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Advances in the development of entry inhibitors for sialic-acid-targeting viruses

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Over the past decades, several antiviral drugs have been developed to treat a range of infections. Yet the number of treatable viral infections is still limited, and resistance to current drug regimens is an ever-growing problem. Therefore, additional strategies are needed to provide a rapid cure for infected individuals. An interesting target for antiviral drugs is the process of viral attachment and entry into the cell. Although most viruses use distinct host receptors for attachment to the target cell, some viruses share receptors, of which sialic acids are a common example. This review aims to give an update on entry inhibitors for a range of sialic-acid-targeting viruses and provides insight into the prospects for those with broad-spectrum potential.

Introduction

Antiviral drugs are highly valuable for slowing down or clearing established viral infections. In addition, most of these drugs can be given prophylactically, which is beneficial for high-risk groups. However, the actual number of treatable viral infections is limited. Of the 219 viruses currently known to be infectious to humans [1], only nine are treatable with antiviral drugs [2]. For this reason, there is an urgent need to investigate further antiviral strategies.

Antiviral drugs can interfere with any step of the viral life cycle. An interesting target for antiviral therapy is the process of viral attachment and entry. However, entry inhibitors have seen limited clinical application, with only seven compounds being approved by the US Food and Drug Administration (FDA) for four virus types: human immunodeficiency virus, varicella zoster virus, herpes simplex virus and respiratory syncytial virus [2]. Moreover, these drugs are not

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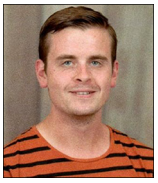
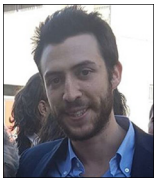


Rick Heida received his master's degree in biomedical sciences at the University of Groningen in the Netherlands in 2017, with a main interest in microbiology and immunology. Currently, he is pursuing his PhD at the department of Pharmaceutical Technology and Biopharmacy at the University of Groningen under the supervision of Henderik W. Frijlink, Anke L.W. Huckriede and Wouter L.J. Hinrichs. His research focuses mainly on the evaluation of antiviral entry inhibitors with broad-spectrum potential and their suitability to be formulated as dry-powder formulations. In line with this, he works on optimizing methods for pulmonary administration of dry-powder formulations in small laboratory animals.

Yoshita C. Bhide completed her PhD in virology and immunology in 2018 at the University of Groningen under the supervision of Anke L.W. Huckriede. Her PhD research focused on improved and cross-protective influenza vaccine evaluation *in vivo*. She is now working as a postdoctoral researcher with Henderik W. Frijlink. Her current research focuses on *in vitro* and *in vivo* evaluation of novel entry inhibitors against influenza viruses. Her research has resulted in several peer-reviewed publications. Being a trained virologist, her research interests are in infectious diseases, especially viral pathogenesis and immune modulation, and the development of vaccines and antivirals.



Matteo Gasbarri received his master's degree in materials science at the Tor Vergata University of Rome. His master thesis focused on flexible perovskite solar cells and was carried out at the Center for Hybrid and Organic Solar Energy (CHOSE). In 2017, he joined Saule Technologies, developing new generation perovskite photovoltaic cells. Since September 2017, he has been a PhD student in the Supramolecular Nanomaterials and Interfaces Laboratory (SuNMIL) at École Polytechnique Fédérale de Lausanne (EPFL), under the supervision of Francesco Stellacci. His research is primarily focused on the development and physicochemical understanding of non-toxic broad-spectrum antiviral nanomaterials.

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commonly prescribed owing to their expense and the availability of more cost-effective alternatives. Therefore, novel ways of targeting viral entry processes should be urgently explored.

Viruses deploy several mechanisms for entry into the host cell, using distinct moieties on the cell membrane as a receptor. Such moieties include sialic acids, which are used as attachment receptor by several viruses. Sialic acids are a family of monosaccharides composed of a nine-carbon backbone. They are typically located at the terminal end of carbohydrate chains, attached to several glycoproteins and glycolipids on the eukaryotic cell membrane. For this reason, sialic acids are highly accessible for protein–ligand and receptor–ligand interactions [3]. Because of their ubiquitous expression on a wide variety of cell types, sialic acids play a part in many physiological processes [4–10,16]. Although they are widely found on mucosal tissue and appear in soluble form in mucosal secretions, the exact tissue distribution of sialic acids in the human body remains largely unknown, and the distribution might vary depending on the physiological conditions and gene expression profile of the individual [11].

Sialic acids exist in different isoforms, depending on chemical substitutions and on the way they are linked to the terminal end of the abovementioned glycoconjugates [12–14]. The most common isoform is neuraminic acid. The two main types of sialic acid found in mammals are two modifications of neuraminic acid: N-glycolylneuraminic acid (Neu5Gc) and N-acetylneuraminic acid (Neu5Ac) [4]. In humans, only Neu5Ac is present because humans lack the ability to produce the enzyme CMP-N-acetylneuraminic acid hydroxylase, which converts the acetyl group into a glycolyl group (Fig. 1) [15].

In addition to their role in normal physiology, sialic acids are involved in the interaction of many pathogens with their host cells. Along with parasites and bacteria, a large number of viruses have been shown to interact with sialic acids for cellular attachment and entry [3]. However, the specific sialic acid derivative that serves as the attachment receptor differs according to the virus strain and depends on the receptor binding site of the viral attachment protein. For this reason, viral tropism is mostly dependent on the location of the attachment receptor. Interestingly, several viruses have been described to be not strictly dependent on sialic acids for attachment and entry, but rather use sialic acids as a co-receptor or enhancer of attachment. At least 16 viral genera have been identified to interact with sialic acids in some way for their entry process, as reviewed elsewhere [11].

Because of the profound role of sialic acids in the viral entry process, an entry inhibitor that prevents virus attachment to sialic acids would have the potential to serve as a broad-spectrum antiviral drug and might therefore be of high therapeutic relevance. This review provides an overview of the epidemiology of a selection of viruses that use sialic acids as their main cellular receptor to initiate entry, and we discuss the current development status of entry inhibitors targeting these viruses. Both sialic-acid-mimicking and sialic-acid-targeting drugs are discussed to highlight the most promising entry inhibitors, which are those with broad-spectrum activity.

Sialic-acid-targeting viruses and the development of entry inhibitors

The following sections provide an overview of the most clinically relevant sialic-acid-targeting viruses for which effective antiviral therapy is urgently needed. We discuss current modes of treatment for these viruses and highlight advances in the development of entry inhibitors, if applicable. The chemical structures of the entry inhibitors described in this review are depicted in Table 1. Because virus–sialic acid binding characteristics at the molecular level have been extensively reviewed elsewhere [13,17], they are not described in detail here.

Influenza viruses

Belonging to the Orthomyxoviridae family, the influenza A virus is one of the most prevalent viral pathogens of our time, frequently causing infections in various mammalian and avian species. Seasonal human influenza epidemics, which are associated with the viruses' susceptibility to undergo antigenic drift [18], are still a major threat to high-risk groups such as the elderly and immunocompromised, accounting for 3 million–5 million cases of severe illness and approximately 500,000 deaths every year [19]. Occasionally, the emergence of new influenza A virus strains can cause even more fatal pandemics [20].

The genome of influenza A and B viruses comprises eight segments of negative-sense, single-stranded RNA, and is enclosed by a lipid envelope [21]. The viral envelope is composed of mainly two proteins, haemagglutinin and neuraminidase, which are both crucial for infection. The primary criterion for the classification of influenza A viruses is based on the subtype of haemagglutinin (H1–H18) and neuraminidase (N1–N11), with the virus being named accordingly (e.g., H1N1) [19]. The trimeric haemagglutinin consists of two smaller subunits: HA-1 and HA-2. HA-1 contains a part of the stalk and forms the globular head domain, which carries the receptor-binding sites for sialic acid and thereby is of utmost importance for viral attachment to the host cell [23]. HA-2 forms

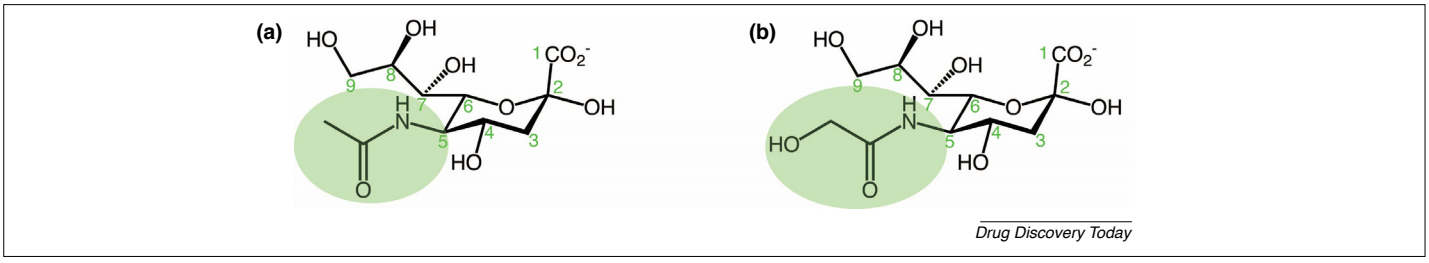


FIGURE 1

Chemical structures of the two sialic acids predominantly present in mammals: **(a)** N-acetylneuraminic acid (Neu5Ac) and **(b)** N-glycolylneuraminic acid (Neu5Gc). The green ellipse highlights the only difference between the two: the substitution on the carbon atom at position 5. Neu5Gc is not present in humans because of a mutation in the gene encoding the enzyme CMP-N-acetylneuraminic acid hydroxylase.

TABLE 1

Structures of compounds discussed in this review

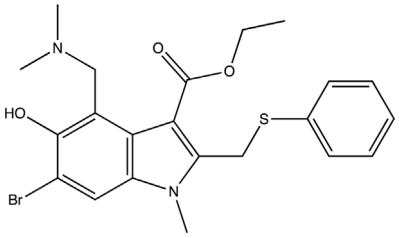
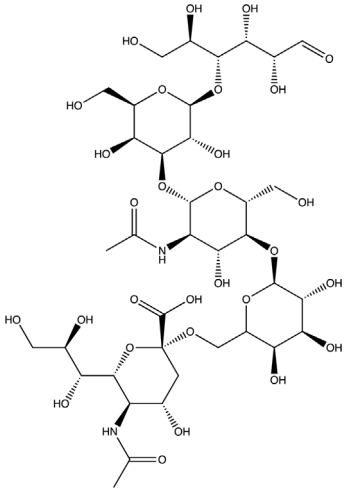
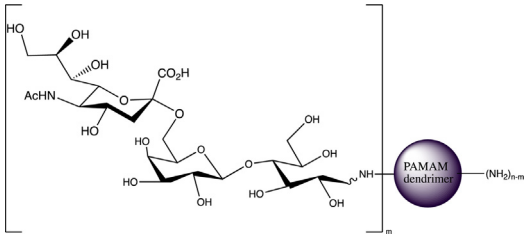
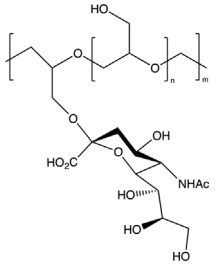
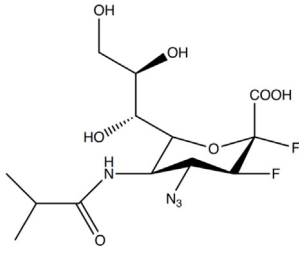
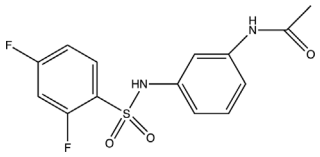
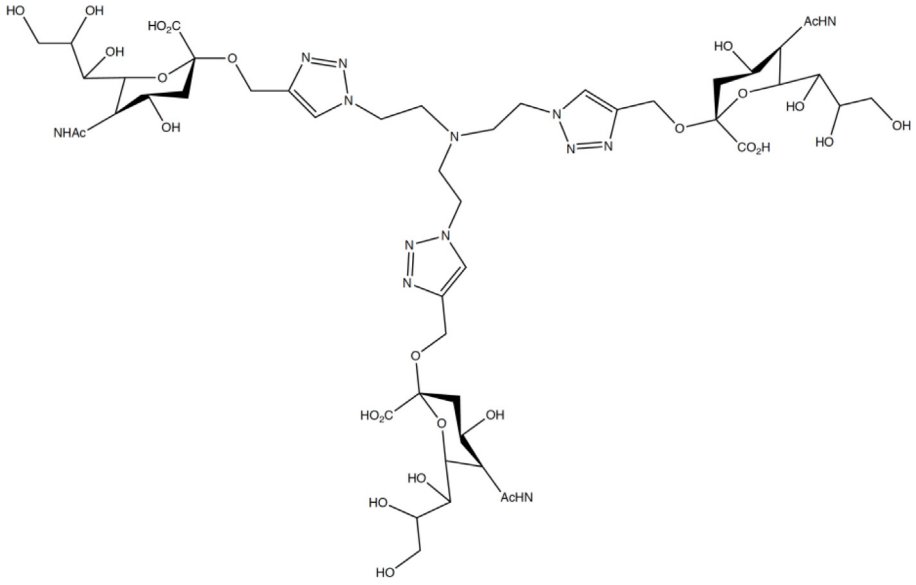
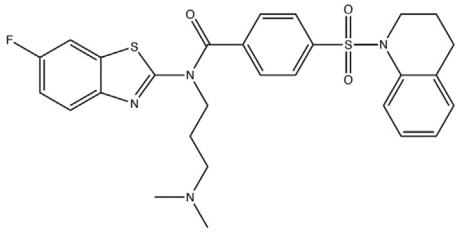
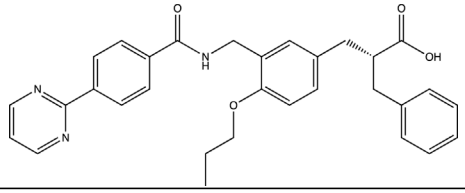
Compound	Structure	*In vitro* activity
Umifenovir (Arbidol)		Influenza [67] EC_{50}: 12.9 μM ± 1.4–30.9 μM ± 0.7[a] Zika [158,191] EC_{50} (vero cells): 12.09 μM ± 0.77 10.57 μM ± 0.74; IC_{50} (A549 cells): 11 μM
DAS-181 (Fludase)	NA	Influenza [62,192,193] IC_{50}: 0.25 nM to 1.0 nM[a]; EC_{50}: 0.02 μM to 0.75 μM[a]
LSTc-bearing liposomes[b]		Influenza [181] IC_{90}: 0.04 μM to 0.98 μM[a]
6′SL-PAMAM conjugates		Influenza [182] IC_{50} range (for different compound variations): 3.4 μM to 220 μM
Linear polyglycerol sialosides		Influenza [183] IC_{50}: 2.35 nM + 0.83
BCX-2798 derivative		HPIV-1 [82] IC_{50}: 0.39 μM

TABLE 1 (*Continued*)

Compound	Structure	*In vitro* activity
PAC-3066		HPIV-3 [84] IC_{50}: 37 μM
17a		Adenovirus-D37 [103] IC_{50} (HCE cell-binding assay): 1.4 nM; IC_{50} (infection assay): 2.9 nM
AY4		JC-polyomavirus [123]: dose-dependent inhibition from 100 μM to 1 mM
EK1		MERS-CoV IC_{50}: 0.11 μM; OC43-CoV IC_{50}: 0.62 μM [134]

[a] Range for several strains of influenza A and/or B.
[b] 6′SL-PAMAM, 6′-sialyllactose-polyamidoamine; HPIV, human parainfluenza virus; LSTc, sialylneolacto-N-tetraose c.

the main part of the stalk, including the transmembrane region and cytosolic tail of the protein, and harbours the 20–23-amino-acid-long fusion peptide (104).

The haemagglutinin protein of human-adapted influenza strains initiates cell entry by binding to the trisaccharide sequence Neu5Ac-α(2-6)-Gal-β(1-4)-GlcNAc, also known as 6′-sialyllactosamine (6′-SLN). This sequence, linking Neu5Ac at the α-2,6 position to galactose, is the predominant sialic-acid-containing sequence on human tracheal epithelium and therefore serves as the primary binding site for human influenza A and B strains [24]. By contrast, avian influenza strains preferentially bind to Neu5Ac, linked at the α-2,3 position to galactose, which is present on the intestinal epithelium of (aquatic) birds (Fig. 2) [25,26]. These differences in linkage specificity between human-adapted and avian-adapted influenza A strains are an important determinant for their host cell tropism. The binding preference of haemagglutinin from avian and mammalian influenza virus strains for differentially linked sialic-acid subtypes, together with the distribution of these sialic acids and their pH stability, serve as a restrictive factor for zoonotic transmission of influenza and determine which species can be infected successfully [27].

After the haemagglutinin binds to sialic acids on the host's respiratory epithelial cells, the virus is internalized by receptor-mediated endocytosis into endosomes. Under acidic conditions, the HA protein undergoes conformational changes that lead to the exposure of the HA-2 fusion peptide, which facilitates the low-pH-

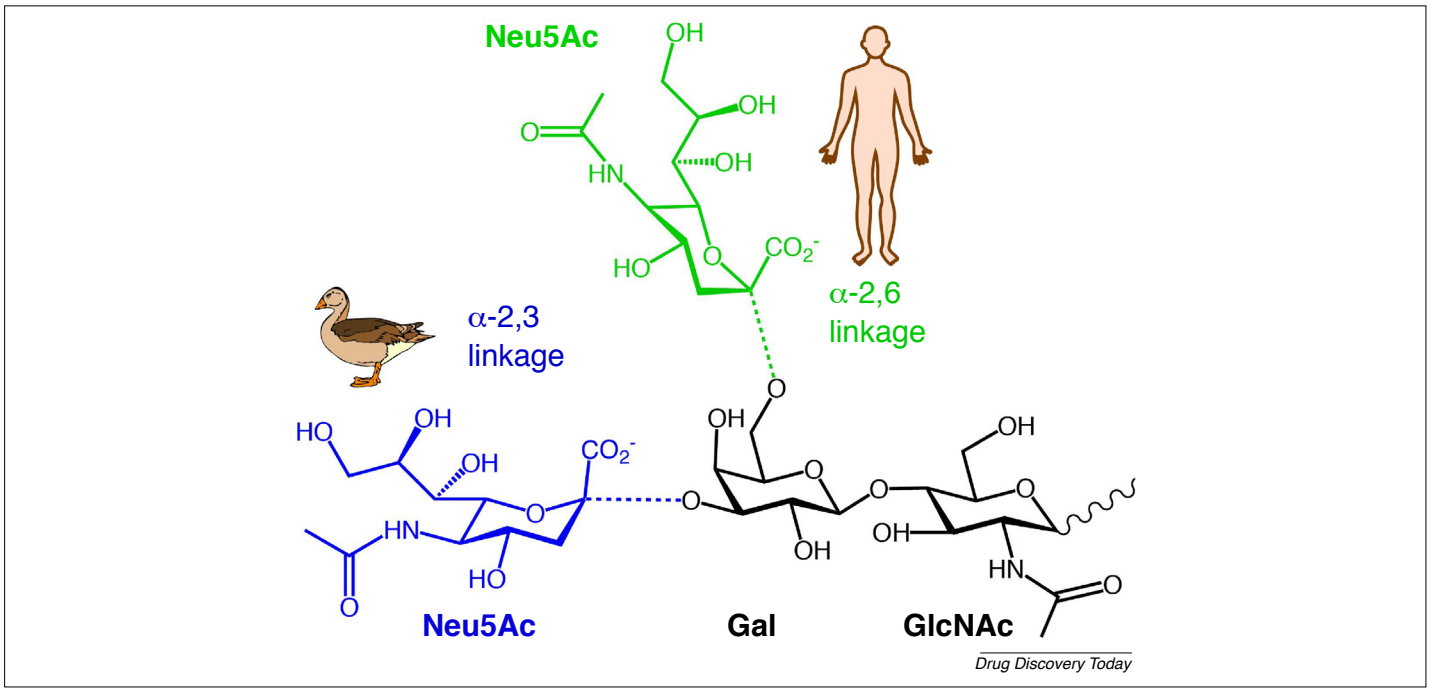


FIGURE 2

Schematic representation of the difference between avian (blue) and human (green) sialic-acid receptors. The only difference is the linkage between N-acetylneuraminic acid (Neu5Ac) and galactose (Gal). In humans, Neu5Ac is linked through an α-2,6 linkage to galactose, whereas for birds, Neu5Ac is linked to galactose via an α-2,3 linkage. In humans, the sequence Neu5Ac-α-(2-6)-Gal-β-(1-4)-GlcNAc is also known as 6′-sialyl-N-acetyllactosamine (6′-SLN).

induced fusion of the viral envelope with the endosomal membrane. This consequently causes an influx of H^+ ions via the M2 ion channel, which in turn leads to the release of the viral ribonucleoprotein into the cytoplasm [28,29]. After replication of the virus has taken place in the host cell, neuraminidase (also known as sialidase) is responsible for the enzymatic cleavage of sialic acid residues, with which it facilitates the release of virions into the extracellular milieu [30].

Prevention of influenza virus infections primarily relies on vaccination. However, vaccines are only moderately effective when they match circulating strains, and they can be ineffective if this is not the case [31]. This has led to interest in the development of influenza-specific antiviral drugs. For the 2019–20 influenza season, four antiviral drugs approved by the FDA were recommended for the treatment of persistent influenza infection in the United States: orally administered oseltamivir phosphate, inhalable zanamivir, intravenously administered peramivir (all of which are neuraminidase inhibitors) and oral baloxavir (which blocks viral RNA transcription) (https://cdc.gov/flu/professionals/antivirals/summary-clinicians.htm#Table1). In the European Union and the European Economic Area, only oseltamivir and zanamivir, sold under their respective trade names Tamiflu and Relenza, have been approved for individuals at a high risk of experiencing complications [33]. Although these drugs have been effective in treating the disease, the emergence of resistance against the currently used antivirals is a major concern for effective treatment and prophylaxis of influenza in the future [34–37]. Therefore, the development of novel drugs or combination therapies that target multiple pathways of the viral replication cycle remains necessary [38].

In this light, entry inhibitors might be a promising alternative. Entry inhibitors for influenza can be generally divided into two categories on the basis of their mechanism of action. The first category includes compounds that interfere with the initial attachment process of the virus. This can be done by directly targeting host sialic acids or by targeting the receptor-binding domain of the virus, using sialic acid analogues as a decoy receptor. Both of these mechanisms disable the ability of influenza-virus particles to bind sialic-acid molecules on the host cell. The other category includes compounds that bind to the haemagglutinin stalk, affecting the fusion of the viral envelope with the endosomal membrane. Although fusion inhibitors do not block the initial attachment, they do block the release of the viral genome into the cytoplasm and thereby interfere with the last step of the viral entry process.

In the past five years, a plethora of novel influenza entry inhibitors have shown potency *in vitro* [39–58]. An overview of compounds that have been further evaluated in the preclinical or clinical phase is given in Tables 2 and 3. Interestingly, only a few of the highlighted entry inhibitors interfere with the initial attachment process of the influenza virus (i.e., with the receptor-binding site of haemagglutinin or with sialic acids). The primary reason for this is that compounds that target the receptor-binding site need to bind the haemagglutinin protein in such a way that they do not lose efficacy on seasonal mutation of the virus, as is the case with neutralizing antibodies from the host. In this context, it should be remarked that haemagglutinin is found in a trimeric form [59]. This implies that it ideally binds to three sialic-acid moieties on the cell surface. Such a concept (i.e., multiple ligands binding to multiple targets) is known as multivalency and is highly important in several biological and pathogenic interactions (Box 1).

BOX 1

The concept of multivalency in biological systems.

To the best of our knowledge, the concept of multivalency has been observed with all viral attachment proteins that have been structurally resolved to date. Multivalency is in fact common in biological systems [187]. It is understood that nature uses it to increase binding strength and consequently selectivity [188,189]. On the basis of this, many receptor analogues have been designed to be multivalent: that is, they were designed as macromolecules containing many repetitive units of the viral receptor to better bind to the virus (Fig. 3). Another advantage of multivalent drugs over monovalent compounds is that a lower dose can be used to elicit the required effect [188]. The design of these compounds further requires the fine-tuning of several parameters such as scaffold geometry and the flexibility of the linkers, their length and their relative distance [182,188,190].

The concept of multivalency is widely used in the development of potentially broad-spectrum sialic-acid-based receptor analogues, which are described in further detail later in this review. Apart from using multivalent drugs to block the receptor-binding site, another strategy is to use monoclonal antibodies that bind to more conserved parts of the HA1 head domain [60]. However, none of the abovementioned compounds has been evaluated in clinical trials.

Besides targeting haemagglutinin to block the attachment process, another way is to directly target host sialic acids. Arguably the most promising influenza entry inhibitor using this method is DAS-181, which is a recombinant fusion protein that can enzymatically cleave terminal sialic acids from carbohydrate chains on the host-cell membrane [61,62]. Because DAS-181 has shown promising results as a broad-spectrum drug, we discuss it later on in this review.

Another study focused on using multivalent proteins derived from the binding domains of bacterial sialidases to target host sialic acids. However, the compounds, which mask the receptor rather than cleaving it, only worked prophylactically, indicating that the virus successfully competed for sialic-acid binding when the same dose was administered therapeutically [63].

Most of the compounds that have been evaluated in preclinical or clinical settings target the stalk domain, the part of the haemagglutinin protein that is involved in the fusion process and is generally more conserved [64]. One interesting example is umifenovir (trade name: Arbidol), a drug that has been licensed in Russia and China and is claimed to have activity against a broad variety of influenza strains [65–68]. The proposed working mechanism is that by interacting with the upper region of the stalk, the drug prevents the low-pH-induced protein rearrangements that are necessary to elicit fusion of the virus with the cell membrane [69]. Other clinically relevant compounds that target the stalk include monoclonal antibodies [70–72]. Of these, five are currently in clinical evaluation (Table 2).

Parainfluenza virus

The Paramyxoviridae family consists of enveloped negative-sense, single-stranded RNA viruses, among which are the parainfluenza viruses. Human parainfluenza viruses (HPIVs) are a common cause of respiratory disease in infants, young children, elderly people and immunocompromised individuals [73]. HPIV infections are the second most prevalent cause of respiratory-disease-related hospitalizations in children under five years of age, after respiratory syncytial virus infection [74]. Symptoms range from mild and cold-like to more severe, including pneumonia and croup. These symptoms can be worse in patients who are already suffering from chronic airway diseases such as asthma and chronic obstructive pulmonary disease [75].

Four types of HPIV have been described: HPIV-1–HPIV-4. Their main outer proteins are haemagglutinin-neuraminidase (HN), which is responsible for attachment to sialic acids, and the fusion protein, which mediates fusion of the virus with the host cell membrane [75]. All HPIV strains are known to bind to sialic acids, preceding cell entry. HPIV-1 and HPIV-3 have been shown to use α-2,3-linked sialic acids, and HPIV-3 also binds to α-2,6-linked sialic acids [76]. This contrasts with avian influenza viruses, which also bind to α-2,3-linked sialic acids but are very rarely transmitted from human to human because they have a different linkage specificity [77]. To our knowledge, the linkage specificity of HPIV-2- and HPIV-4-binding sialic acids has not yet been described.

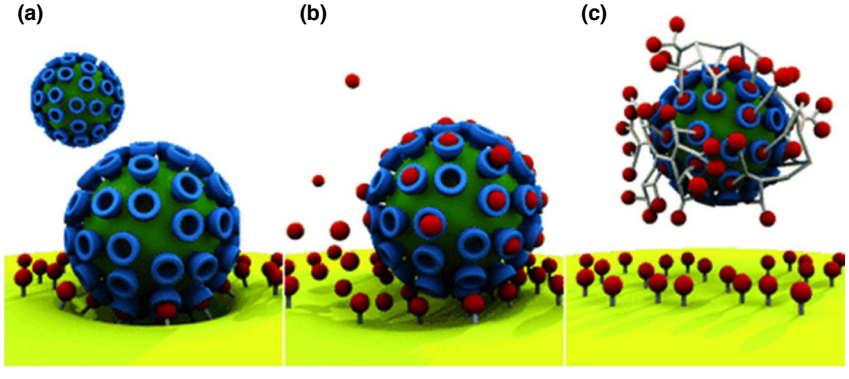


FIGURE 3

TABLE 2

Influenza entry inhibitors in the clinical phase

Compound	Type	Sponsor	Proposed target region	Clinical status	Trial identifier	Refs
DAS-181	Sialidase	Ansun Biopharma	Host sialic acid	Phase IIb study completed assessing safety and efficacy in otherwise healthy individuals infected with influenza A	Phase I: NCT00527865; NCT01173224; NCT01651494 Phase II: NCT01037205; NCT01740063; NCT04298060	[61,62,159,192]
CR6261	Monoclonal antibody	US National Institute of Allergy and Infectious Diseases	HA[a] stalk region	Phase II study completed assessing efficacy of the drug in healthy individuals challenged with H1N1 compared to placebo	Phase I: NCT01406418 Phase II: NCT02371668	[194,195]
Umifenovir	Indole derivative	Pharmstandard	HA stalk region	Phase III study recruiting in China assessing the safety and efficacy of the drug in combination with oseltamivir in hospitalized influenza patients; phase IV study with unknown status	Phase III: NCT03787459 Phase IV: NCT01651663	[67,69,196]
CT-P27	Monoclonal antibody	Celltrion	HA stalk region	Phase IIb study recruiting assessing the safety and efficacy of the drug in influenza-A-infected patients compared to placebo	Phase I: no data posted Phase II: NCT02071914; NCT01740063; NCT03511066; KCT0002211	NA
MEDI8852	Monoclonal antibody	MedImmune LLC	HA stalk region	Phase IIb withdrawn (owing to delay in site enrolment timelines)	Phase I: NCT02350751 Phase II: NCT02603952; NCT03903718	[70,197,198]
MHAA4549A	Monoclonal antibody	Genentech	HA stalk region	Phase II study completed assessing efficacy of the drug in hospitalized influenza-A-infected patients compared with oseltamivir	Phase I: NCT01877785; NCT02284607 Phase 2: NCT01980966; NCT02623322; NCT02293863	[71,199,200]
VIS-410	Monoclonal antibody	Visterra	HA stalk region	Phase II study recruiting to assess safety and efficacy of the drug in hospitalized influenza-A-infected patients compared with oseltamivir	Phase I: NCT02045472 Phase II: NCT02989194; NCT02468115; NCT03040141	[72,201–203]

[a]HA, haemagglutinin.

TABLE 3

Influenza entry inhibitors in the preclinical phase

Compound	Type	Proposed target region	Preclinical relevance	Refs
Sialic acid-functionalized Qβ-bacteriophage capsids	Sialic-acid-functionalized bacteriophage capsid	Receptor binding site of HA[a]	*In vitro* and *in vivo* protection against two H3N2 strains	[184]
IY7640	Small molecule inhibitor	HA stalk region	*In vitro* protection against H3N2, H1N1 and influenza B; *in vivo* efficacy against H1N1	[204]
JNJ4796	Small molecule inhibitor	HA stalk region	Protected mice against lethal and sublethal H1N1 influenza challenge after oral administration	[205]
Carbinoxamine maleate; chlorpheniramine maleate	Histamine antagonist	Endocytic pathway	Showed *in vivo* protection after challenge with H7N9 influenza A virus	[206]
1428A33/1; 1428B5/1; F3A19	Monoclonal antibody	Receptor binding site of HA	*In vivo* efficacy against the A(H1N1) pdm09 strain	[60]
Dialtizem	Calcium channel blocker	Voltage-gated Ca^{2+} channel $Ca_v1.2$	*In vivo* efficacy against H1N1	[207]
S-KKWK	Lipopeptide	HA stalk region	Prevented HA-2 rearrangements and subsequent membrane fusion of several H1N1 strains and H3N2 *in vitro*, and protected mice from lethal infection with H1N1	[208]
Linear polyglycerol sialosides	Linear polyglycerol sialosides	Receptor binding site of HA	*In vitro* protection against H3N2 and both avian H3N2 and avian H7N1; *in vivo* protection against H3N2	[183]
Urumin	Frog-derived peptide	HA stalk region of H1-type HA	*In vitro* efficacy against several H1N1 strains; *in vivo* protection against lethal H1N1 influenza A virus infection	[209]
3′-SL- and 6′-SL-linked PAMAM dendrimers	PAMAM conjugates	Receptor binding site of HA	*In vivo* protection against lethal H1N1	[41,182]
Multivalent carbohydrate-binding modules	Sialidase derivatives	Host sialic acid	*In vivo* protection against lethal H1N1	[63]

[a]HA, haemagglutinin; PAMAM, polyamidoamine; SL, sialyllactose.

Because vaccination strategies have not yet proven to be effective in eliminating the disease [78], patients suffering from HPIV infection could benefit from antiviral therapy. Currently, no drugs have been approved for treating HPIV infections. Because HPIV needs sialic acids for entry, research has been done regarding the development of suitable antivirals for blocking this mechanism. The HN inhibitor and sialic-acid derivative BCX-2798 has been proven to effectively protect mice against challenge with a 90% lethal dose of a chimeric strain of HPIV-1, in which the fusion and HN proteins of Sendai viruses were replaced by those of HPIV. Moreover, the compound had prophylactic potential against chimeric HPIV-3 [79–81]. BCX-2798 was chemically modified by Eveno *et al*. to bind covalently to a key catalytic component of the HN protein of HPIV-1. Hereafter, HPIV-1 replication *in vitro* was reduced by 13-fold compared with its parent compound [82]. Research into the compound is still ongoing.

Recently, the small molecule CM9 was found to interact with the HN protein *in vitro* and *in vivo*, causing the fusion protein to undergo its conformational change prior to the actual attachment of HPIV-3 to cells, which led to a block in viral infectivity [83]. It was found that the molecule interacts with the second sialic-acid-binding site of the HN protein without disturbing the receptor-binding capacity of the virus. This proof-of-concept study led to the design of another compound, named premature activating compound-3066 (PAC-3066), which targets the same second sialic-acid-binding site, but is 100 times more effective *in vitro* than its predecessor [84]. Aside from these studies, only the recombinant sialidase DAS-181 has made it into clinical trials.

Adenovirus

Human adenoviruses (AdVs) are non-enveloped double-stranded DNA viruses with a size range of 90–100 nm. They belong to the family of Adenoviridae and are divided into seven different species, AdV-A to AdV-G, which can be further classified into more than 60 different subtypes [85]. Young children are the most susceptible to AdV infection, and account for roughly 80% of cases. This is mainly due to their lack of humoral immunity against the virus [86].

A wide variety of symptoms can arise following adenoviral infection, including gastrointestinal and ocular inflammation. Most commonly, however, symptoms manifest in the respiratory tract, and they vary from common cold-like symptoms, such as sneezing and coughing, to pneumonia and bronchitis [87]. One of the less common symptoms is epidemic keratoconjunctivitis (EKC), or inflammation of the ocular surface tissue, which is caused by AdV-B1, AdV-E and, in the more severe form, by AdV-D subtypes. EKC is mostly characterized by eye redness, itchiness, soreness and excessive tearing [85]. Although some antiviral drugs, such as the DNA polymerase inhibitor cidofovir, have been reported to be helpful in treating AdV-related disease in people who have received an organ transplant [88,89], no drug has been made available to the clinic because the virus is mostly self-

limiting and can be cleared within 3 weeks [85]. However, susceptible patient groups might benefit from broad-spectrum antiviral drugs.

The two main receptors for adenovirus attachment are the coxsackievirus-adenovirus receptor (CAR) on epithelial cells [90] and the ubiquitously expressed membrane cofactor protein (or CD46) [91]. After binding, a second interaction with αV-integrin is necessary for initiation of endocytosis [92,93]. Of all adenovirus strains that have been described to cause EKC, AdV-D37, AdV-D8 and AdV-D19a account for most severe disease [85]. Interestingly, all of these strains have been reported to interact with sialic-acid residues for binding to the host cell [94–96]. The reported adenoviruses all share a prevalence for α-2,3-linked sialic acids, which contrasts with the requirement of human influenza viruses to solely bind α-2,6-linked sialic acids [97]. This distinction can be explained by AdVs having a tropism for ocular tissue, where α-2,3-linked sialic acids are abundant, rather than respiratory epithelium. The ocular tropism of the above adenoviruses also explains the onset of symptoms affecting the eyes [98]. The AdV-D37 spike protein knob domain has been found to contain three sialic-acid-binding sites, all of which are involved in the interaction with sialic-acid residues that are attached to a GD1a glycan motif on host corneal epithelial cells [99]. The importance of this interaction was highlighted by the fact that after pretreatment of these cells with GD1a-based monoclonal antibodies and soluble GD1a, inoculation with AdV-D37 did not lead to infection. The implications of these findings for the *in vivo* setting have not yet been fully elucidated [99].

In 2005, Johansson and colleagues were the first to design multiple multivalent 3′-sialyllactose derivatives, conjugated to human serum albumin, which were able to effectively bind to AdV and inhibit infection in a model of human corneal epithelial cells [100]. Furthermore, they found that the multivalent molecules yielded a 1000-fold higher inhibitory effect than the monovalent form, which can be explained by the fact that AdV uses several of its fibre proteins for entry into the host cell. The same group confirmed their findings by using sialic-acid conjugates instead of 3′-siallylactose and showed that treatment with this compound led to virus aggregation [101]. On the basis of these results, Spjut *et al*. designed trivalent sialic acid-based inhibitors that bound to all three of the AdV-D37 sialic-acid-binding sites on its knob domain. The most promising compound, ME0322, was a potent inhibitor of AdV-D37 *in vitro* [102]. Based on this, an even more potent trivalent compound was designed, which was highly effective in cell-attachment and -infection assays [103]. In addition to binding CAR, the gastroenteritis-causing AdV-G52 was recently found to bind α-2,8-linked polysialic acids, which are a unique type of post-translationally modified sialic acid that is usually found in brain tissue [104]. Also, AdV-D26 has recently been shown to rely on sialic acids for cell entry [105]. Future insights into the binding mechanisms of these viruses might pave the way for new types of potentially broad-spectrum entry inhibitors for sialic-acid-dependent AdV subtypes.

Polyomavirus

Polyomaviruses include a variety of non-enveloped double-stranded DNA viruses [106]. Their icosahedral-shaped capsid consists of three proteins, namely VP1, which is the viral attachment protein and makes up most of the viral capsid [107], VP2 and VP3, which are probably involved in insertion of the viral genome into the host cell nucleus [108]. Around 14 different human polyomaviruses have been identified [109]. The two strains that are most infectious to humans are BK virus (BKV) and JC virus (JCV), both of which can be further divided into different subtypes [107]. The newly discovered Merkel cell polyomavirus was recently added to this list [110].

Polyomavirus infections are highly prevalent among the global population. An estimated 80% of humans are infected in their lifetime, with the first infection usually occurring in early childhood [107]. Mostly, the virus latently resides in the kidneys and peripheral blood, making the infection asymptomatic or mildly symptomatic for the majority of infected individuals [111]. However, in immune-deficient individuals, the virus might start to replicate again and cause specific disease. In this context, BKV is a common cause of nephropathy, mostly occurring in people who have undergone a kidney transplant [112,113]. By contrast, recurring infections with JCV can lead to progressive multifocal leukoencephalopathy. It is thought that JCV infiltrates the brain tissue via B cells in the blood, from where it can cause a lytic infection in myelin-producing oligodendrocytes and in astrocytes [114,115]. In humans, Merkel cell polyomavirus has been found to be the primary cause of Merkel cell carcinoma, which is a highly aggressive form of skin cancer [110,116].

Different types of human polyomavirus interact with different sialic-acid residues on host cells. JCV has been found to interact with α-2,6-linked sialic acids [117,118], as well as with α-2,3-linked sialic acids [119]. BKV was found to interact with α-2,3-linked sialic acids only [120]. Merkel cell polyomavirus has also been found to rely on α-2,3-linked sialic acids for attachment, although it can use α-2,6-linked sialic acids as well [121,122].

With knowledge of the receptors used for attachment, it might be possible to design antiviral drugs based on the viral entry mechanism. Using computational screening, Yatawara *et al*. identified four different compounds that blocked JCV infectivity in an *in vitro* setting involving the astrocyte cell line SVGA. Of those, the small-molecule inhibitor AY4 was the most effective inhibitor of infection, albeit with low affinity. AY4 was shown to directly bind to the VP1 attachment protein to block viral attachment [123]. Because the options for treating polyomavirus infections and related diseases are limited, the development of novel antiviral compounds is important.

Coronavirus

Coronaviruses (CoVs) belong to the Coronaviridae family and are enveloped, positive-sense, single-stranded RNA viruses. The abundant spike (S) glycoprotein functions as the viral attachment protein. The S protein is composed of two subunits, S1 and S2: S1 contains the receptor-binding site, whereas S2 contains the fusion machinery [124]. CoVs are divided into four subtypes (α, β, γ and δ). Of these, only certain α-CoVs (CoV-229E and CoV-NL63) and β-CoV (OC43-CoV, HKU1-CoV, SARS-CoV, MERS-CoV and the recently emerged SARS-CoV-2) infect humans, and are all thought to be of zoonotic origin [124–126]. CoVs have emerged unexpectedly three times over the past two decades, with the most recent example being the SARS-CoV-2 pandemic, which causes respiratory disease designated as COVID-19 [126]. Overall, most

CoVs cause mild respiratory disease varying from cough to fever. However, symptoms can be worse, especially in susceptible groups such as elderly people and individuals with an underlying illness. To manage the current pandemic and prevent future epidemics or pandemics, the development of novel antiviral compounds has become urgent.

Many clinical trials of potential antiviral compounds such as nucleoside analogues and corticosteroids have failed [127]; thus, other strategies for treating patients are needed to prevent future outbreaks. In that respect, entry inhibitors that target the viral receptor or receptor-binding site of the CoV S protein might be of therapeutic value. Several of the β-CoVs have been shown to interact with sialic acid moieties for cell entry processes. Both OC43-CoV and HKU1-CoV specifically target 9-*O*-acetylated sialic acids [124,128,129], which are modified sialic acids that are thought to have an important role in many biological and pathological processes [130,131]. Attachment occurs via the receptor-binding site in the S1 domain of the S protein [124,132,133]. Recently, the small molecule EK1, derived from the HR2 domain of the OC43-CoV spike protein, was found to elicit high fusion-inhibitory activity against various human CoVs, underlining the potential for its development as a broad-spectrum drug [134]. MERS-CoV interacts with dipeptidyl peptidase 4 as its receptor to enter the cell [135,136], using α-2,3-linked sialic acids as a co-receptor for attachment [137,138]. Although monoclonal antibodies that target the sialic-acid-binding S1a domain might provide a synergistic effect in combination treatment regimens, sialic acids do not seem to be essential for MERS-CoV infectiveness [139].

Enterovirus D68

Enterovirus D68 (EV-D68) is a member of the Picornaviridae family, which is a large family consisting of non-enveloped, single-stranded RNA viruses [140]. Unlike most other enteroviruses, EV-D68 infects the upper respiratory tract, primarily causing disease in the paediatric population [141]. In most patients, the virus causes symptoms similar to those of the common cold. However, in some cases, infections are accompanied by acute flaccid myelitis (AFM), a polio-like disorder that is characterized by lesions in the spinal cord, which can eventually cause paralysis [142,143]. Most of these patients suffer from underlying respiratory disease, have a history of organ transplantation or are immunocompromised in another way [144]. However, whether there is a causal relationship between the virus and AFM pathogenesis is still a matter of debate [141,145,146]. The emergence of more severe cases, together with recent outbreaks, have led to an increased interest in the virus and in disease pathogenicity.

In recent years, EV-D68 has been found to exploit sialic acids for attachment and for cell entry [147]. It was found that these sialic acid molecules are terminally linked via α-2,6-linkers [148] to intercellular adhesion molecule 5 (ICAM5), which is found in the upper respiratory tract as well as on neurons [149], with the latter providing a possible link to the pathogenesis of AFM. Interestingly, it was found that EV-D68 can also exploit α-2,3-linked sialic acid in the lower respiratory tract for infection, increasing the probability of the virus successfully infecting its host [150]. Owing to this, interest in the development of entry inhibitors for the currently untreatable infection has increased. Rhoden *et al.* were among the first to test existing antivirals for the treatment of EV-D68 in a cell-based assay, one of which was DAS-181. They found that the drug was able to reduce the viral cytopathic effect on infection with three EV-D68 strains isolated from the 2014 outbreak, and one prototype strain [151]. However, it is unclear whether these results can be translated to an *in vivo* system, partly owing to the lack of suitable animal models.

Zika virus

The Zika virus (ZIKV) is a single-stranded, enveloped RNA virus in the Flaviviridae family. It is transmitted via mosquitoes, but once it has infected a host, it can be transmitted through the congenital and perinatal route, and it can be sexually transmitted [152]. Infection with ZIKV can affect all age groups and is usually asymptomatic or mildly symptomatic, generally resolving within 2 weeks. However, several reports have indicated that ZIKV infection of pregnant women can cause congenital brain abnormalities of the foetus such as microcephaly, in which the foetus has an abnormally small brain, leading to severe cognitive and motor deficits [153,154]. Although the virus has caused infections only sporadically in the past, several outbreaks have emerged since 2007, which shows the need for preventive measures [152]. People are usually advised to use mosquito nets or DEET-containing insect repellents to prevent initial transmission, but pregnant women who have become infected might benefit from antiviral therapy.

ZIKV has been shown to infect a range of cells from the reproductive tract, as well as certain brain cell types, including neuronal cells from the foetal brain [155]. The primary surface protein of ZIKV is the envelope (E) protein. It is thought that the E protein interacts with several cellular attachment factors before cell entry, with each individual interaction contributing to its binding avidity to the host cell [156]. Once the binding strength has reached the threshold, the virus is internalized via clathrin-mediated endocytosis.

ZIKV was recently found to use α-2,3-sialic acids for cell entry, supposedly as a factor contributing to ZIKV internalization after attachment has taken place [157]. In a study conducted by Fink *et al.*, it was found that the anti-influenza drug umifenovir caused inhibition of ZIKV infection in a range of cell types, including vaginal and cervical epithelial cells. Although the authors propose that the drug inhibits viral entry, the exact mechanisms of inhibition are unknown [158]. Additional insights into the entry mechanism and specific attachment factors of ZIKV might lead to specific therapy for pregnant women at risk of infection.

Antiviral drugs with broad-spectrum potential

Although most studies on antiviral therapies focus on virus-specific pathways, it might be more promising to search for drugs that affect mechanisms common to multiple viruses: for example, the mechanism of viral entry into the host cell. To develop such broad-spectrum entry inhibitors in the context of sialic acid-targeting viruses, they should either target the sialic-acid residues or mimic them by making use of drugs that function as decoy receptors to block the virus from binding to the host cell. Below, both strategies are discussed.

Sialic-acid-targeting drugs

One of the potentially broad-spectrum antiviral drugs that target the entry pathway is DAS-181, a recombinant neuraminidase

analogue that cleaves sialic-acid residues from carbohydrate chains on host epithelia. In this way, virus binding to the host cell can be indirectly inhibited. More specifically, DAS-181 is a fusion protein that contains both the catalytic domain of the sialidase enzyme of *Actinomyces viscosus* and the anchoring domain of human amphiregulin [61]. Because the drug is administered via inhalation, it prevents entry of the virus directly at the airway epithelium, which is thought to keep adverse systemic side effects to a minimum [159]. DAS-181 is considered to be a promising candidate for the treatment of infections with sialic-acid-targeting viruses that are transmitted via the respiratory tract because it targets the host receptor rather than the virus, and thereby circumvents any losses of efficacy related to viral mutations.

Recently, a randomized double-blinded phase IIb clinical trial has been completed regarding the safety and therapeutic efficacy of DAS-181 for treatment of influenza (ClinicalTrials.gov identifier: NCT01740063). Results so far have indicated that the inhaled drug is generally well tolerated for up to 7 days [159,160]. Longer treatment periods have shown to lead to symptoms related to systemic reabsorption and to induce DAS-181-specific antibodies. A new phase IIb clinical trial has been scheduled to assess the efficacy, safety and pharmacokinetics of DAS-181 in people who have been hospitalized with severe influenza and require supplemental oxygen (NCT04298060). In a sub-cohort of the study, DAS-181 efficacy will also be tested in hospitalized patients who have been infected with other sialic-acid-dependent viruses.

In addition, DAS-181 has shown to be effective for HPIV infections. In preclinical studies, administration of the drug has been shown to lead to a significant reduction in the number of HPIV-infected cells *in vitro* and *in vivo* [161]. Furthermore, the drug has successfully passed a phase II clinical trial in which its efficacy was investigated in immunocompromised patients with parainfluenza infection (NCT01644877) [162]. DAS-181 was also shown to have therapeutic potential in the treatment of HPIV-infected individuals who recently underwent haematopoietic stem cell transplantation [163]. A phase III clinical trial has started recruiting participants for evaluating the treatment of human parainfluenza infections in hospitalized, immunocompromised patients (NCT03808922). These developments have led the FDA to designate DAS-181 as both a fast-track and breakthrough therapy.

Aside from the promising advances regarding its use for the treatment of influenza and parainfluenza infections, DAS-181 has shown high therapeutic efficacy against EV-D68 at concentrations in the nanomolar range [151]. However, concerns have been raised owing to the finding that isolated strains of EV-D68 might evade the drug because they are capable of infecting desialylated cells, although it is not known whether these strains actively circulate in the human population [150].

The cleavage of viral attachment receptors seems like a promising strategy, but it is uncertain whether the removal of sialic-acid residues from the respiratory epithelium leads to adverse side effects. Concerns over the use of neuraminidase analogues are primarily based on the hypothesis that neuraminidase treatment makes the patient more prone to secondary infections by bacteria such as *Streptococcus pneumoniae* [164–166]. This possibility was strengthened after treatment with the neuraminidase inhibitor oseltamivir in influenza-infected mice was reported to lead to a decrease in cases of secondary bacterial pneumonia [165].

Additional concerns were raised over the possibility that cleavage of sialic-acid residues by neuraminidase might expose cryptic receptors that are necessary for the opportunistic bacteria to adhere [167,168]. This hypothesis has been tested in *in vivo* colonization studies with *S. pneumoniae* in influenza-infected mice, which showed that DAS-181 had no effect on bacterial growth or might even reduce the risk of acquiring a secondary bacterial infection [169].

However, because sialic acids are necessary for several physiological processes, the long-term effects of their removal should be considered carefully. Moreover, another limitation might be that the removal of sialic acids by sialidases could lead to the emergence of highly resistant escape mutants that do not strictly depend on the sialic-acid interaction.

Notwithstanding these concerns, DAS-181 is currently the only compound in advanced clinical development that tackles sialic-acid-targeting viruses by targeting the cellular receptor itself.

Sialic-acid analogues

Another strategy for broad-spectrum targeting is to mimic the cellular receptor. This can be done by using compounds that function as a decoy, thereby competing with the cellular receptor to bind the virus (i.e., competitive-binding inhibitors). A number of studies have investigated this possibility for treating sialic-acid-targeting-virus infections, especially infections with influenza viruses.

Early studies of the use of sialic-acid-containing compounds to inhibit viral infection primarily investigated naturally occurring soluble substances that contain sialic-acid molecules, such as egg white, serum and respiratory mucus [170]. These substances were shown to be able to compete with cell-associated sialic acids for the binding of influenza virus. It should be noted that the inhibitory capacity of these molecules was found to be highly dependent on several parameters such as size, rigidity and accessibility to haemagglutinin, as well as on the susceptibility of the substance to being neutralized by neuraminidase molecules [170]. Other interesting examples of molecules that are thought to contribute to the defence mechanism of the host are mucins [171,172] and surfactant proteins [173–176], which are produced by cells of the respiratory tract as a component of mucus and contain a wide variety of carbohydrates, including sialic acid. These compounds, which are part of the innate immune system, can function as naturally occurring decoy receptors against intruding sialic-acid-targeting pathogens, leading to aggregation of the virus particles and an enhanced ability of phagocytic cells to recognize such particles, as has been shown recently [176].

As well as naturally occurring molecules, several synthetic sialic-acid analogues have been tested against influenza, albeit with limited success. The efficacy of these drugs increased markedly with the development of the first multivalent inhibitors, although this success varied for different strains of influenza [170]. To overcome the problem of strain-dependent efficacy of the inhibitors, research focused on moieties that are essential to most influenza strains, such as 6′-SLN (Fig. 2). Synthetic compounds containing 6′-SLN epitopes were shown to effectively inhibit influenza A virus infection in mice, both prophylactically and

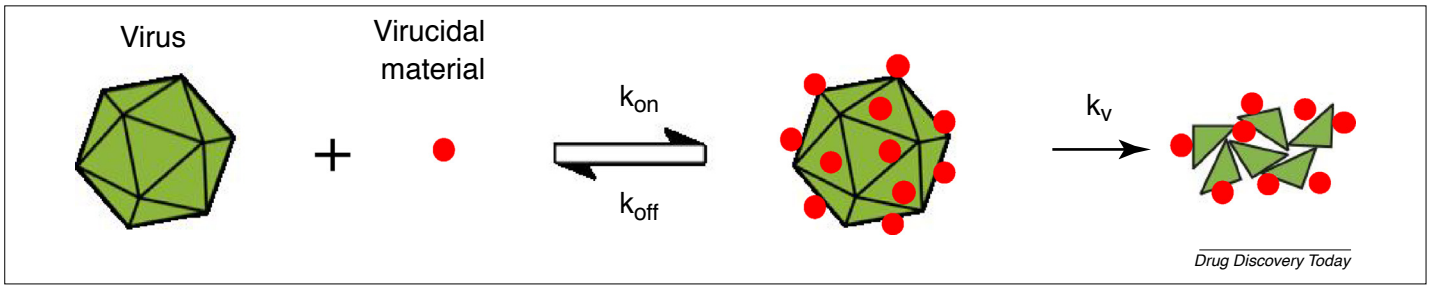


FIGURE 4

A schematic representation of the virucidal mechanism of action. The virucidal drug first interacts with the virus with kinetic constants K_{on} and K_{off}. If the compound used binds with an irreversible mechanism, a local increase in pressure on the virus leads to its inactivation, driven by a kinetic constant K_v.

therapeutically [177,178]. In a series of studies conducted by Papp *et al.*, sialic-acid-functionalized nanoparticles were shown to effectively inhibit influenza virus infection *in vitro* by binding to viral haemagglutinin in a multivalent manner [179,180]. Another application of multivalent receptor analogues was introduced by Wang and colleagues, who functionalized liposomes with sialyl-neolacto-N-tetraose c (LSTc) glycans. The compounds, which acted like polymeric receptor decoys, were able to inhibit infection with several influenza strains, both *in vitro* and *in vivo* [181].

To optimize drugs that target the receptor-binding site of influenza, Kwon and colleagues designed multivalent 6′-sialyllactose-polyamidoamine (6′SL-PAMAM) conjugates with well-defined linker spacings to match the spacing of HA molecules on the viral envelope, and were therefore able to inhibit infection of mice with a lethal dose of H1N1 [182]. On the basis of this, Günther *et al.* recently fine-tuned the design of these conjugates to bear more functional groups, and were able to confirm the efficacy of the compounds against various human and avian influenza strains [41].

Other interesting compounds that target the receptor-binding site are linear polyglycerol sialosides. By optimizing the ligand densities, these compounds were shown to prevent influenza infection in mice in a superior fashion compared with dendritic polyglycerol sialosides. The authors state that the efficacy of dendritic polyglycerol sialosides was probably affected by steric shielding [183]. Another interesting application of multivalent receptor decoys for the treatment of influenza was introduced recently by Lauster and colleagues, who used bacteriophage capsids functionalized with sialic-acid moieties as a decoy for the virus. With ligand arrangements matching the distance of the receptor-binding sites of trimeric influenza haemagglutinin, the systems were able to multivalently bind to influenza virions, thereby efficiently blocking viral attachment *in vitro*, *ex vivo* and *in vivo* [184].

Although the concept of sialic-acid-receptor analogues is promising, not many studies have been conducted on this idea in recent years. This lack of interest could be partially attributed to the poor translatability of *in vitro* results to the clinical setting. The low *in vivo* effectiveness of sialic-acid-receptor analogues is intrinsic to their mechanism of action; such compounds are competitive binding inhibitors that interact with the virus, thus preventing the infection just above a certain concentration. On dilution, a usual condition in the *in vivo* setting, the reversible interaction is lost, and the drug releases an intact virion that can restart an infection cycle, which explains the lack of efficacy *in vivo*. As such, the drug has a so-called virustatic effect.

Arguably, a superior approach would be based on a virucidal mechanism of action. A virucidal drug is able to irreversibly inhibit a virus, so that the inhibition is retained even on dilution (Fig. 4). Compounds that are based on a virucidal mechanism of action enhance the properties of a standard competitive inhibitor because they permanently inactivate the virus. Examples of virucidal compounds are bleach, disinfectants and strong acids, which are all used to deactivate viruses *in vitro* but are also extremely toxic. Therefore, they are not suitable as drug candidates.

One way of designing non-toxic virucidal drugs is to couple decoy receptors to nanoparticles via long, flexible and hydrophobic linkers. The validity of this concept has been shown for heparan sulfate proteoglycans (HSPGs), which are common receptors for many viruses. The modified compounds were shown to bind to several HSPG-targeting viruses in a multivalent manner, leading to irreversible viral deformation, probably owing to a local increase in pressure [185].

Recently, this approach has been successfully translated to sialic-acid-targeting viruses using natural molecules as a core. Specifically, β-cyclodextrins were modified with long and hydrophobic linkers, bearing either 6′-SLN or 3′-SLN as functional group. These compounds were able to irreversibly inhibit different strains of human and avian influenza in the nanomolar range. The results were confirmed *ex vivo* and validated *in vivo* [186].

Taken together, sialic-acid-based receptor analogues might hold promise as entry inhibitors for sialic-acid-dependent viruses, especially for influenza. The possibility of using monosaccharides (such as Neu5Ac) could pave the way for broad-spectrum antiviral drugs with a virucidal mechanism of action.

Concluding remarks and perspectives

Because the number of approved antiviral drugs is limited and new virus strains continue to emerge, the urgency of developing novel therapeutic strategies remains high. Despite numerous attempts to find efficient antiviral drugs, in the end, many of them will fail in phase II or III studies: a frequent occurrence in drug development. Therefore, more effort should be put into the development of potent, preferably broad-spectrum antivirals.

In addition, the administration route should be considered. For example, respiratory-virus infections are likely to be treated most optimally with antivirals that are administered via the

respiratory tract. Because several viruses share a common attachment receptor, we think that focusing on the mechanism of viral entry could open doors to the development of compounds with broad-spectrum activity. Many viruses use sialic-acid residues as their receptor, so this review has focused on the process of viral entry of these sialic-acid-targeting viruses and the current status of entry-inhibitor development, including those which might have broad-spectrum activity. Although numerous studies have been conducted on entry inhibitors targeting this group of viruses, most of the compounds studied have strain-specific targets. Therefore, we think that receptor-targeting compounds that directly interfere with the attachment of the virus to sialic acids, as well as multivalent sialic acid receptor analogues, deserve more attention, because they might have the potential to broadly combat a wide variety of sialic-acid-targeting viruses.

Competing interests

F.S. is the inventor on patent number WO 2018/015465 A1—Virucidal compounds and uses thereof. The author declares no other competing interests.

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