase. The PA chain contains the endonuclease active site[82] for cap-snatching[76] and interacts with the PB1 chain. PB1 has the RdRP domain that has not been structurally characterized. Interactions of PB1 with both PA and PB2 have been structurally characterized, and these intersubunit interactions are potential targets for drug discovery.[83] Newly synthesized polymerase proteins are transported into the nucleus as PB2 and PB1-PA complex. A recent study reports inhibitors of PB1-PA dimerization that inhibit nuclear import of PB1-PA and growth of different influenza A viruses, including oseltamivir-resistant isolates, at low micromolar concentrations.[84] The PB2 chain contains (i) the cap-binding domain, (ii) nuclear localization sequence (NLS)[85] (therefore, PB2 is transported into the nucleus independently and assembles with PB1-PA in nucleus),[86] and (iii) mutations, in particular at amino acid position 627 in the C-terminal domain of PB2,[87] which impact the host adaptation[88] and virulence[89] of influenza A virus. Associated mutations D701N, R702K, and S714R in the NLS region appear to improve PB2 binding with importin α molecules[90] in the cytoplasm, which enhances nuclear import of PB2 and consequently improves the viral replication. Understanding of the biology and molecular interactions of influenza polymerase that are fast evolving is likely to uncover additional targets for drug discovery.[91] However, the two major catalytic activities, i.e., cap snatching and RdRP, are very attractive targets for small molecule inhibitors as discussed in the following two sections.

INHIBITING mRNA CAP SNATCHING

After invading a host cell, a virus exploits host functions and entities for copying viral genes and producing viral proteins. Protein production in eukaryotic cells requires transcription of viral mRNAs that usually contain a ^{m7}GpppN-cap structure (**13**)[92] at the 5′-end and a poly(A) tail[93] at the 3′-end. Processing of both termini is essential for mRNA stability and translation efficiency. Different types of viruses use different mechanisms to cap their mRNAs.[94] The polymerase of influenza A and other orthomyxoviruses hijacks a 5′-capped RNA fragment (^{m7}GpppNpN$_{(10-13)}$) from a host pre-mRNA by a mechanism called "cap snatching" to initiate transcription (Figure 6a), whereas influenza vRNAs contain genetic information for adding a poly(A) sequence at the 3′-end of viral mRNAs. The PB2 subunit (PB2$_{cap}$, residues 318−482) binds the 5′-cap (Figure 6b).[95] The crystal structure of PB2$_{cap}$ in complex with a ^{m7}GTP revealed that the binding of 5′-cap is assisted by a hydrophobic sandwich between the residues Phe325 + Phe404 and His357 and polar interactions with Glu361 (Figure 6c). The pocket in the cap-binding domain of PB2 appears to be a favorable drug target; however, a ^{m7}GTP-mimic may also be recognized by cellular cap-binding proteins[96] and cause selectivity and cytotoxicity issues.

The capped pre-mRNA is cleaved by the endonuclease active site present at the N-terminal domain of PA[82] (PA$_N$, residues 1−197). Crystal structures of H5N1 PA$_N$[97] and H3N2 PA$_N$[98] revealed a conserved deep cleft. H3N2 PA$_N$ structure also revealed chelation of two cations (Mn^{2+}) at the active site, whereas H5N1 PA$_N$ structure had only one metal ion (Mg^{2+}), leading to a controversy over the catalytic mechanism of phosphodiester bond cleavage.[99] Well before any understanding of the structure or even the location of the endonuclease active site/domain in influenza polymerase, the endonuclease activity was targeted based on the hypothesis that the endonuclease cleavage associated with cap snatching would share a common cation-dependent catalytic reaction mechanism with other exo/endonucleases.[100] Several chemical classes of inhibitors (**14**,[101] **15**,[102] **16**;[103] Figure 7) of influenza endonuclease activity were developed in the past by Merck, Roche, and other pharmaceutical

dx.doi.org/10.1021/jm300455c | *J. Med. Chem.* 2012, 55, 6263−6277

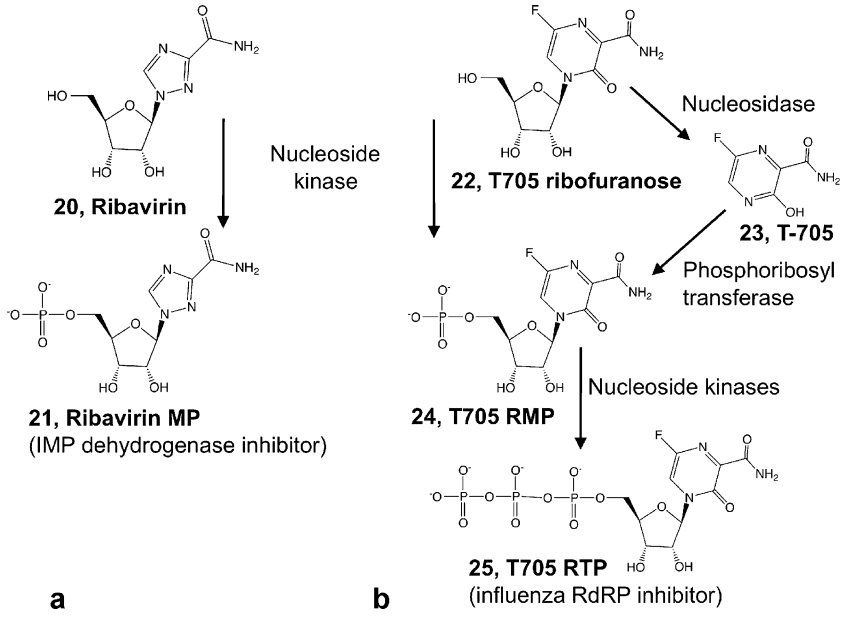

Figure 8. Nucleotide analogue and RNA-dependent RNA polymerase (RdRP) inhibitors of influenza A polymerase. (a) Ribavirin (**20**) is converted by cellular nucleoside kinase to ribavirin monophosphate (MP) (**21**), which acts as an IMP dehydrogenase inhibitor in the pathway of GTP production in the cell. (b) T-705 (**22**, **23**) is the only inhibitor of influenza RdRP that is in clinical trial. T705 RMP (**24**), the monophosphate form, is not an effective inhibitor of IMP dehydrogenase; however, T705 RMP is converted by nucleoside kinases into T705 RTP (**25**), which inhibits the RdRP.

companies. All the inhibitors share a common metal chelation mode (**17**), and alterations of the metal binding moieties led to complete loss of inhibition, suggesting that a two-metal ion chelation (**17**) at the active site is an absolute requirement for the binding of these inhibitors. Some of the diketo acid derivatives could be optimized (**18**,[101] **19**[104]) to selectively inhibit cap-dependent influenza virus transcription and virus yield at IC_{50} of ~1 μM. Compound **19** inhibited influenza infection in mice and validated that the cap-snatching endonuclease is an antiviral target.[104] These studies also demonstrated that alterations of the metal binding moieties led to complete loss of inhibition, suggesting a two-metal ion chelation (**17**) at the active site. The crystal packing in the H3N2 PA_N structure[98] did not permit binding of an endonuclease inhibitor; however, Cusack's group subsequently showed the binding of **14** to the endonuclease domain of a *Bunyaviridae* RNA polymerase.[105] In fact, the diketo acid compounds were found to inhibit HIV integrase by chelating the two metal ions at the active site that pioneered the discovery of integrase inhibiting HIV drug raltegravir.[106] Binding of inhibitors to endonuclease,[105] integrase,[107] and RNase H[108] enzymes shares a common metal-chelation geometry, **17**. The structurally characterized deep cleft in influenza endonuclease may help in designing inhibitors with higher binding affinity. Renewed interest in targeting influenza endonuclease activity has helped in the discovery of new inhibitors[109] and high-throughput enzyme assays.[110] Structural characterizations of the binding of inhibitors to influenza endonuclease may make it possible to optimize such inhibitors to achieve high potency and specificity.

INFLUENZA RNA SYNTHESIS AND RdRP INHIBITORS

Nucleoside analogues are commonly used for treating viral infections such as HIV, herpes simplex virus (HSV), and hepatitis B (HBV).[111] Nucleoside analogues are converted into (i) mono-, di-, and triphosphate forms by cellular nucleoside kinases, (ii) then compete with dNTP for incorporation into the 3′-end of a growing nucleic acid strand by a polymerase, and (iii) block further elongation of the nucleic acid. Nucleoside/nucleotide drugs are highly successful in treating HIV infection. In fact almost all anti-AIDS drug combinations use one or two nucleoside/nucleotide analogue(s). In contrast, there is no approved RdRP-inhibiting drug for treating flu infection. Ribavirin (**20**, 1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide; Figure 8)[112] is a nucleotide-mimicking antiviral drug that is used against several RNA viruses including HCV and human respiratory syncytial virus (RSV).[113] The predominant mode of action by ribavirin is to block GTP synthesis in cells by inhibiting the cellular enzyme inosine 5′-monophosphate (IMP) dehydrogenase.[114] Ribavirin (Figure 8) is converted intracellularly into its monophosphate form ribavirin MP (**21**) that inhibits conversion of IMP to xanthosine monophosphate in the GTP synthesis pathway, leading to inhibition of RNA synthesis in cells. However, ribavirin may also function as a nucleoside analogue if converted intracellularly into its triphosphate (TP) form, which may be a reason why ribavirin is not an effective RdRP inhibitor. Enzymatically, poliovirus RNA polymerase could bind ribavirin TP and incorporate ribavirin into RNA primer strand;[115] however, ribavirin does not effectively inhibit poliovirus in cell culture, suggesting that ribavirin MP is not efficiently converted into ribavirin TP by cellular nucleoside kinases.

A nucleoside analogue T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide, favipiravir, **22**, **23**,[116] Figure 8) is currently in clinical trials by Toyama Chemical Co., Ltd. (Japan) as an anti-influenza drug candidate. In contrast to **21** (ribavirin MP), T-705 RMP (**24**) is not an effective inhibitor of IMP dehydrogenase. T-705 is converted intercellularly into its triphosphate form T-705 RTP (**25**) which acts as a nucleoside inhibitor of influenza RdRP (Figure 8), and importantly, T-705 does not inhibit synthesis of cellular RNA or DNA.[117] Compound **25** (T705 RTP) appears to act as a purine analogue and was shown to be active against oseltamivir-sensitive or -resistant H1N1, H3N2, and H5N1 influenza A strains both in cell culture and in animal models.[116,118] Compound **22** (T-705) appears to be more

dx.doi.org/10.1021/jm300455c | *J. Med. Chem.* 2012, 55, 6263−6277

effective than oseltamivir in inhibiting (i) multicycle infection in plaque reduction assays,[119] and (ii) late stage(s) of viral infection in mice models.[120] A combination of **4** (oseltamivir, 1 mg kg^{-1} day^{-1}) and **22** (20 mg kg^{-1} day^{-1}) is significantly effective for the survival of influenza A infected mice compared to mice treated with the individual compounds.[121] Discovery and development of **22** as an anti-influenza candidate drug establish the potential for developing viral RdRP inhibitors as flu dugs. Recently, some uridine-based nucleoside triphosphate analogues have demonstrated anti-influenza activity; however, their monophosphates do not effectively inhibit influenza A in cells because of inefficient conversion of the nucleoside monophosphates into their active triphosphate metabolites.[122] Future research and development efforts may help develop potent nucleoside analogues against influenza.

Apart from inhibition of specific RdRP, cap-binding, and endonuclease activities, influenza polymerase may also be inhibited by allosteric inhibitors. A high-throughput viral reduction screening of a library of over a million compounds identified inhibitors of polymerase and NP.[14c] The NP inhibitors inhibited H1N1 virus growth in cell culture and protected infected mice. A recent study describes a quinoline derivative that inhibits viral replication at submicromolar concentration.[123] The compound inhibits the polymerase in a cap-dependent way; however, the precise molecular mechanism of inhibition is not clear. Small molecules that block binding of NP molecules and/or restricts switching between the transcription and replication modes of the polymerase[124] would inhibit virus growth. Interaction of NP molecules with the polymerase complex apparently plays a critical role in switching the polymerase into replication mode.[78] Also, small viral (sv) RNA fragments of 22–27 nucleotides produced by influenza A trigger the polymerase to switch into replication mode and have a significant impact on vRNA and not cRNA production.[125] These findings suggest that svRNA or NP induced switching of polymerase to replication mode is a potential target for drug discovery.

CONCLUSIONS

New antivirals are needed to overcome resistance to current influenza drugs. Recent studies have enhanced our understanding of M2 inhibition by adamantane drugs and resolved the issue of how they bind M2. Current information on the role of drug resistance and compensatory mutations on NA to restore viral fitness while maintaining resistance to oseltamivir may aid discovery of new NA-inhibiting drugs. Influenza A proteins NP, NS1A, and the RdRP are important and emerging targets for drug discovery. Like other fast mutating viruses such as HIV and HCV, influenza A tends to develop antiviral resistance under drug pressure. Antiviral combinations amantadine + oseltamivir[126] and oseltamivir + T-705[121] have shown therapeutic benefits in infected mice. Therefore, discovery of new drugs targeting different entities of influenza A may provide wider options of synergistic drug combinations for improved prophylactic and therapeutic benefits.

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Notes

The authors declare no competing financial interest.

Biography

Kalyan Das received his Ph.D. degree from the Indian Institute of Technology (IIT), Bombay, India, in 1991. His research for the past two decades has been focused on understanding the structural basis of, and molecular mechanisms associated with, resistance to antivirals and antibiotics and the application of this information to drug development. He currently holds the positions of Research Professor, Rutgers University, the State University of New Jersey, and scientific cofounder of Prodaptics Pharmaceuticals, Inc., a nascent life sciences venture that applies techniques in structural biology and protein chemistry to enhance rational drug design, discovery, and development.

ACKNOWLEDGMENTS

I thank Aaron Shatkin for his critical input on the manuscript and Juan Ortín for the permission to reproduce Figure 1b.

ABBREVIATIONS USED

cRNA, complementary RNA; DCP, dodecylphosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycerophosphocholine; HA, hemagglutinin; NA, neuraminidase; NEP, nuclear export protein; NS1, nonstructural protein 1; NS2, nonstructural protein 2; RdRP, RNA-dependent RNA polymerase; RNP, ribonucleoprotein particle; TM, transmembrane; vRNA, viral RNA

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