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Medicinal chemistry strategies for discovering antivirals effective against drug-resistant viruses†

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During the last forty years we have witnessed impressive advances in the field of antiviral drug discovery culminating with the introduction of therapies able to stop human immunodeficiency virus (HIV) replication, or cure hepatitis C virus infections in people suffering from liver disease. However, there are important viral diseases without effective treatments, and the emergence of drug resistance threatens the efficacy of successful therapies used today. In this review, we discuss strategies to discover antiviral compounds specifically designed to combat drug resistance. Currently, efforts in this field are focused on targeted proteins (*e.g.* multi-target drug design strategies), but also on drug conformation (either improving drug positioning in the binding pocket or introducing conformational constraints), in the introduction or exploitation of new binding sites, or in strengthening interaction forces through the introduction of multiple hydrogen bonds, covalent binding, halogen bonds, additional van der Waals forces or multivalent binding. Among the new developments, proteolysis targeting chimeras (PROTACs) have emerged as a valid approach taking advantage of intracellular mechanisms involving protein degradation by the ubiquitin-proteasome system. Finally, several molecules targeting host factors (*e.g.* human dihydroorotate dehydrogenase and DEAD-box polypeptide 3) have been identified as broad-spectrum antiviral compounds. Implementation of herein described medicinal chemistry strategies are expected to contribute to the discovery of new drugs effective against current and future threats due to emerging and re-emerging viral pandemics.

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1. Global epidemiology of viral infection

Viral infections constitute a major threat to human health. The recent outbreak and ongoing pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is claiming thousands of lives, while causing havoc in health systems across the world.[1,2] However, there are other viruses that also cause serious and deadly diseases. Examples are influenza, dengue, Ebola disease, chikungunya, and respiratory diseases caused by other coronaviruses such as SARS-CoV-1 or the Middle East respiratory syndrome coronaviruses (MERS).[3,4] Some of these viruses show high mortality rates, while lacking effective treatments. Currently, for example, it is estimated that there are approximately 390 million cases of dengue virus infection each year.[5,6] On the other hand, it is very difficult to eradicate viruses producing chronic infectious diseases, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), or herpes simplex virus (HSV) which may infect more than half of the world's population. In 2019, around 1.7 million were newly infected with HIV across the world.[7] In addition, it has been estimated that 3.7 billion people under age of 50 (67% of the world's population) are currently infected with HSV-1, while HSV-2 infections affect around 417 million people aged 15–49 (11%).[8]

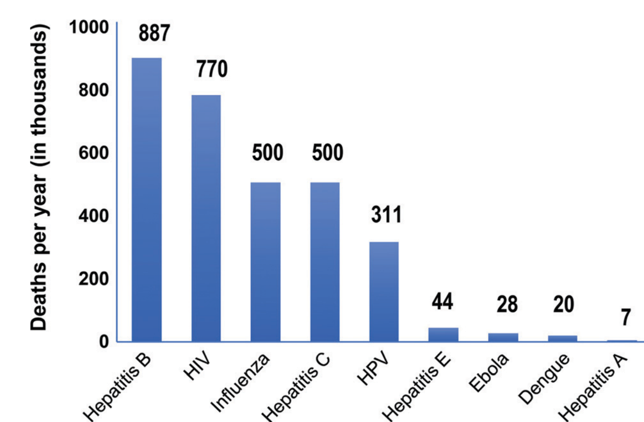

Fig. 1 Estimated number of deaths per year (in thousands) caused by some pathogenic viruses across the world. Data taken from ref. 9. HPV, human papillomavirus.

The spiritual and material losses of mankind are immense, while current research and development of antiviral drugs cannot meet all major clinical needs. Fig. 1 provides the annual deaths produced by some of the most relevant pathogenic viruses, based on data released by the World Health Organization (WHO).[9]

1.1 Human immunodeficiency virus (HIV)

HIV attacks the human immune system and weakens the body's defences against infections and certain cancers. As the virus destroys and impairs immune cell function, infected persons become progressively more and more susceptible to a larger number of infections, cancers and other diseases, which are usually harmless when the immune system is intact.[10] More

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than 32 million people have died from acquired immune deficiency syndrome (AIDS), as of the end of 2019.[7] However, between 2000 and 2018, new HIV infections decreased by 37% and HIV-related deaths decreased by 45% due to antiretroviral intervention. It is estimated that antiretroviral treatment has saved approximately 13.6 million lives, removing AIDS from the top 10 global causes of death.[11] However, there is still a demand for more effective anti-HIV therapies able to fight drug-resistant strains. A substantial increase of transmitted drug resistance (TDR) has been observed in many countries,[12–14] and a relatively high prevalence of drug-resistant strains at the initiation of treatment has been documented in some regions, most notably in sub-Saharan Africa.[13] Thus, in 2016, the prevalence of resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs) in southern and eastern Africa and Latin America was close to the 10% threshold recommended by the WHO for changing first-line antiretroviral therapies.[15]

1.2 Hepatitis virus

Hepatitis is defined as inflammation of the liver tissue, often caused by viral infection. There are five major hepatitis viruses, referred to as types A, B, C, D, and E. These five types of viruses are amongst the most attractive for drug development due to their huge burden of disease, and their potential for outbreaks and transmission.[16] In particular, HBV and hepatitis C virus (HCV) infect hundreds of millions of people, resulting in chronic diseases, including cirrhosis and liver cancer.[17]

According to the WHO, nearly 257 million people worldwide, about 4% of the world's population, are chronically infected by HBV (defined as HBsAg positive).[18] In addition, about 686 000 people die each year from HBV infection, as a result of acute or chronic hepatitis and related complications.[19] It is estimated that around 71 million people worldwide are infected with HCV, and in 2016, approximately 399 000 people died of HCV infection.[11] Both HBV and HCV infections are mostly chronic, if not treated. In HBV infection, long-term therapies based on only a few antiviral drugs have contributed to an increase in prevalence of resistant strains.[20]

1.3 Influenza virus

Seasonal influenza is an acute viral infection that can be transmitted through direct person-to-person contact, rapidly spreading in human populations. With the increased movement of people around the world, influenza virus strains tend to diversify, while the prevalence of drug resistance increases, turning the influenza virus epidemic into a global health concern.[21] It is estimated that every year the influenza epidemic involves approximately 3 to 5 million severe cases worldwide, and around 290 000 to 650 000 deaths related to respiratory diseases.[22]

1.4 Other viruses

Currently, there are no drugs available to treat other important viral infections. Examples are Ebola virus,[23] hantaviruses,[24] Zika virus,[25] enterovirus 71,[26] chikungunya virus,[27] West Nile virus,[28] and respiratory syncytial virus.[29] For these viruses, the development of preventive and therapeutic drugs is urgent and a priority over any other consideration on their potential for development of drug resistance. In this scenario, host factors could be useful targets of antiviral intervention for these emerging viruses,[30,31] particularly if their replication and pathogenicity could be blocked by using broad spectrum antiviral drugs.

2. Antiviral therapy and resistance

2.1 Approved drugs for antiviral therapy

Synthesized in the late 1950s, idoxuridine (5′-iodo-2′-deoxyuridine) was the first approved antiviral agent in 1962.[32] It was used to treat eye and skin infections caused by HSV. For three decades, progress in antiviral therapy was slow until the approval of zidovudine, the first antiretroviral drug used to combat HIV infections and AIDS-related diseases.[33] Since then, we have witnessed impressive advances in antiviral therapy leading to the approval of about one hundred drugs,[34,35] although these compounds target only 9 virus families. About one-third of the approved drugs are used against HIV. The others are used alone or in combination for the treatment of infections caused by HBV, HCV, herpesvirus or influenza virus.[34] Based on their chemical structures, marketed drugs can be divided into small molecules, peptides, proteins, and oligonucleotides, where the first group is represented by more than 60 different compounds.[35] Nevertheless, viral infections are still difficult to cure, and resistance is a major threat to the success of antiviral therapy.[36]

2.2 Resistance

The emergence of resistance-associated mutations in the viral genome and the concomitant loss of susceptibility to antiretroviral drugs are major hurdles for therapy success, and an enormous limitation for the prevention and treatment of viral infections.[36] Drug resistance contributes to viral persistence in human populations and puts additional limits on the efficacy of antiviral drugs.

Drug resistance can emerge due to the pre-existence of viruses with reduced susceptibility to the antiviral agents, or appears in the presence of the drug.[36] Naturally selected mutant strains appear under drug pressure during viral treatments. Their relevance for antiretroviral therapies has been underlined by many studies carried out during the last 30 years. The virus will mutate under natural conditions, and the mutations generated under drug selection pressure usually lead to resistance and therefore, survival of the virus. In viruses, prokaryotes and eukaryotes, mutation rates are negatively correlated with the size of their genomes.[37] Among viruses, RNA viruses (*e.g.* retrovirus, poliovirus, and influenza virus) have higher mutation rates than DNA viruses (*e.g.* herpesviruses).[38]

In the absence of antiviral drugs, immune pressure facilitates the selection of viral strains that appear, while the virus propagates in individuals and human populations. This occurs in influenza virus infections[39] and many other viruses. Subtle and gradual changes lead to antigenic drift, and sudden and major changes can cause antigenic shift due to genetic recombination

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or reassortment, particularly when viral genomes are made of two or more different nucleic acid molecules.[40] These genetic changes may lead to the emergence of new influenza viruses, which can cause pandemics like the Spanish flu in 1918 or the 2009 H1N1 swine flu pandemic.[41] One of reasons for the relatively easy development of resistance is that many approved drugs target viruses with high mutation rates, and often with low genetic barriers to resistance. Therefore, the discovery and development of drugs against new targets and new mechanisms is of great relevance.

HIV-1 variants with resistance mutations can be transmitted from antiretroviral treatment-experienced people through sex, blood, or vertically (mother-to-child transmission). Transmitted drug resistance can also serve as a source for the continued spread of drug-resistant variants.[42] Viruses such as HBV, which has a similar transmission route to HIV, may also spread resistance in a similar manner. In the next sections we will briefly discuss mutations and mutational pathways leading to antiviral drug resistance, and those occurring during therapy for HIV, HBV, HCV and influenza virus.

2.3 HIV-1

2.3.1 Replication cycle of HIV-1. The HIV replication cycle involves a series of steps starting from virus-cell recognition and viral entry to budding of an immature virion that is converted to a mature virus with infectious capacity[43] (Fig. S1, ESI†). Recognition and binding of viral surface glycoprotein gp120 to T lymphocytes expressing the CD4 receptor requires the assistance of a cell surface co-receptor, namely CCR5 or CXCR4.[44] Recognition and binding triggers HIV envelope and cell membrane fusion.[45] After fusion, the capsid containing the viral genome (single-stranded viral RNA) is released into the cell.[46] Then, the single-stranded RNA is converted into double-stranded DNA by the viral reverse transcriptase (RT).[47,48] This proviral double-stranded DNA enters the host cell nucleus and is integrated into the host DNA in a reaction catalysed by the HIV integrase.[49] The integrated DNA serves as a template for the production of mRNAs including viral genomes that are synthesized by host RNA polymerase II. Obtained mRNAs are translated into viral proteins that are packaged at the cell membrane to generate an immature virion, which is then converted to a mature infectious virus, after proteolytic processing of viral polyproteins by the HIV protease.[50]

2.3.2 Approved drugs for HIV-1. HIV undergoes many successive replication cycles and continuously infects T cells, while debilitating and destroying the human immune system. Therefore, theoretically, if enzymes and proteins involved in the replication cycle are inactivated, viral replication can be prevented and damage produced by viral propagation can be stopped.[51] As of today, about 30 antiretroviral drugs have been approved by the U.S. Food and Drug Administration (FDA) (Table 1). These molecules target the HIV replicative cycle at five different steps: (i) receptor and coreceptor recognition, and attachment; (ii) fusion; (iii) reverse transcription; (iv) integration; and (v) maturation.[35,52–54]

2.3.3 Transmitted drug resistance in HIV-1 infection. HIV has an extraordinary replication capacity, completing a round of infection to maturation cycle in about 1.2 days.[55] It has been estimated that during full-blown AIDS, the number of virions produced each day is above 10^{10}. In addition, assuming a mutation rate of 10^{-4} and a genome size of around 10 000 nucleotides, it is predicted that HIV produces one mutation per replication cycle.[56] These data provided strong support for the use of combination therapies to treat HIV infections. Highly active antiretroviral therapy (HAART) was introduced in the mid-1990s as a combination of three or more antiretroviral drugs with different viral targets. Current combination therapies against HIV-1 involve the use of two nucleoside reverse transcriptase inhibitors (NRTIs), plus one additional drug that could be a non-nucleoside reverse transcriptase inhibitor (NNRTI), a protease inhibitor or an integrase inhibitor.[57] These treatments are highly successful, while the emergence of resistance has been largely reduced in developed countries. However, long-term drug exposure and very often, suboptimal drug treatments have facilitated the emergence of drug-resistant strains, that can be eventually transmitted to naïve or infected individuals.[58]

In 2007, the prevalence of HIV-1 transmitted drug resistance (TDR) in China was 6.25%, relatively low compared with

Table 1 FDA-approved drugs for HIV treatment

HIV replication step	Type of inhibitor	Representative drugs
Recognition and binding	CCR5 co-receptor antagonist	Maraviroc
	CD4 inhibitor	Fostemsavir[a], ibalizumab[b]
Fusion	Membrane fusion inhibitor	Enfuvirtide
Reverse transcription	RT inhibitor	NRTIs: abacavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir[c], zalcitabine, zidovudine
		NNRTIs: delavirdine, doravirine, efavirenz, etravirine, nevirapine, rilpivirine
Integration	Integrase inhibitor	Bictegravir, dolutegravir, elvitegravir, raltegravir
Maturation	Protease inhibitor	Amprenavir, atazanavir, darunavir, fosamprenavir[d], indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, tipranavir

[a] Fostemsavir (commercial name Rukobia) is an attachment inhibitor that was approved in July 2020 and binds HIV-1 gp120 preventing its binding to CD4. [b] Ibalizumab (commercial name Trogarzo) is an approved monoclonal antibody directed against the CD4 receptor of T cells. [c] Tenofovir can be provided as tenofovir disoproxil fumarate or tenofovir alafenamide. [d] Amprenavir and fosamprenavir represent the same active inhibitor, although fosamprenavir is a prodrug containing an extra phosphoryl group.

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developed countries. TDR showed a downward trend (from 4.75% to 3.75%) from 2001 to 2011, but showed a rapid growth after 2011 until 2017 (increasing from 3.75% till 6.25%). This rise was mainly driven by NNRTI resistance (2.25% in 2001, 1.75% in 2011, and 5.0% in 2017), while NRTI and protease inhibitor resistance remained stable.[59] Overall, NRTI-related mutation (M184V/I) and NNRTI-related mutations (K103N/S, Y181C/I and G190A/S) account for the largest proportion of resistance-associated mutations in treated and untreated patients across the world. Many of these amino acid substitutions confer high level resistance to lamivudine, efavirenz and/or nevirapine.[48]

An analysis of HIV-1 TDR prevalence carried out in Europe in 2005 showed that 1 out of 10 patients naïve for antiretroviral therapy (10.4%) were infected with virus containing at least one mutation associated with drug resistance.[60] Prevalence of NRTI, NNRTI and protease inhibitor resistance mutations were estimated at around 7.6%, 2.9%, and 2.5%, respectively. In 2017, the European Union surveillance system for HIV-1 drug resistance reported that 14.5% of the infected individuals carried viruses with resistance to at least one antiretroviral drug.[61] Prevalence of NRTI, NNRTI and protease inhibitor resistance mutations were estimated at 8.6%, 5.1%, and 2.0%, respectively.

A declining trend in the prevalence of drug resistance was observed in the U.S. between 2006 and 2017, going from 48.9% to 39.3%, when considering viral strains containing single or multiple mutations associated with resistance to NRTIs, NNRTIs or protease inhibitors. This trend was more evident in patients having resistance-associated mutations to two or three drug families (from 43.3% in 2006 to 17.1% in 2017), although the prevalence of TDR increased for HIV variants containing a single drug resistance-associated mutation (from 40.0% to 52.9%).[62]

Table 2 summarizes HIV-1 TDR frequencies in several regions of the world.[13,59,61,62] In general, we observe a large variability in the data, but the general trend is that the prevalence of drug resistance decreases for older therapies. However, the prevalence of resistance mutations is higher for those drugs frequently used in recent years. Most notably, NNRTI resistance is common due to the extensive use of efavirenz and other drugs over many years and their relatively low genetic barrier to resistance.[63] This also reminds us that in the competition with the development of drug resistance, drug design efforts should also focus on the development of novel agents targeting resistant viruses.

Table 2 HIV TDR in several countries and regions. Data were taken from ref. 57–61

TDR	Year[a]	Overall (%)	NRTI (%)	NNRTI (%)	Protease inhibitor (%)
China	2017	6.25	0.7	5.0	0.5
Europe	2017[b]	14.5	5.1	8.6	2.0
U.S.	2017	39.3	19.2	27.4	6.2
Sub-Saharan Africa	2000–2013	2.8	0	1.4	0
South/Southeast Asia	2000–2013	2.9	1.0	0.8	0.5
North America	2000–2013	11.5	5.8	4.5	3.0
Upper-income countries of Asia	2000–2013	5.6	3.5	1.1	1.6
Latin America/Caribbean	2000-2015	7.7	4.0	3.6	1.7

[a] Reported values correspond to the median overall TDR prevalence for year intervals obtained from data collected along the time interval considered. [b] The overall TDR in Europe was obtained from an aggregated report including 1417 cases.[61] TDR data for NRTI, NNRTI and protease inhibitors in Europe were obtained from a surveillance study carried out in 2017 with 1680 individuals.[61]

2.4 HBV

2.4.1 The replication cycle of HBV. In HBV, adsorption, penetration, unpacking, repair, transcription, translation, capsid protein assembly, DNA replication, and release of viral particles constitute major steps in its infection cycle[64–66] (Fig. S2, ESI†). HBV binds reversibly and non-specifically to heparan sulfate proteoglycans on the surface of hepatocytes, while the viral surface antigen recognizes the receptor sodium taurocholate co-transporting polypeptide (NTCP).[67] Then, the virus penetrates into hepatocytes, releasing the nucleocapsid into the cytoplasm. The viral capsid containing the partially double-stranded DNA and the polymerase is disassembled and transported to the nucleus on microtubules. The DNA is transferred through the nuclear pore, where host DNA polymerases generate fully double-stranded viral DNA that can be transformed into highly stable covalently closed circular DNA (cccDNA).

The cccDNA is used as a template to generate a pre-genomic RNA and three mRNAs that are translated into viral proteins, including the capsid core protein and the viral RNA-dependent DNA polymerase. The core protein in the cytoplasm self-assembles with pre-genomic RNA and viral polymerase to form the nascent primary viral nucleocapsids. New viral capsids can be disassembled again and directed into the nucleus for recycling, or secreted out of cells to begin a new cycle of infection.

2.4.2 Approved drugs for HBV and acquired drug resistance. Current clinical therapies against HBV are mainly based on the combination of immunomodulators and nucleos(t)ide analogues.[68,69] However, all drugs approved as anti-HBV agents develop resistance of different degrees.[70,71] The target of nucleos(t)ide analogues is the viral DNA polymerase, an RT lacking the proofreading activity found in eukaryotic DNA polymerases.[48] Approved nucleos(t)ide inhibitors of HBV replication are lamivudine, telbivudine, entecavir, adefovir dipivoxil and tenofovir prodrugs (tenofovir disoproxil and tenofovir alafenamide) (Fig. 2). Major mutational patterns conferring resistance to these analogues are associated with amino acid substitutions in the HBV RT, most notably rtL180M/rtM204(*I*/*V*) for lamivudine, telbivudine and entecavir, and rtA181V/rtN236T for adefovir and tenofovir.[71] Based on the structural homology between HBV RT and HIV-1 RT, it is assumed that these amino acid substitutions affect binding of the corresponding triphosphorylated nucleos(t)ide analogues, leading to the development of resistance and cross-resistance. Fig. 3 summarizes the accumulation of resistance to approved nucleos(t)ide analogues after treating naïve patients for five consecutive years.[72–76]

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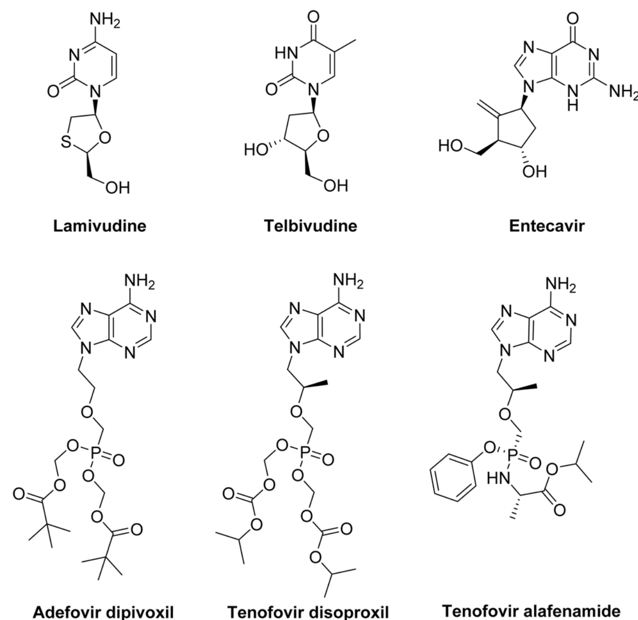

Fig. 2 Chemical structures of nucleoside/nucleotide analogues approved by the FDA for treating HBV infection.

2.4.3 Anti-HBV agents in clinical trials. None of the approved therapies (*i.e.* interferons plus nucleos(t)ide analogues) can cure HBV completely.[77] Interferon can boost immunity, but it is poorly tolerated, with a low cure rate and many side effects, while nucleos(t)ide analogues are well tolerated, but have to be used during long periods of time and are likely to develop resistance.[78] Therefore, research focused on the discovery of safe and efficient drugs with a low tendency to select for resistant variants is a priority, as well as searching for inhibitory compounds with other mechanisms of action.[79] Table 3 shows a list of anti-HBV drugs in clinical trials acting on unexploited targets or with novel mechanisms of action.[78,80–83]

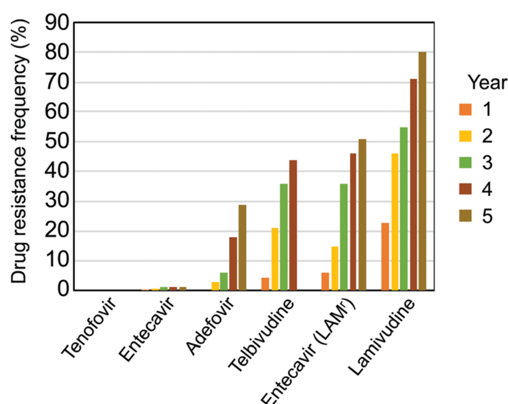

Fig. 3 Cumulative annual incidences of resistance in HBV-infected drug-naïve patients treated with nucleos(t)ide analogues. Tenofovir and telbivudine data are shown for 3 and 4 years, respectively. LAM^r: lamivudine-resistant. Data were taken from clinical trial reports published in ref. 72–76.

2.5 HCV

2.5.1 Replication cycle of HCV. HCV selectively infects hepatocytes and causes liver disease.[84–86] The HCV is an enveloped virus with an icosahedral core that contains a positive-sense single-stranded genomic RNA. HCV entry occurs *via* endocytosis, in a process involving the participation of viral glycoproteins E1 and E2, and cell-surface molecules CD81, LDL receptor, SR-BI, DC-SIGN, claudin-1, and occludin (Fig. S3, ESI†). After entering the cell, the positive-strand RNA genome is released into the cytoplasm and translated into a single polyprotein, which is processed by viral and host proteases to produce three structural (core protein and envelope glycoproteins E1 and E2) and seven non-structural proteins (NS1 (p7), NS2, NS3, NS4A, NS4B, NS5A and NS5B). NS5B is an RNA-dependent RNA polymerase, responsible for the replication of the genomic RNA of 9600 nucleotides.[87] The first step in HCV replication leads to the synthesis of a negative-strand RNA. This molecule serves as a template to obtain positive-sense single-stranded HCV genomic RNA. This RNA can then be translated into new viral proteins, recycled as template for further RNA replication, or assembled to form new viral particles. Cellular secretory pathways involving the participation of very low-density lipoproteins or the endoplasmic reticulum through the endosomal-sorting complex are important for the release of mature HCV virions.

2.5.2 Approved drugs for HCV. Non-structural proteins play many important functions in the life cycle of HCV, thus becoming ideal targets for directly acting antiviral agents.[88] NS3 is a multifunctional protein with a serine-protease domain and a helicase-NTPase domain.[89] NS4A is a cofactor for NS3 protease activity, synergistically involved in polyprotein cleavage. NS5A participates in RNA binding and replication,[90] while NS5B is the RNA-dependent RNA polymerase that plays a pivotal role in HCV genome replication.[87] For many years, therapies against HCV were based on the use of interferon combined with ribavirin, but very often treatments were interrupted due to serious side effects.

With the approval of boceprevir and telaprevir about ten years ago, HCV NS3/4A serine protease inhibitors (administered together with interferon/ribavirin) were found to increase up to 70% the rates of sustained virologic response in treatment-naïve individuals.[91] Then, the discovery and development of sofosbuvir as a potent and effective anti-HCV drug inhibiting the viral polymerase (approved in 2013) became a major breakthrough that allowed the introduction of interferon-free therapies and facilitated the eradication of HCV from infected individuals[92,93] (Table 4).

The third family of anti-HCV agents includes NS5A inhibitors, such as daclatasvir, that were discovered after large high throughput screening assays using HCV replicons. HCV NS5A is a protein of unknown enzymatic function and its identification as a target of antiviral agents was based on the selection of resistant HCV replicons that contained mutations in the NS5A-coding sequence.[94] In contrast to HIV and HBV infections, current therapies against HCV are able to eradicate the virus.[95]

Successful HCV eradication treatments are based on the use of two or three directly acting agents (Table 4) with different

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Table 3 Representative antiviral agents against HBV currently in clinical trials

Drug	Mechanism or target	Company	Clinical stage
Hepcludex (formerly myrcludex B)	Entry inhibitor	Hepatera and Myr GmbH	Phase III
Morphothiadin (GLS4)	Capsid protein inhibitor	HEC Pharma	Phase II
ABI-H0731	Capsid protein inhibitor	Assembly bioscience	Phase II
ABI-H2158	Capsid protein inhibitor	Assembly bioscience	Phase I
JNJ56136379	Capsid protein inhibitor	Janseen	Phase II
RO7049389	Capsid protein inhibitor	Roche	Phase I
Rep 2139	HBsAg inhibitor	Replicor	Phase II
Rep 2165	HBsAg inhibitor	Replicor	Phase II
GSK3389404	Antisense oligonucleotide	GlaxoSmithKline	Phase II
GS 4774	Therapeutic vaccine	Globeimmune and Gilead	Phase II
INO-1800	Therapeutic vaccine	Inovio	Phase I
HB-110	Therapeutic vaccine	Ichor Medical and Genexine	Phase I
TG1050	Therapeutic vaccine	Transgene	Phase I
HepTcell	Therapeutic vaccine	Altimmune	Phase I
GS 9620	TLR-7 agonist	Gilead	Phase II
RO6864018	TLR-7 agonist	Roche	Phase II
RO7020531	TLR-7 agonist	Roche	Phase I
Vesatolimod (GS-9620)	TLR-7 agonist	Gilead	Phase II
Selgantolimod (GS-9688)	TLR-8 agonist	Gilead	Phase II
AIC 649	TLR-9 agonist	AiCuris	Phase I
SB9200	RIG-1 and NOD2 agonist	Spring Bank	Phase II
EYP001	FXR agonist	ENYO Pharma	Phase II
CRV431	Targeting HBx	ContraVir	Phase I
Nitazoxanide	Targeting HBx	Romark	Phase II
JNJ-3989	RNA interference	Janssen	Phase II

mechanisms of action, sometimes combined with ribavirin.[96] These combination therapies improve treatment efficiencies, while reducing the emergence of drug resistance. However, risk of failure is not completely avoided, particularly in cases of advanced liver fibrosis, or in patients infected with drug-resistant variants.[97,98]

2.5.3 Genotypic resistance to approved anti-HCV agents. The reduced antiviral activity of NS3/4A protease inhibitors in different HCV genotypes seems to be related to the natural occurrence of resistance-associated mutations in some genotypes.[100] For example, S122R confers moderate resistance to simeprevir, and is a natural variant of HCV genotype 2; D168Q, confers high-level resistance to simeprevir, and is found in all HCV genotype 3 isolates; and Q80K is a natural variant in HCV genotype 5 and a frequent polymorphism in genotype 1a.[101,102] Q80K reduces viral susceptibility to simeprevir, but has a minor impact on faldaprevir (discontinued) and asunaprevir resistance.[103,104]

The prevalence of natural variants resistant to NS5A inhibitors in therapy-naïve individuals infected with HCV genotype 1 was estimated to be 0.3–2.8% by population sequencing in different studies.[97] HCV genotype 1b variants containing L31M that confer moderate levels of resistance to daclatasvir and ledipasvir were found in 2.1–6.3% of the infected patients, whereas the most frequently observed resistance-associated substitution (*i.e.* Y93H) had a prevalence of 3.8–14.1%. Y93H confers moderate to high-level resistance to first generation NS5A inhibitors, such as daclatasvir, ledipasvir or ombitasvir.[97]

Table 4 Approved directly acting antiviral agents against HCV infection[97–99]

Target protein	Generation or category	Approved drugs	Genotype	Resistance barrier
NS3/4A protease inhibitor	First generation	Telaprevir Boceprevir	Genotype 1	Low
	Second generation	Simeprevir Asunaprevir Vaniprevir Danoprevir	Genotype 1	Moderate
	Third generation	Voxilaprevir Glecaprevir Grazoprevir Paritaprevir	Pan-genotypic	High
NS5B polymerase inhibitor	Nucleoside	Sofosbuvir	Pan-genotypic	High
	Non-nucleoside	Dasabuvir	Genotype 1	Moderate
NS5A serine protease inhibitors	First generation	Daclatasvir Ledipasvir Ombitasvir	Pan-genotypic	Low
	Second generation	Velpatasvir Pibrentasvir Elbasvir	Pan-genotypic	High

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Natural resistance to approved non-nucleoside inhibitors of the NS5B RNA polymerase has been estimated around 0.2–3.1% in patients infected with HCV genotype 1. Two major resistant variants (Leu414 and Ile423) and seven minor variants (Asn316, Val421, Phe445, Leu482, Ala494, Ala499 and Gly556) were found as natural polymorphs in selected genotypes.[105–107] Specifically, Leu414 and Ile423 were found in 36.8% of the HCV genotype 4 and in all HCV genotype 5 sequences, respectively.[108]

2.5.4 Phenotypic resistance to directly acting antiviral agents. Resistance is usually related to a change of shape in the binding site of the directly acting antiviral agent within the viral target protein. Depending on the structure of the inhibitor, resistance-associated mutations can have different effects on phenotypic drug resistance and treatment efficacy. For example, Q80K is an amino acid substitution conferring low-level resistance to simeprevir,[109,110] that could lead to therapy failure of treatments including pegylated interferon α plus ribavirin. However, the same mutations seem to have a reduced impact if simeprevir is co-administered with the NS5B polymerase inhibitor sofosbuvir. In combination therapies involving two or three directly acting antiviral agents, therapy response is strongly associated with the resistance barrier and decreased viral susceptibility in phenotypic assays due to the pre-existence of drug resistance-associated mutations.[97]

Resistance barriers of first generation NS3/4A protease inhibitors are very low, and there is extensive cross-resistance between different drugs.[111] Second and third generation antiviral agents have improved potencies, pharmacokinetic profiles and physicochemical characteristics, although their resistance-associated amino acid substitutions are similar to those of the first-generation inhibitors, with major mutations occurring at positions 155 and 168.[112] In the case of first generation NS5A inhibitors, short-term monotherapy studies have revealed their low genetic barrier to resistance, leading to rapid selection of resistance-associated mutations. Second-generation NS5A inhibitors also have a higher genetic barrier to resistance.[113] Dasabuvir, a non-nucleoside inhibitor of NS5B RNA polymerase is inactive against HCV genotypes 2, 3 and 4, and is considered as a drug with a low genetic barrier to resistance. In contrast, sofosbuvir is a pan-genotypic inhibitor binding at the conserved active site of the polymerase, and is rather resilient to the development of drug resistance.[99]

In summary, available combination therapies including two or three directly acting antiviral agents (Table 5) have remarkable advantages, most notably, high curative rates, short duration of the antiviral treatment and minimal adverse effects. Although drug resistance should not be ignored, current regimens have reduced chances of treatment failure, while decreasing the probability of drug resistance development.[114,115]

Table 5 Approved combinations of directly acting antiviral agents against HCV and their targeted genotypes[116]

Drug combination	HCV genotypes
Ledipasvir/sofosbuvir	1, 4, 5, 6
Daclatasvir/sofosbuvir	1, 3
Velpatasvir/sofosbuvir	All 6 genotypes
Grazoprevir/elbasvir	1a, 1b, 4
Glecaprevir/pibrentasvir	All 6 genotypes
Voxilaprevir/velpatasvir/sofosbuvir	All 6 genotypes

2.6 Influenza virus

2.6.1 Replication cycle of influenza virus. Influenza virus replication takes place shortly after infecting host cells.[117–119] The viral hemagglutinin is a glycoprotein located on the viral surface that binds and recognizes the sialic acid receptor on the host cell. After cell recognition, the virus is endocytosed and transported inside the host cell within an endosome (Fig. S4, ESI†). The acidic environment of the endosome is important for inducing conformational changes in the hemagglutinin that facilitate the membrane fusion process, while opening the M2 ion channel. This opening acidifies the viral core and triggers the release of the viral ribonucleoprotein complex, containing the negative-stranded viral RNA. The complex is transported into the nucleus, where the viral RNA must be converted into a positive strand RNA to serve as a template for the production of viral RNAs. Meanwhile, non-structural viral proteins block the production of host cell mRNA, and viral mRNAs use the host cell's translation system to synthesize viral proteins. Viral proteins are recruited by the viral RNA to form new ribonucleoproteins that are exported out of the nucleus. New viruses assemble at the cell surface and are released by the receptor-cleaving neuraminidase proteins of influenza A and B viruses or the hemagglutinin-esterase-fusion protein of influenza C viruses.[120,121]

2.6.2 Drug resistance in influenza virus. Antiviral drugs are expected to be important for treating epidemic as well as eventual influenza pandemics.[122] Adamantane derivatives (amantadine and rimantadine) were licensed about 50 years ago to treat influenza virus infection.[123] These drugs disrupt the transmembrane domain of the viral M2 protein, a proton channel required for infection.[124] However, their use for treatment or prophylaxis of influenza A is not recommended due to their high levels of resistance caused by the long-term, widespread and/or large-scale use.[125] Fortunately, after the loss of efficacy shown by adamantane derivatives, neuraminidase inhibitors were found to be active against all human influenza viruses.[126,127]

Two neuraminidase inhibitors (oseltamivir and zanamivir) have been globally approved, although laninamivir and peramivir have been licensed by regulatory authorities in some Asian countries.[128,129] Until recently, the prevalence of resistance to approved neuraminidase inhibitors has remained relatively low.[130] However, almost all seasonal H1N1 strains transmitted in 2008–2009 were resistant to oseltamivir.[131] These results indicate that neuraminidase inhibitor resistance should be monitored to provide timely guidance for clinical management and potential drug prevention, particularly in the event of a serious pandemic outbreak.[132]

2.6.3 Therapeutic agents for influenza virus. The development of high-level resistance to adamantane derivatives and the widespread resistance to oseltamivir and zanamivir are a source of concern fueling the development of new antiviral agents with novel mechanisms of action and exploiting alternative viral targets. Recently, baloxavir marboxil has been

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approved as the first inhibitor of the viral acid protein endonuclease.[133–135] In addition, there are drugs such as ribavirin, favipiravir and arbidol hydrochloride (Table 6) considered as broad-spectrum inhibitors of viral replication that have shown inhibitory effects on influenza viruses.[136]

3. Strategies for antiviral drug design against resistant viruses

3.1 Elements of the interaction between drug and target

In clinical practice, antiviral drug combinations (*e.g.* HAART in the case of antiretroviral therapy) are recommended instead of monotherapies in order to avoid the rapid selection of drug-resistant strains.[36] Guidelines applied for HCV eradication also recommend combinations of two or three drugs acting on different targets to combat the infection (Table 5). However, undesired drug interaction and cumulative toxicity in the kidneys and liver are problems associated with the combined use of drugs. Drug resistance is usually developed by the acquisition of mutations that produce amino acid substitutions in the target protein that reduce its binding affinity for the antiviral agent. Different solutions have been proposed depending on the elements considered in the analysis of interactions between drug and target: targeted proteins,[138] drug conformations,[139] binding sites,[140] interaction forces[141] and intracellular mechanisms triggered by protein binding, such as those involving the ubiquitin-proteasome system.[142] Medicinal chemistry strategies to fight antiviral drug resistance and to develop antiviral drugs based on the different elements involved in drug–target binding are widely used in the development of therapeutic agents against several viruses. In this review, we provide examples of each strategy, explaining their rationale and discussing their applications in drug design and development.

3.2 Multi-target drug design strategies

The discovery and development of drugs with multiple targets have opened new possibilities for the treatment of diseases involving multiple genes or molecular targets, such as malignancy of different tissues and organs,[143–145] nervous system diseases (depression,[146–148] Alzheimer's disease,[149] *etc.*), and other pathological conditions.[150] In contrast with the classical combination therapies involving the use of two or more drugs acting on different processes, the goal of multi-target drug design is to integrate in the same molecule of functions and structures directed against two or more targets. Thus, based on structural similarity, the pharmacophores of two or more ligands can be connected, superimposed or fused to obtain a ligand that can act on two or more targets.[151] These multi-target drugs are likely to interact with their target with lower affinity than classical single-target drugs. However, unlike single-target drugs, they may be able to interact with multiple components of a functional complex.[138] Increasing the number of targets can overcome the limitations of drugs aimed at a single site, and improve their therapeutic efficacy, by preventing the emergence of drug resistance and reducing the incidence of undesired adverse effects.[150]

3.2.1 HIV RNase H and polymerase dual-target inhibitors. HIV RT has two domains, the DNA polymerase domain and the RNase H domain with distinct enzyme functions essential for HIV replication.[47,48] The FDA-approved NRTIs and NNRTIs are both DNA polymerase inhibitors. RNase H inhibitors have not yet been developed into therapeutic agents, while the RNase H domain remains an unexploited target of antiviral intervention.[152,153] The comparison and analysis of the structures of available HIV RNase H inhibitors[154–157] (Fig. 4) showed that these compounds have chelating groups (in red) for competitively binding to the divalent metal in the enzyme's active site, as well as hydrophobic aromatic groups (in green) for potent and selective RNase H inhibitory activity. Vernekar *et al.*[157] used a molecular hybridization strategy to design a series of hydroxyisoquinoline-1,3-dione derivatives, which are dually active against the RT RNase H and DNA polymerase at submicromolar to low micromolar concentrations. Moreover, this new skeleton also maintained significant dual inhibitory activity against mutant strains containing amino acid substitutions Y181C and L100I/K103N

Table 6 Influenza therapeutics approved for clinical use and representative candidates in preclinical and advanced clinical trials[137]

Category	Mechanism of action	Drug	Target virus	Status
Specific influenza virus inhibitors	Neuraminidase inhibitors	Oseltamivir Zanamivir Peramivir Laninamivir	Influenza virus A and B	Approved Approved Approved Phase III
	Hemagglutinin-mediated fusion inhibitor	JNJ4796	Influenza virus A	Preclinical
	M2 ion channel inhibitors	Amantadine Rimantadine	Influenza virus A	Both discontinued due to high levels of resistance and undesired toxic effects
	Acidic protein (PA) endonuclease inhibitor	Baloxavir marboxil	Influenza virus A and B	Approved in the U.S.
	Basic protein 2 inhibitor	JNJ-63623872 (VX787)	Influenza virus A	Phase II
Broad-spectrum antiviral agents	Fusion inhibitor	Arbidol hydrochloride	Influenza virus A and B	Approved in China and Russia
	RNA-dependent RNA polymerase inhibitor	Favipiravir	Influenza virus A and B	Approved in Japan
	Unknown target	Nitazoxanide	Influenza virus A and B	Phase III

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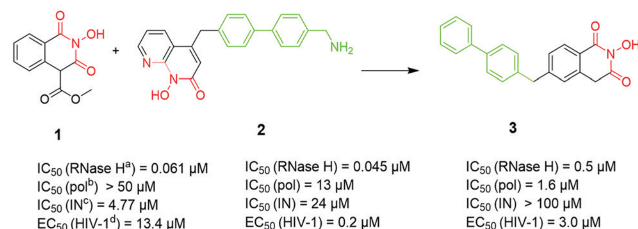


Inhibitory effect of compound **3** against RNase H and DNA polymerase activities of NNRTI-resistant HIV-1 RT mutants

Compound	Y181C mutant IC_{50} (μM)			L100I/K103N mutant IC_{50} (μM)		
	RNase H HTS-1[e]	RNase H HTS-2[f]	pol	RNase H HTS-1[e]	RNase H HTS-2[f]	pol
3	0.9	1.1	0.7	0.7	1.0	0.5

Fig. 4 Structures and activities of compounds **1–3** and design of dual-target inhibitors of HIV-1 RT's RNase H and DNA polymerase. Enzyme inhibition data and efficacy against viruses were taken from ref. 156 and 157. [a] HIV-1 RT RNase H; [b] HIV-1 DNA polymerase; [c] HIV-1 integrase; [d] antiviral efficacy against HIV-1; [e] internal cleavage activity of HIV RT RNase H; [f] DNA 3′ end directed cleavage activity of HIV RT RNase H.

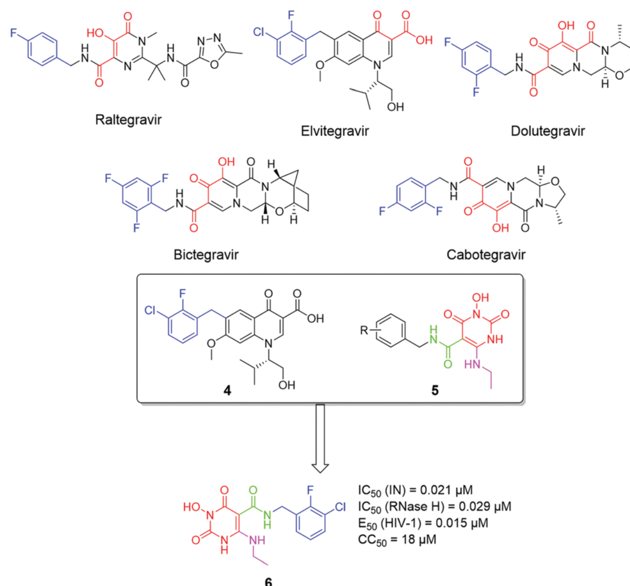
(Fig. 4). Although their inhibition mechanism is not clear, molecular docking analysis confirmed that compound **3** could bind to the RNase H active site.

3.2.2 HIV RNase H and integrase dual-target inhibitors. The HIV integrase is a viral enzyme that catalyses the insertion of the proviral DNA into the host cell genome. The viral integrase is absent from human cells, while being necessary for HIV replication.[49,158] Therefore, integrase inhibitors are expected to be valuable drugs with strong viral specificity. Currently, there are four approved integrase inhibitors: raltegravir, elvitegravir, dolutegravir and bictegravir, and a fifth one (cabotegravir) is in advanced clinical trials (Fig. 5).[159] These drugs contain two domains with different structural characteristics: a diketo acid or its bioelectronic isostere (in red), and a hydrophobic terminal benzyl moiety (in blue), and are structurally similar to RNase H inhibitors (Fig. 5).

Wu *et al.*[160] integrated the pharmacophores of integrase and RNase H inhibitors to obtain a dual target inhibitor with a 3-hydroxytoluene-2,4-dione-5-*N*-benzene skeleton. A similar *N*-hydroxypyrimidinone skeleton was designed by combining the chelating group of compound **5** (in red) and the hydroxypyrimidone moiety found in raltegravir. The unique formamide was introduced at the C5 position to improve integrase inhibition, while an alkylamino group was added at C6 to confer low nanomolar inhibitory activity. Compound **6** showed dual inhibitory activity against wild-type (WT) RNase H and integrase, but in addition, it was also a potent inhibitor of integrase variants containing mutations associated with resistance to approved inhibitors, such as Y143C and N155H, as well as the double and triple mutants G140S/Q148H and G140S/Y143H/Q148H.

3.3 Conformation-based drug design

3.3.1 Conformation variability and drug positioning in the binding pocket. NNRTIs are often included in combination therapies against HIV-1.[161,162] Six NNRTIs have been approved



Resistance profile of **6** against raltegravir-resistant HIV-1 clones.

HIV-1 clone with major mutation	Fold resistance[a]			
	6	AZT[b]	RPV[c]	DLV[d]
Y143C	0.4	1.4	170	2.0
N155H	2.4	0.7	14	1.8
G140S/Y143H/Q148H	6.3	0.9	220	9.6
G140S/Q148H	13	0.5	145	12

Fig. 5 Chemical structures of approved HIV-1 integrase inhibitors, and compounds **4–6**, designed to obtain multi-target drugs. The table in the bottom panel shows the relative antiviral activity of compound **6** and representative antiretroviral drugs against a panel of resistant HIV isolates with mutations associated with resistance to integrase inhibitors. Data taken from ref. 160. [a] Fold-resistance is defined as EC_{50} (mutant)/EC_{50} (WT); [b] AZT, 3′-azido-3′-deoxythymidine (zidovudine); [c] RPV, rilpivirine; [d] DLV, delavirdine.

by the FDA (shown in Fig. 6). Nevirapine and delavirdine are considered as first generation NNRTIs. Single amino acid substitutions affecting six or seven residues of the NNRTI binding pocket are known to confer high-level resistance to these drugs, with Y181C being the most frequently found in the clinical setting.[63]

Efavirenz is also an inhibitor with a low genetic barrier, but is effective against mutant viruses containing Cys at position 181. However, resistance to efavirenz is usually conferred by the mutation K103N, which is also a commonly transmitted mutation in countries where efavirenz has been extensively used for treating HIV infections.[12,13] Second-generation NNRTIs are represented by etravirine and rilpivirine, and show efficacy against many resistant HIV-1 strains. These two NNRTIs are considered as the most efficient in current antiretroviral therapies, and show a relatively high genetic resistance barrier although mutations at position 138 decrease their efficacy.[163] In addition to the NNRTIs mentioned above, doravirine has been recently approved for use in combination therapies. Although doravirine has a low genetic barrier, it retains activity against the most frequently transmitted NNRTI mutations, K103N, E138K, Y181C and G190A.[164] The structures of the six approved NNRTIs and

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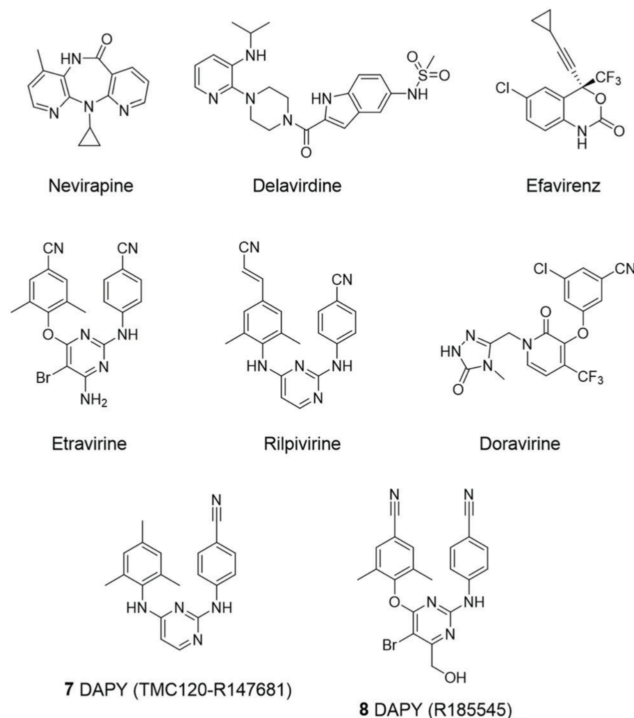

Resistance profile of nevirapine, delaviridine, efavirenz, etravirine and compounds 7 and 8 against HIV-1 mutant strains associated with NNRTI resistance.

	WT	L100I	K103N	V106A	Y181C	Y188L	G190A
Nevirapine	0.085	0.638	2.467	2.41	5.351	> 100	3.465
Delaviridine	0.016	3.467	1.697	2.245	1.336	0.178	0.038
Efavirenz	0.001	0.038	0.039	0.36	0.002	0.138	0.011
TMC120-R147681 (7)	0.001	0.016	0.004	0.003	0.008	0.04	0.001
Etravirine	0.002	0.003	0.001	0.002	0.006	0.003	0.001
R185545 (8)	0.004	0.002	0.004	0.002	0.006	0.006	0.001

Fig. 6 Chemical structures and resistance profiles of approved NNRTIs and DAPY analogues **7** and **8**. The bottom panel shows resistance profiles (EC_{50}s in μM) of HIV-1 mutant strains containing specific amino acid changes in the NNRTI binding pocket. The colours represent different potencies: red, $EC_{50} > 0.1$ μM; blue, $0.1 > EC_{50} > 0.01$ μM; and white, $EC_{50} < 0.01$ μM. Data were taken from ref. 165.

their efficacy against clinically relevant HIV strains are shown in Fig. 6.

Structural studies[165] have found that the reason why etravirine, rilpivirine and their derivatives (compounds 7 and 8 in Fig. 6) are effective inhibitors of resistant strains of HIV-1 could be related to the fact that these inhibitors are able to bind the RT in multiple conformations, avoiding the effect of amino acid substitutions occurring at the NNRTI binding site. These studies have shown that diarylpyrimidine (DAPY) analogues can adapt to changes in the NNRTI binding pocket in several ways: (i) DAPY analogues can bind in at least two conformationally different modes; (ii) for a given binding mode, the torsional elasticity ("swing") of the DAPY analogues allows for many conformational variants; and (iii) the

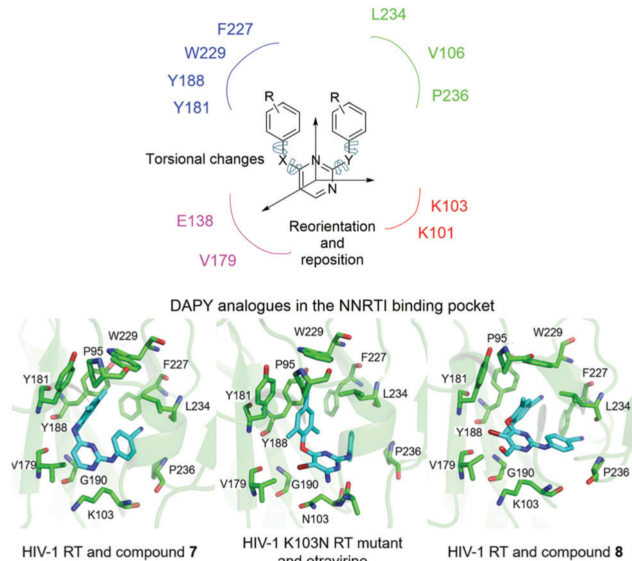

Fig. 7 Torsional changes in the NNRTI binding pocket. Flexible inhibitors like DAPY analogues with flexible chemical bonds, which allow torsional changes, can avoid mutation resistance by reorientation and repositioning in the binding pocket. The bottom panel shows the HIV-1 NNRTI binding pocket and relevant interactions with different inhibitors. Images were obtained using Pymol software (www.pymol.org), and the crystal structures of HIV-1 RT/TMC120-R147681 (**7**) (PDB code 1S6Q), K103N mutant RT/TMC125-R165335 (etravirine) (PDB code 1S5V) and HIV-1 RT/R185545 (**8**) (PDB code 1SUQ).

compact design of the DAPY analogues allow significant repositioning and reorientation (translation and rotation-like shaking) within the pocket (Fig. 7). These proposals are supported by the crystal structures of HIV-1 RT bound to DAPY compounds (etravirine, **7** and **8**), as shown in Fig. 7.

The adaptability of DAPYs to bind the HIV-1 NNRTI binding pocket seems to be crucial for their efficacy against the WT RT and enzymes containing drug resistance mutations. Elements favouring the conformational flexibility of these inhibitors (such as the torsional flexibility of strategically-positioned chemical bonds) can be helpful for designing drugs effective against rapidly mutating targets.

3.3.2 Conformation conservation through circularization strategies. An important area of anti-HCV research has been focused on the discovery of pan-genotypic HCV-NS3/4A protease inhibitors. Among efforts to achieve this goal, Neelamkavil *et al.*[166] optimized a P2 quinoline moiety by introducing a spirocyclic-proline based on the previously discovered compound MK-5172 (**9**) shown in Fig. 8. The spirocyclization of the quinoline moiety is expected to improve van der Waals contacts with amino acid residue His57 in an unmodified catalytic region, while the structural rigidity and conformational constraint favour a conformation with biological activity. In addition, the favourable bioactive conformation may reduce the entropy cost of binding, while increasing the efficacy of the molecule against WT and mutant strains. As a result, compound **10** is 80 times more potent than compound **9** against HCV genotypes 1b and 3a. Furthermore, compound **10** is also more effective against selected mutant strains both in enzyme inhibition and antiviral efficacy assays (Fig. 8).

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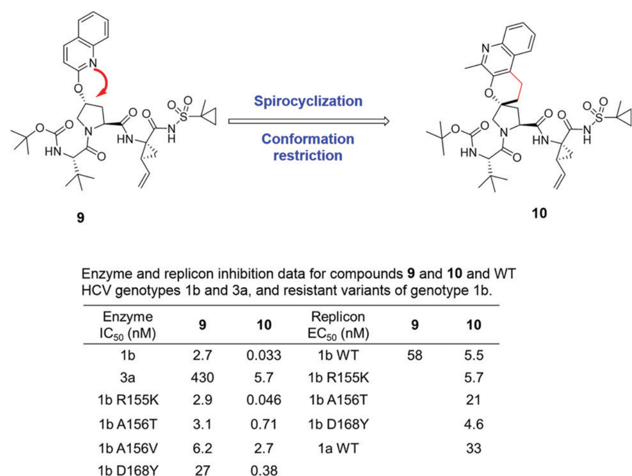

Enzyme and replicon inhibition data for compounds **9** and **10** and WT HCV genotypes 1b and 3a, and resistant variants of genotype 1b.

Enzyme IC_{50} (nM)	9	10	Replicon EC_{50} (nM)	9	10
1b	2.7	0.033	1b WT	58	5.5
3a	430	5.7	1b R155K		5.7
1b R155K	2.9	0.046	1b A156T		21
1b A156T	3.1	0.71	1b D168Y		4.6
1b A156V	6.2	2.7	1a WT		33
1b D168Y	27	0.38			

Fig. 8 Optimization of compound **9** based on a circularization strategy that maintains the conformation of the molecule. The inhibitory effects and antiviral efficacies of compounds **9** and **10** against selected HCV strains are shown at the bottom. Data were taken from ref. 166.

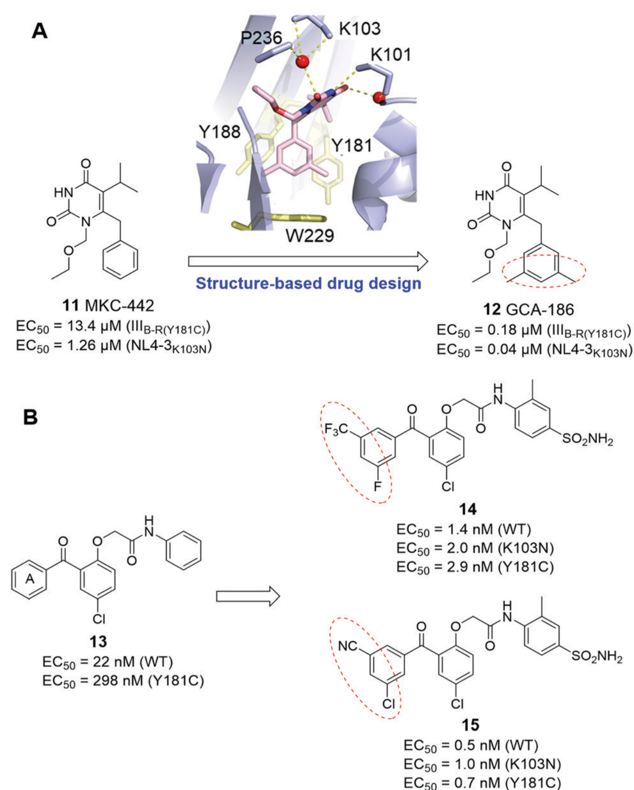

Fig. 9 Optimization of compounds **11** (A) and **13** (B) targeting the conserved Trp229 residue in HIV-1 RT. The image in panel (A) showing compound **12** bound to the HIV-1 NNRTI binding pocket was obtained using Pymol (PDB code 1C1B). EC_{50} values shown in the figure were obtained from ref. 167 and 168.

3.4 Drug design strategy based on multiple binding sites

3.4.1 Drug design strategy targeting conserved regions. Y181C, a common NNRTI resistance mutation found in HIV-1 RT, reduces viral susceptibility to the NNRTI MKC-442 (emivirine) (**11**).[167] The interaction between the aromatic group of compound **11** and the surrounding residues of RT (Tyr181, Tyr188, Phe227 and Trp229) is absolutely required for binding (Fig. 9a). Moreover, the crystal structures of various NNRTIs bound to HIV-1 RT have shown that these conserved aromatic groups are important for binding. In order to reduce the effect of the Y181C mutation in drug binding, the volume of GCA-186 (**12**) relative to compound **11** was increased by introducing two methyl groups in the aryl moiety of **11**. An increased interaction with the surrounding highly conserved Trp229 was expected, while reducing the role of Tyr181 in binding stabilization.

Molecular simulations show that the methyl groups at the 3′ and 5′ positions of the 6-benzyl group of **11** can be comfortably located in the deep hydrophobic region above Trp229. Experimental data showed that after the introduction of *m*-dimethyl groups, the inhibitory effect of the obtained compounds on the Y181C mutant was significantly improved. The susceptibility of the Y181C RT to compound **11** was reduced 3000 times, but the activity of **12** against the Y181C mutant was reduced only 180 times. In addition, the K103N mutation increased resistance to compound **11** by more than 1000-fold, while **12** showed only a 40-fold reduction in inhibitory activity (EC_{50} = 40 nM).[167]

Other NNRTIs with a benzophenone skeleton share the same binding pocket and a similar binding mode than compound **11**. In order to reduce the effect of the Y181C substitution on the molecule backbone binding to the target protein, Romines *et al.*[168] followed a similar approach by introducing a polar aprotic group (*e.g.* a halogen atom) and a small bulk substituent (*e.g.* a cyano group) in the A ring of compound **13** (Fig. 9b). This small *meta*-substituent was expected to enter the hydrophobic cavity adjacent to the Tyr181 and Tyr188 side chains, increasing the interaction with the conserved region of HIV-1 RT. Interactions with the adjacent Tyr188 will then reduce the dependence on Tyr181 substitutions in the same way as previously discussed for compounds **11** and **12**. Modification of the A loop of compound **13** increased the antiviral activity against both WT and mutant strains (*e.g.* K103N), and particularly against mutant strains containing Y181C. Thus, compounds **14** and **15** were 100 and 400 times more potent than compound **13**, respectively.

3.4.2 Targeting new binding sites. Binding pockets are usually defined by key interacting residues, but in addition, they contain alternative binding sites that can be eventually exploited in drug design. These additional sites might be helpful to design new compounds that would eventually overcome resistance.[169] Such a strategy requires precise structural biology research. When designing the drug structure, it is necessary to understand the specific shape of the binding pocket, the distribution of residues, and the precise location of the amino acid conferring drug resistance. Taking the binding mode of the lead compound as a reference, subsequent analysis should explore all potential interactions between the molecule and any other potential binding sites relevant for drug resistance.

With the development of structural biology, crystal structures of HIV-1 RT complexed with many NNRTIs have been

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resolved. Structural similarities between ligands and their modes of interaction with the target protein show that DAPY analogues contained four pharmacophores (Fig. 10b).[170] The left-wing aryl group is a hydrophobic moiety that interacts with the surrounding benzene rings of Tyr188 and Trp229. The right-wing aryl group locates at a solvent–protein interface known as tolerant region 1. The N atom of the core pyrimidine, together with the right-wing N atom, establishes hydrogen bonds with the surrounding amino acids Lys101 and Lys103. A narrow cleft between the side chains of Glu138 and Val179, and the left side of the pyrimidine ring defines what is known as the tolerant region 2. The optimization of DAPY analogues according to the four-point pharmacophore model was mainly concentrated on the two tolerant regions, aimed to generate additional interaction forces with new binding sites.

New DAPY compounds have been designed to exploit the new binding site corresponding to the tolerant region 2. Huo *et al.*[175] connected an N-morpholine ring to the pyrimidine ring of etravirine through a long amide chain, fitting the narrow shape of the tolerant region 2 (Fig. 10a). The obtained compound **16** showed improved activity against WT HIV-1, and six strains carrying frequent NNRTI resistance mutations.

The exploration of tolerant region 2 as a target for improving the efficacy of etravirine and other DAPY derivatives rendered molecules that inhibited the replication of NNRTI-resistant HIV-1 strains. These results showed that targeting new binding sites within the same binding pocket was a valid strategy to overcome drug resistance. Further research on new binding sites in the NNRTI binding pocket was focused on tolerant region 1. A variety of substituted aryl hetero-N-cyclic amines were introduced in the cyanobenzyl moiety of etravirine to improve interactions in tolerant region 1 (Fig. 10b).

This approach led to the discovery of several excellent molecules, including compounds **17–21**.[170–174] Crystallographic studies of these new compounds showed that tolerant area 1 involves interactions with a hairpin loop extending from Pro225 to Pro236, before reaching the new binding site. Several compounds obtained through this approach showed favourable and drug-like pharmacokinetic properties and water solubility, and were generally active against NNRTI-resistant HIV-1 strains[176] (Fig. 10b).

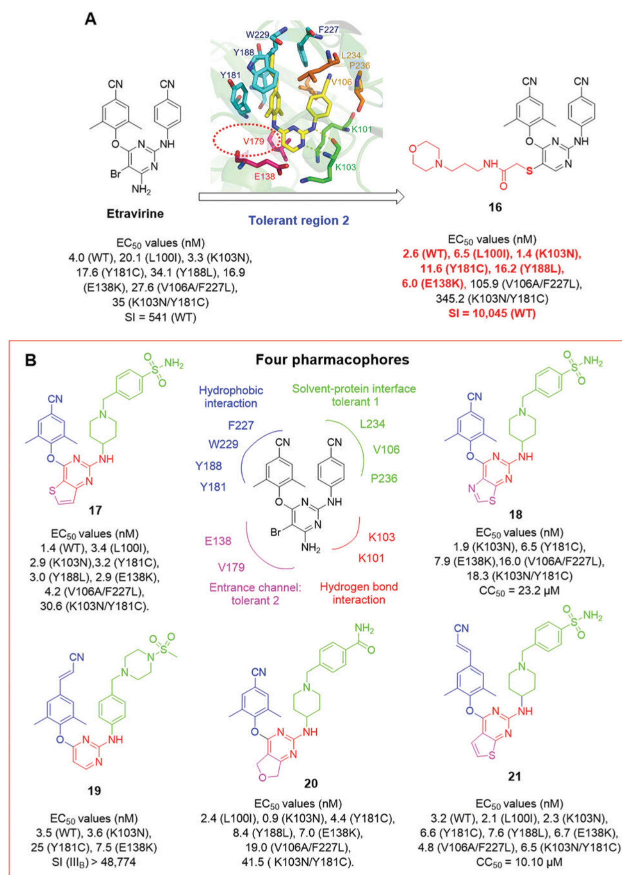

Fig. 10 Drug design strategy exploiting new interactions in a defined binding pocket. (A) Design and antiviral activities of compound **16**, obtained after optimization of etravirine binding to HIV-1 RT (PDB code 4KFB), by targeting tolerant region 2 adjacent to its binding site. Improved potencies against WT and resistant variants are highlighted in red. The antiviral efficacies against HIV-1-resistant variants of etravirine and compound **16** were taken from ref. 165. (B) Key pharmacophores and major binding interactions defined for a DAPY analogue complexed with HIV-1 RT (central) including a hydrophobic interaction region in blue, a solvent–protein interface tolerant region 1 in green, the entrance channel tolerant region 2 in pink, and a hydrogen bond region in red. The structures of compounds **17–21** in adjacent panels represent alternatives aimed at discovering novel interactions in the NNRTI binding pocket. Shown EC_{50} values were taken from recently published ref. 170–174.

Influenza virus neuraminidase is an important surface antigen glycoprotein, and an attractive target for prevention and treatment of seasonal and pandemic flu.[177] Nine crystal structures of influenza A virus neuraminidase have been disclosed. A look at the structures reveals that these enzymes can be crystallized in two major conformations that differ in the positioning of the flexible 150-loop. This loop contains residues 147–152 and can be in an open or close conformation. The open 150-loop forms a larger cavity adjacent to the binding site of oseltamivir. Modifying oseltamivir to increase the affinity for the 150-cavity can make the inhibitor effective against mutant neuraminidase bearing the oseltamivir resistance mutation H274Y.

The 150-cavity allows the interaction with some high-affinity molecules mainly through hydrophobic contacts. The introduction of hydrophobic groups into oseltamivir at the C5 position provides additional interactions with the hydrophobic residues surrounding the 150-cavity (Fig. 11). Compounds **22**[178] and **23**[179] obtained by using this strategy maintained the same inhibitory activity as oseltamivir against the most common influenza virus subtypes, and were highly active against oseltamivir-resistant strains. Compound **23** was 50 times more potent than oseltamivir in assays carried out with the H5N1 strain carrying the oseltamivir resistance mutation H274Y (IC_{50} values of 1630 nM and 27.9 nM for oseltamivir and compound **23**, respectively).

3.4.3 "Substrate envelope" hypothesis. The definition of the "substrate envelope" hypothesis[52] refers to an inhibitor with a shape similar to the substrate. The van der Waals surfaces of these inhibitors do not protrude from the substrate

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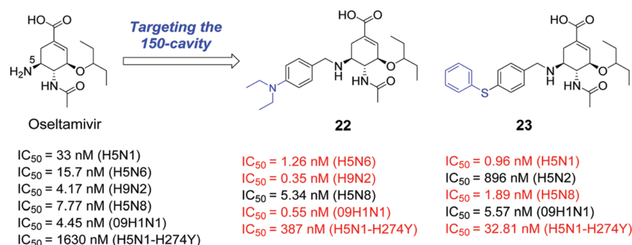


Fig. 11 Chemical structures and antiviral activities of oseltamivir and its derivative compounds **22** and **23**. Influenza virus strains are indicated between parentheses. Values in red indicate those cases where compounds **22** and **23** showed increased potency in comparison with oseltamivir. Data taken from ref. 178 and 179, respectively.

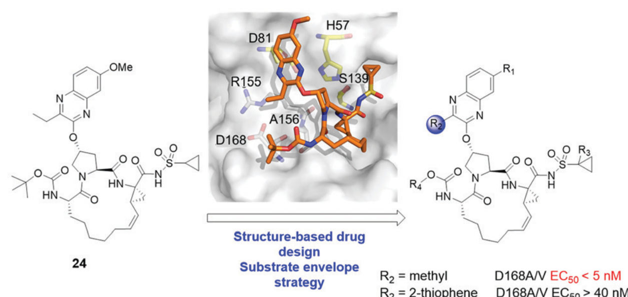


Fig. 12 Chemical structures and optimization of derivatives of compound **24** based on a substrate envelope strategy, based on the binding mode of compound **24** and HCV WT NS3/4A protease (PDB code 5EPN).

envelope, and their binding is rarely affected by mutations in the target protein.[180] The rationale for the substrate-envelope constraint is that it prevents designed inhibitors from making interactions beyond those required by substrates and thus limits the availability of mutations tolerated by substrates but not by designed inhibitors.[181]

Drug design strategies based on the substrate envelope hypothesis have been applied to HCV NS3/4A protease inhibitors in an effort to overcome resistance. All HCV NS3/4A protease inhibitors are known to lose potency as a result of emergence of single-amino acid substitutions in the viral protease, especially at Arg155, Ala156, and Asp168.[182,183] Among them, D168A and D168V are known to be the most frequent amino acid substitutions in patients failing therapy with these inhibitors. High-resolution crystal structures of protease inhibitors bound to WT and mutant HCV NS3/4A proteases[184] showed that the large heterocyclic P2 portion of the protease inhibitor falls outside of the substrate-binding region, that is, the substrate envelope.[185] In addition, structures showed extensive interactions with residues Arg155, Ala156, and Asp168.[186] Resistance to HCV NS3/4A protease inhibitors was explained by the disruption of the electrostatic interaction between Arg155 and Asp168 due to amino acid substitutions at any of these positions.

Matthew *et al.*[187] used a substrate envelope model to design and synthesize a series of analogues with different substitutions at P2 quinoxaline moieties based on lead compound **24** (Fig. 12). The P2 quinoxaline derivative with a small hydrophobic substituent at the 3-position exhibits good activity against drug-resistant strains, with EC_{50} values of less than 5 nM against mutants D168A and D168V. On the other hand, larger substituents at the 3-position (such as thiophene ring) did not show promising activity against resistant strains with EC_{50} values above 40 nM.

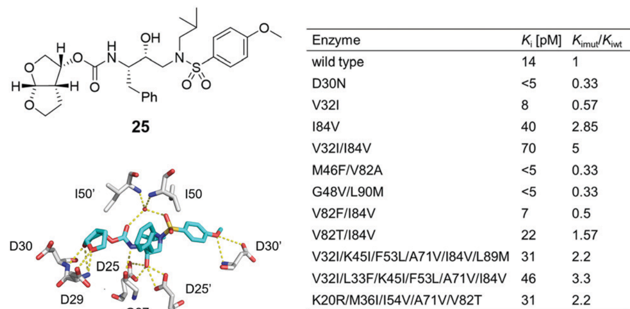
3.5 Interaction forces

3.5.1 Drug design strategies based on multiple hydrogen bonds or robust hydrogen bonds with a high degree of covalency.

Amino acid substitutions conferring resistance are not expected to alter the overall structure of the active site in a significant manner, since its precise geometry is required to maintain its function. Mutant enzymes containing resistance-associated amino acid substitutions show minimal distortion in the structure of their active site to maintain catalytic activity, and therefore, viral replication capacity. In this basically unchanged skeleton of the protein, increasing enzyme–drug interaction forces as much as possible is important to maintain affinity and overcome resistance caused by amino acid substitutions in the ligand binding site.[188]

Structural analysis of various mutant and WT HIV-1 proteases revealed little changes in the backbone architecture of the mutant protease's active site.[189,190] Therefore, Ghosh *et al.*[188] focused on promoting extensive hydrogen bonding interactions between inhibitors and target protein backbone atoms (Fig. 13). Compound **25** was designed to increase hydrogen bonding with surrounding amino acids, both by increasing their number and coverage within the inhibitor binding site.[191] The obtained molecule would work as a "molecular crab" that firmly holds the protein backbone. Due to this enhanced reticulated hydrogen bonding, compound **25** has excellent inhibitory activity against a wide range of HIV-1 resistant proteases, with IC_{50} values in the low picomolar range.

Boric and boronic acids are frequently used as functional groups in the formation of covalent complexes. They produce a reversibly covalent interaction with nucleophiles in the target



Enzyme	K_i [pM]	K_{imut}/K_{iwt}
wild type	14	1
D30N	<5	0.33
V32I	8	0.57
I84V	40	2.85
V32I/I84V	70	5
M46F/V82A	<5	0.33
G48V/L90M	<5	0.33
V82F/I84V	7	0.5
V82T/I84V	22	1.57
V32I/K45I/F53L/A71V/I84V/L89M	31	2.2
V32I/L33F/K45I/F53L/A71V/I84V	46	3.3
K20R/M36I/I54V/A71V/V82T	31	2.2

Fig. 13 Chemical structure, crystallographic conformation and resistance profile of compound **25**. The right panel shows inhibitory constants (K_i) for WT HIV-1 protease and a series of mutant enzymes containing amino acid substitutions associated with drug resistance. Data taken from ref. 188. The image showing the conformation of **25** within the HIV-1 protease binding pocket has been obtained using Pymol and PDB file 3I7E.

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protein.[192] Although it is rarely used as a non-covalent recognition reagent, the two hydroxyl groups contained in the boric acid moiety can act as hydrogen bond donors and acceptors. These hydroxyl groups have four lone pairs and two hydrogen bond donors, thus providing six opportunities to form hydrogen bonds. These characteristics are particularly valuable when designing protein ligands effective in counteracting drug-resistant selection pressures. The combination of hydrogen bond donors and acceptors in a boric acid group as well as their covalency facilitate the formation of multiple hydrogen bonds that can be relatively strong. Multiple hydrogen bonds enhance the affinity for the ligand (or inhibitor) and facilitate binding in the presence of drug resistance mutations.

Windsor *et al.*[193] included boric acid groups to generate hydrogen bonds with a high degree of covalency in order to generate HIV-1 protease inhibitors with high affinity for the enzyme. Replacing the aniline moiety of darunavir (K_i = 10 pM) (Fig. 14) with phenylboronic acid (compound **29**) resulted in a remarkable 20-fold increase in affinity for WT proteases (K_i = 0.5 pM) and 25-fold increase for the D30N mutant protease (K_i = 0.4 pM), while the relative affinity of compounds **25–28** varied less than two-fold in comparison with darunavir.[193] In addition, the crystallographic analysis showed that the boronic acid group of compound **29** facilitates the formation of three hydrogen bonds, more than the amino group of darunavir or any other analogues (as shown in Fig. 14).[193–197] The hydrogen bond between boric acid and the carbonyl group of Asp30 in WT HIV-1 protease (or Asn30 in the mutant enzyme) is very small ($r_{O\cdots O}$ = 2.2 Å). Natural bonding orbital (NBO) analysis revealed that the interaction energy between boric acid and WT protease was 69.8 kcal mol^{-1}. Such a large interaction energy and short hydrogen bond indicate a high degree of covalency in the BOH*OC hydrogen bond. Moreover, boric acid uses its robust hydrogen bonding potential to maintain the affinity for drug-resistant HIV-1 proteases, such as the D30N mutant.

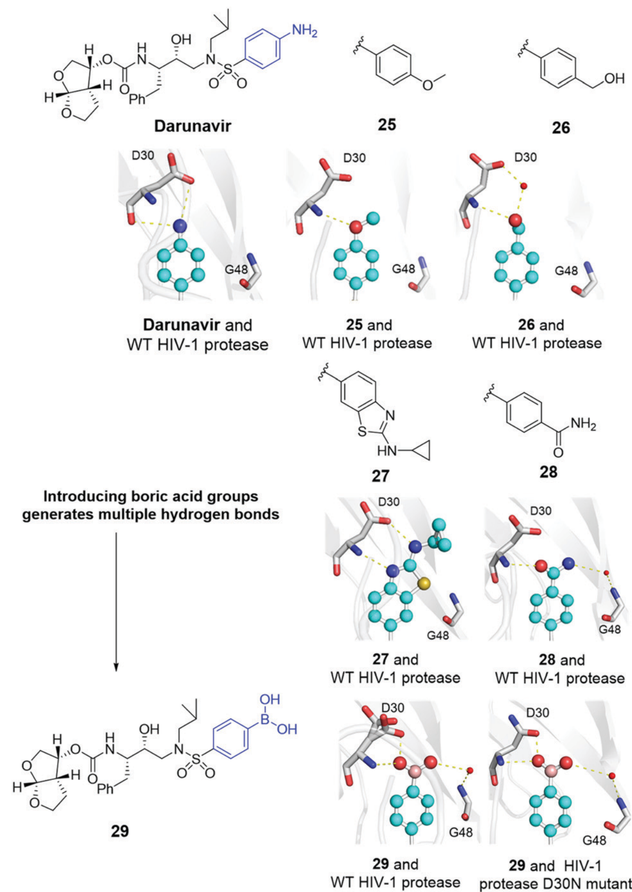

Fig. 14 Structures and conformations of darunavir and compounds **25–29** in the HIV-1 protease substrate binding site. Partial views of the HIV-1 WT protease substrate binding site, containing darunavir (PDB code 4HLA), and compounds **25** (PDB code 2I4U), **26** (PDB code 3O9G), **27** (PDB code 5TYR), **28** (PDB code 4I8Z), and **29** (PDB code 6C8X), as well as HIV-1 mutant D30N protease bound to compound **29** (PDB code 6C8Y). Images have been obtained using Pymol. The yellow dashed lines represent hydrogen bonds, and the red spheres represent water molecules. The aniline moiety in the darunavir chemical structure is shown in blue.

3.5.2 Drug design strategies based on covalent binding. Covalent inhibitors usually recognize first the target protein through non-covalent and reversible interactions, and then form an irreversible covalent bond. After the covalent bond is generated, the drug can maintain its inhibitory activity indefinitely until the target disappears. Therefore, covalent inhibitors can theoretically overcome resistance, taking advantage of reduced drug exposure and prolonged action.

Chan *et al.*[198] designed a covalent inhibitor targeting HIV-1 RT Cys181. The crystal structure of lead compound **30** bound to WT HIV-1 RT showed that the chlorine atom of compound **30** faces Tyr181 and is close to the amino acid side chain.[199] Therefore, by substituting the chlorine atom in the lead compound by a small active group (acrylamide), researchers were able to specifically target Cys181.

After target recognition, the obtained derivative binds non-covalently and reversibly, but then the electrophilic warhead attacks the side chain of Cys181, undergoing a Michael addition reaction that forms the covalent bond. This bond could be verified by crystallography analysis (Fig. 15). Compared to non-covalent inhibitors, the covalent modification of Cys181 not only reduces but also inhibits in a complete and permanent manner the activity of HIV-1 mutants containing Cys181, including single and double mutants, such as, for example, K103N/Y181C. Virus susceptibility assays carried out in cell cultures showed that RT activity was completely eliminated after three days, as expected from the inactivation of the virus in the presence of the covalent agent.[198]

3.5.3 Halogen bonds in drug discovery. A halogen bond results from the attraction between the electrophilic region of the halogen atom and the nucleophilic region on a halogen bond acceptor. These bonds are widely present in drug molecules containing halogen atoms and amino acid residues containing O, N, and S atoms. Drug–target halogen bond interaction is common and can be used in drug design.[200,201]

In order to find effective drugs against HIV-1 drug-resistant strains, Bollini *et al.*[202] searched for consistent high-scoring hits in docking studies involving multiple RT structures,

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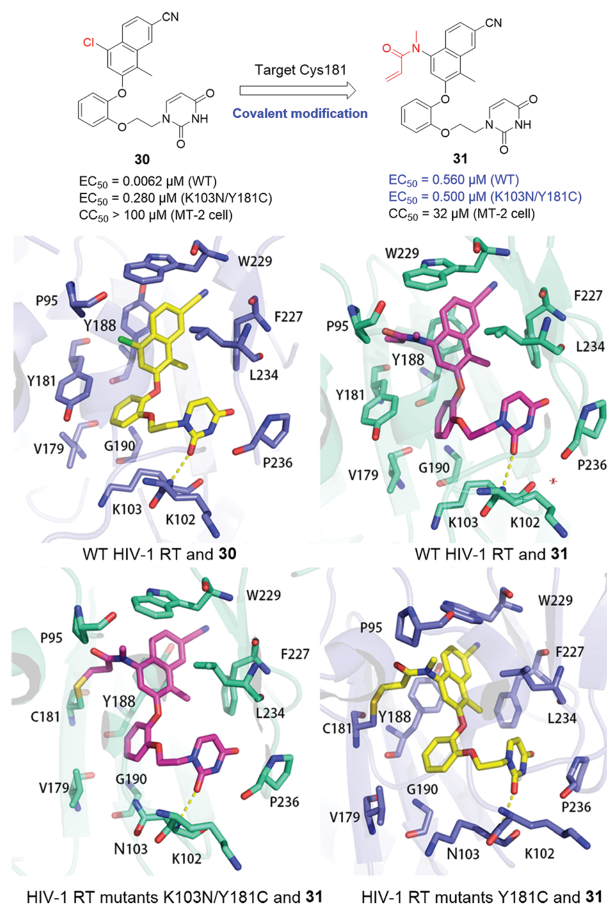

Fig. 15 Covalent modification of compound **30** and crystal structures of complexes of WT HIV-1 RT bound to compounds **30** (PDB code 5TER) and **31** (PDB code 5VQR), and HIV-1 RT mutants K103N/Y181C and Y181C bound to compound **31** (PDB codes 5VQY and 5VQV, respectively). Antiviral potencies of compounds **30** and **31** against HIV-1 WT and mutant strains were taken from ref. 198.

including WT and mutant enzyme variants containing the Y181C substitution (Fig. 16). Two million compounds of the ZINC library were docked with crystal structures of WT and mutant Y181C HIV-1 RTs, as well as WT RTs presenting different conformations of the side chain of Tyr181. Nine compounds were selected. Three of them showed inhibitory activity against one or more HIV-1 strains with EC_{50} values in the range of 5-12 μM.[202] Then, compound **32,** which exhibited the strongest inhibitory activity, was subjected to computationally guided optimization to obtain compound **33**. This antiviral molecule showed picomolar activity and efficacy against NNRTI-resistant HIV-1 strains.[203]

Based on the structural and biological basis of the potent activity of compound **33**, it was found that the Cl atom (Fig. 16) of compound **33** facilitated the formation of a halogen bond with the carbonyl group of Pro95. Pro95 and Trp229 are highly conserved in HIV RTs, and therefore, this additional interaction might be important to counteract drug resistance.[202]

3.5.4 van der Waals forces in drug design. Islatravir (4′-ethynyl-2-fluoro-2′-deoxyadenosine, EFdA or MK-8591)

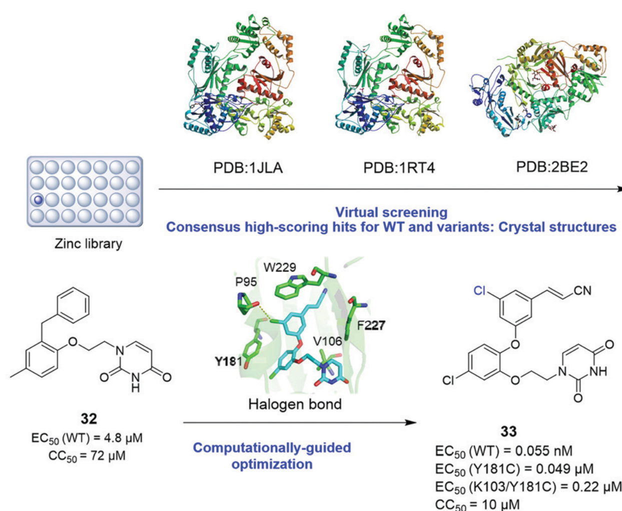

Fig. 16 Discovery and optimization of compound **33**. The crystal structure in the bottom panel shows compound **33** bound to the HIV-1 NNRTI binding pocket. The halogen bond between Cl atom (in blue) of compound **33** and Pro95 is represented with a dashed line. The image has been obtained using Pymol (PDB file 4H4M). The antiviral potencies of compounds **32** and **33** were taken from ref. 200 and 202.

(**34**)[204] and its derivatives with modified 4′-groups are potentially promising long-acting HIV-1 NRTIs, which can inhibit some NRTI-resistant HIV-1 strains (Fig. 17). Antiviral activity assays and structural analysis revealed that key structural features for effective inhibition of the replication of drug-resistant HIV-1 strains reside in the 4′-ethynyl and 4′-cyano moieties of these molecules. These groups can establish strong interactions in the nucleotide binding pocket of HIV-1 RT, even in the presence of multiple amino acid substitutions associated with resistance. In contrast, 4′-methyl groups have no significant impact on the inhibitory activity against drug-resistant strains.

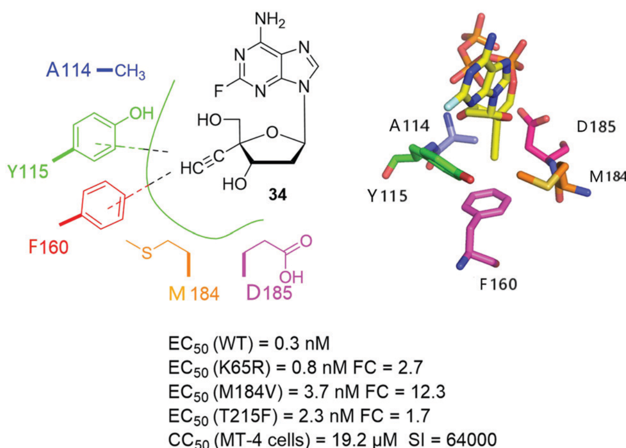

Fig. 17 Binding mode and interaction between islatravir (compound **34**) and HIV-1 RT. The EC_{50} values were taken from ref. 204. FC represents fold-change in resistance, and is defined as EC_{50} (mutant)/EC_{50} (WT). CC_{50}, 50% cytotoxic concentration. SI, selective index.

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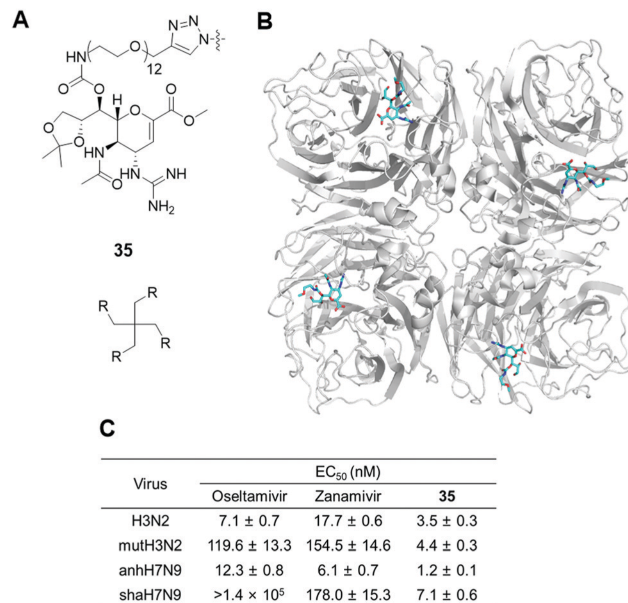
Structural analysis revealed that EFdA and 4′-ethynyl-NRTIs could establish strong van der Waals interactions with key residues of the RT, in contrast with other NRTIs. These interactions were still maintained in mutant RTs containing the amino acid substitution M184V. Islatravir triphosphate was effectively incorporated into DNA by the HIV-1 RT, with an efficiency comparable to those of natural dNTPs. Islatravir monophosphate interacts strongly with HIV-1 RT nucleotide binding residues Ala114, Tyr115, Phe160, Met184 and Asp185. However, once incorporated it blocks the translocation process required for DNA synthesis.

3.5.5 Multivalent drug design strategies. Multivalent interactions involve the simultaneous binding of multiple ligands on one biological entity. For example, multivalent ligands can bind to one or more receptors increasing its efficacy by exhibiting additional properties, not shown by monovalent interactions (*e.g.* receptor clustering). More specifically, multivalent interactions exhibit higher affinity, strength, stability and binding specificity than the corresponding monovalent interactions.[205,206]

For target proteins with multiple binding sites, the development of multivalent drugs may have more unique advantages than monomeric inhibitors, such as improved binding between multiple ligands and receptors, and extended residence time at the interacting site.[207] Although the mutation of the target may reduce the affinity between the monomer drug molecule and the target protein, in general, the drug molecule can still maintain the ability to occupy the binding site. A multivalent drug may compensate for the affinity decrease through multiple binding. Multivalent drug design strategies are generally used for targets and drugs that meet several requirements.[208] First, reported target proteins with multivalent site interactions generally exist on the surface of cells, bacteria or viruses. Second, the target protein density on the surface should be appropriate. Third, preferably the target protein itself is in the form of a multimer. Finally, the linking chain of a single ligand connected by a covalent bond cannot interfere with the binding of the ligand and the receptor.[208]

One target that meets all of these requirements is the neuraminidase of the influenza virus. Fu *et al.*[209] designed a tetrameric form of an inhibitor to facilitate its accommodation and binding to a tetrameric form of neuraminidase. Zanamivir was selected as the monomeric inhibitor due to its structural similarity to the substrate. The 6-hydroxyl group of zanamivir was selected as the monomer attachment site to ensure that all functional groups participate in the interaction with the active site of the enzyme, and in this way avoid a loss of binding affinity (Fig. 18). Finally, flexible polyethylene glycol chains were used to link zanamivir monomers. Zanamivir tetramers showed improved antiviral activity compared to monomers, occupying the four neuraminidase subunits simultaneously. Although resistant mutants containing the amino acid substitutions E119V/I222L or R294K had reduced binding affinity for neuraminidase inhibitors, the tetrameric derivative (compound **35**) showed higher activity against oseltamivir- and zanamivir-resistant mutants (Fig. 18).



Virus	EC_{50} (nM)		
	Oseltamivir	Zanamivir	**35**
H3N2	7.1 ± 0.7	17.7 ± 0.6	3.5 ± 0.3
mutH3N2	119.6 ± 13.3	154.5 ± 14.6	4.4 ± 0.3
anhH7N9	12.3 ± 0.8	6.1 ± 0.7	1.2 ± 0.1
shaH7N9	>1.4 × 10^5	178.0 ± 15.3	7.1 ± 0.6

Fig. 18 Multivalent drug development of oseltamivir derivatives. (A) Chemical structure of compound **35**. The monomer is shown on the left panel. (B) Crystal structure of compound **35** bound to influenza virus (strain shaH7N9) neuraminidase tetramer (PDB file 5JYY). The tetrameric assembly is generated from crystallographic symmetry, since only one monomer was identified from electron densities in crystal structures. The image shows a top view of the structure. (C) Antiviral activity of compound **35** against WT and mutant strains of influenza virus. Listed strains are: H3N2, A/Moscow/10/99 strain; mutH3N2, mutant H3N2 A/Moscow/10/99 strain, containing neuraminidase mutations E119V/I222L that confer resistance to oseltamivir; anhH7N9, H7N9 A/Anhui/1/2013 strain; shaH7N9, H7N9 A/Shanghai/2/2013 strain resistant to zanamivir. EC_{50} values were taken from ref. 209.

3.6 PROTAC strategy

Proteolysis targeting chimeras (PROTACs) have opened up a new field of drug discovery, utilizing cell's own protein degradation mechanisms to selectively cleave and eliminate proteins involved in human diseases, either from an infectious pathogen or causing malignant processes.[142,210] PROTAC molecules are bifunctional compounds that can simultaneously bind the target protein and the E3 ubiquitin ligase. The target protein is then ubiquitinated by the E3 ligase, and degraded by the proteasome complex (Fig. 19a). Compared with ordinary small molecule inhibitors, the PROTAC strategy has interesting advantages.[211] It has a wide range of targets including some undruggable ones. The dosage is small and the amount of catalyst required is minimal. Good pharmacodynamic properties ensure low drug exposure reducing the possibility of off-target effects as well as toxicity and unwanted side effects. PROTAC technology is used to treat diseases caused by abnormal expression of pathogenic proteins. From an antiviral perspective, the PROTAC strategy has a special advantage in that it can directly degrade target proteins (*i.e.* viral proteins), while avoiding resistance caused by mutations or overexpression of the target protein.

The PROTAC strategy has been used by de Wispelaere *et al.*[212] to design a series of bifunctional molecules aimed to degrade HCV proteases, showing that these compounds were

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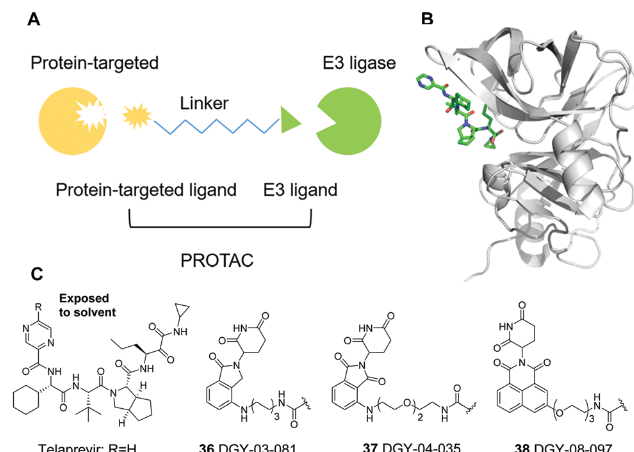

Fig. 19 PROTAC strategy in the development of anti-HCV agents. (A) Schematic outline of the PROTAC strategy, involving two ligands connected by a linker. (B) Crystal structure of telaprevir bound to HCV NS3/4A protease. Image obtained using Pymol and the structure retrieved from PDB file 3SV6. The pyrazine ring of telaprevir is located on the interface exposed to the solvent. (C) Structure of telaprevir and cereblon binders (R substituent) used for targeting HCV protease to the E3 ubiquitin ligase complex.

effective inhibitors in antiviral assays. In their study, telaprevir was chosen as a protease binding ligand and the high-resolution crystal structure of telaprevir bound to the HCV NS3/4A protease was used to identify a suitable linking site for the E3 ligase. The crystal structure shows that the pyrazine ring of telaprevir is on the solvent exposure interfaces. Therefore, this ring was used as the linking site to avoid steric clashes with the target protein (Fig. 19b). Different linkers were used to join the protease inhibitor with cereblon ligands (*e.g.* compounds **36** (DGY-03-081), **37** (DGY-04-035) and **38** (DGY-08-097)). Cereblon (encoded by the *CRBN* gene) is a component protein of the ubiquitin ligase complex (Fig. 19c).

The designed molecule retains the ability to inhibit the HCV protease, while directing the enzyme to the ubiquitin proteasome pathway for degradation. The obtained compound retained antiviral activity against telaprevir-resistant mutant HCV, as demonstrated with HCV replicons containing the amino acid substitutions V55A or A156S in the NS3/4A protease.

4. Novel targets and strategies of antiviral intervention

4.1 Lethal mutagenesis

An increase of the viral replication error rate above a critical threshold can lead to the loss of genetic information in a process termed ''error catastrophe''. Lethal mutagenesis is an antiviral strategy by which mutagenic agents are used to extinguish the virus by the accumulation of mutations.[213] Nucleoside analogues (*e.g.*, 5-azacytidine, 5-hydroxydeoxycytidine and 5-fluorouracyl) have been shown to effectively increase viral mutation rates in different RNA viruses, while decreasing their infectivity in cell culture experiments.[214–218] Interestingly, the mutagenic effect of approved nucleosides such as ribavirin and favipiravir has been shown in different viruses. These nucleosides can mispair with natural nucleotides during RNA synthesis and introduce mutations in the RNA strand. Ribavirin increases G → A and C → U mutation frequencies, while favipiravir facilitates A → G and U → C transitions.[213]

The mutagenic effect of favipiravir was initially demonstrated in human influenza virus in cell culture and murine norovirus *in vivo*,[219,220] but has also been shown in flaviviruses, filoviruses and more recently, SARS-CoV-2.[221–223] EIDD-1931 (β-D-N^4-hydroxycytidine) is another mutagenic ribonucleoside analogue effective against several viral families including alphaviruses (*e.g.* chikungunya virus) and human pandemic coronaviruses.[224–227] Molnupiravir, its orally bioavailable prodrug (β-D-N^4-hydroxycytidine-5′-isopropyl ester; MK-4482/EIDD-2801), is currently in phase II/III clinical trials. Animal studies have shown that molnupiravir blocks SARS-CoV-2 transmission in ferrets when administered intranasally.[228] Safety concerns due to the mutagenic potential of these compounds have to be seriously considered, and therefore, rigorous dosing and toxicity studies will be required for approval of this and other related compounds.

4.2 Host-targeting antivirals and their advantages

The current antiviral strategies are mainly directed at targeting viral proteins or host factors.[229] Virus-targeting antivirals can directly or indirectly interfere with viral protein function. The antiviral mechanisms discussed above are mainly directed against viral macromolecules (*e.g.* enzymes, and receptors), and include attachment inhibitors (*e.g.* fostemsavir), protease inhibitors (HIV-1 and HCV protease inhibitors), polymerase inhibitors (*e.g.* NRTIs and NNRTIs for HIV-1), and integrase inhibitors (HIV-1 integrase strand transfer inhibitors). Indirect virus-targeted antiviral drugs mainly block the function of some important biological complexes, such as the replication and transcription complexes[230] or ribonucleoprotein complexes.[231] Interfering with interactions involving host factors might be important to impair pathogen replication or persistence, or to enhance the protective immune response against pathogens.[31]

Drugs targeting viral proteins usually show high specificity, but rapidly mutating viruses can facilitate the development of resistance. Although drugs targeting host proteins may be potentially cytotoxic or have an impact on the human immune system, their appeal cannot be ignored. First, they usually show broad-spectrum activity and are not prone to resistance. In addition, they can be used immediately in the clinic, a fact that has enormous importance in large viral outbreaks, especially when development and production of specific antiviral agents is difficult, as recently shown for SARS, Ebola disease, Zika virus infections, and more recently, COVID-19. Second, drugs targeting host proteins and cellular processes might be able to avoid the combination of multiple antiviral drugs, thereby reducing treatment complexity and drug–drug interactions.[232] Third, multiple genotypes or phenotypes can be inhibited effectively at the same time.[233] Fourth, broad-spectrum antiviral drugs are of great value for some viruses that infect only a small number of individuals,

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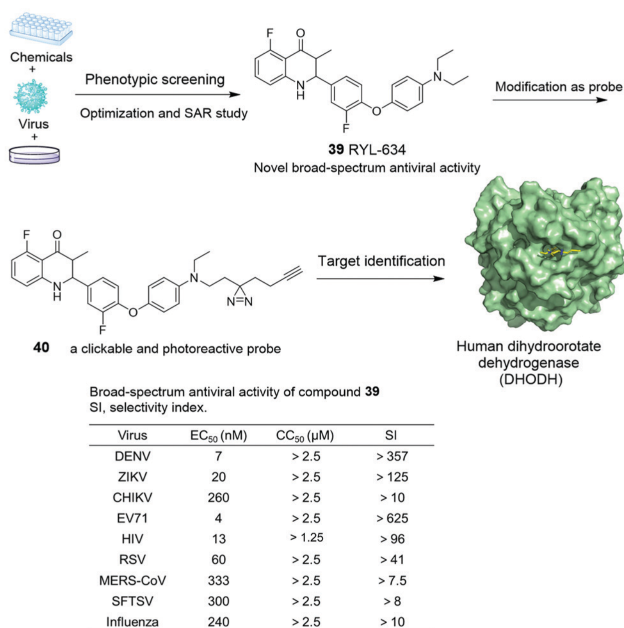
due to the high costs of production and development of drugs that make them unprofitable for pharmaceutical companies.

Overall, broad-spectrum antiviral drugs are very attractive, although ribavirin is the only approved drug showing an antiviral effect against different types of viruses (typically RNA viruses).[234] The key to the development of broad-spectrum antiviral drugs targeting host proteins is to clarify the common link and mechanism of action between virus and host. In the following sections we present a couple of examples of efforts directed towards this goal.

4.2.1 Human dihydroorotate dehydrogenase (hDHODH). A library of around 200 biaryl-substituted quinolones was initially used to identify an inhibitor of the NADH-ubiquinone oxidoreductase of *Plasmodium falciparum*, as an effective antimalarial drug.[235] Then, researchers used the same library to screen for antiviral agents with a phenotype-based screening method.[236] In the first round of screening, five compounds with antiviral activity were identified. Structure–activity relationship studies and optimizations were performed to obtain the most active molecule, named RYL-634 (compound **39** in Fig. 20).

Further research revealed that this molecule was active against HCV, dengue virus, Zika virus, chikungunya virus, enterovirus EV71, HIV, respiratory syncytial virus, MERS-CoV, Huaiyangshan banyangvirus (formerly known as SFTS virus), and influenza virus.[236] Compound **39** exhibits strong inhibitory effects on human pathogenic viruses and acceptable toxicity towards host cells. Viral strains resistant to compound **39** were not selected under drug pressure. These results showed that compound **39** had great potential for development as a novel



Broad-spectrum antiviral activity of compound **39**
SI, selectivity index.

Virus	EC_{50} (nM)	CC_{50} (μM)	SI
DENV	7	> 2.5	> 357
ZIKV	20	> 2.5	> 125
CHIKV	260	> 2.5	> 10
EV71	4	> 2.5	> 625
HIV	13	> 1.25	> 96
RSV	60	> 2.5	> 41
MERS-CoV	333	> 2.5	> 7.5
SFTSV	300	> 2.5	> 8
Influenza	240	> 2.5	> 10

Fig. 20 Discovery and target identification of compound **39**. Compound **39** shows novel broad-spectrum antiviral activity against multiple viruses. Abbreviations: DENV, dengue virus; ZIKV, Zika virus; CHIKV, chikungunya virus; EV71, enterovirus 71; RSV, respiratory syncytial virus; SFTSV stands for severe fever with thrombocytopenia syndrome virus. Antiviral activity data were taken from ref. 237.

broad-spectrum antiviral drug. However, the mechanism of action of compound **39** was not defined. Researchers designed and synthesized compound **40** as a clickable and photoreactive probe to find out that the target protein of compound **39** was the human dihydroorotate dehydrogenase (DHODH).

The application of several methods including drug affinity responsive target stability (DARTS) or activity-based protein profiling (ABPP), among others, confirmed those findings. Human dihydroorotate dehydrogenase (DHODH) was identified as the target responsible for the antiviral activity, using various methods such as drug affinity-responsive target stability (DARTS) or activity-based protein profiling (ABPP), among others. Interestingly, the potential of DHODH inhibitors had been previously showed in cancer, immune regulation, and antimalarial therapies.[237] Therefore, compound **39**, characterized as a novel type of DHODH inhibitor, is expected to have other therapeutic uses, in addition to its antiviral potential.

S416 is another potent human DHODH inhibitor with broad-spectrum antiviral activity against RNA viruses, such as influenza A virus, Zika virus, Ebola virus, and particularly against SARS-CoV-2. This compound showed an EC_{50} of 17 nM and an SI > 10 500 against SARS-CoV-2, with a favorable pharmacokinetic profile.[238] Human DHODH is rate-limiting enzyme in *de novo* pyrimidine biosynthesis, and supplies uridine and cytosine required for RNA synthesis. Despite being promising molecules, the efficacy of human DHODH inhibitors as antiviral drugs is limited by salvage pathways involving recycling of pre-existing nucleosides from food or other nutrition, and relatively high uridine levels in plasma. However, it is reasonable to assume that *de novo* nucleotide biosynthesis rather than salvage pathways is more critical for RNA virus replication.[238]

4.2.2 Human DEAD-box polypeptide 3 (DDX3). DDX3 is an ATPase/RNA helicase, and a human host factor required for the replication of some DNA or RNA viruses, including some emerging or challenging human pathogens such as HIV-1,[239] HCV,[240] dengue virus,[241] and West Nile virus.[242] Brai *et al.*[243] optimized known DDX3 inhibitors such as compound **41** to obtain a novel series of DDX3 inhibitors (Fig. 21). In the absence of a crystal structure of human DDX3, a homology model was helpful to define the inhibitor binding pocket. Based on the modelled structure, a series of compounds were designed and synthesized. Compound **42** showed broad spectrum antiviral activity against WT and resistant HIV-1 strains, HCV, dengue virus and West Nile virus without relevant cytotoxicity. Pharmacokinetic and toxicity studies confirmed safety and bioavailability of compound **42**, suggesting that DDX3 inhibitors can be further explored and developed for the treatment of HIV/HCV co-infections, emerging viral diseases and also to fight drug-resistant strains in HIV-infected patients.[243]

Virtual screening based on the homology model was carried out to discover novel DDX3 inhibitors with antiviral activity. Compound **43** was identified as the most promising molecule based on its solubility in water and its inhibitory activity in enzymatic assays (IC_{50} = 0.36 μM).[244]

Docking analysis of two promising compounds (**42** and **43**) facilitated the design of derivatives using a molecular hybridization

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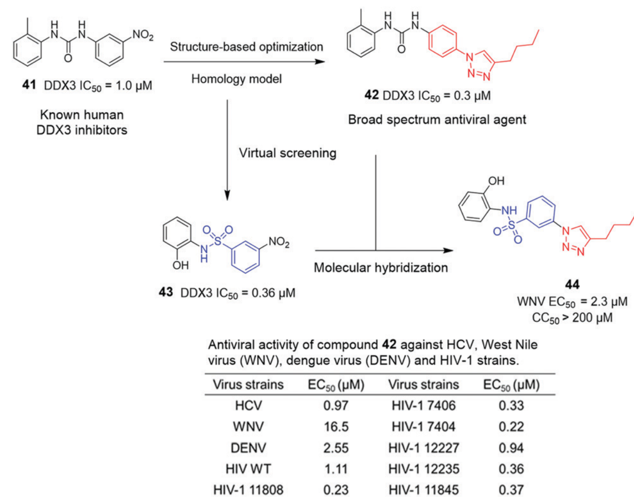

Antiviral activity of compound **42** against HCV, West Nile virus (WNV), dengue virus (DENV) and HIV-1 strains.

Virus strains	EC_{50} (μM)	Virus strains	EC_{50} (μM)
HCV	0.97	HIV-1 7406	0.33
WNV	16.5	HIV-1 7404	0.22
DENV	2.55	HIV-1 12227	0.94
HIV WT	1.11	HIV-1 12235	0.36
HIV-1 11808	0.23	HIV-1 11845	0.37

Fig. 21 Design and optimization of DDX3 inhibitors as broad-spectrum antiviral agents. Antiviral activities of compound **42** are shown in the bottom panel. HIV-1 strains are designated according to their NIH AIDS Reagent Program catalogue number, and correspond to strains resistant to protease inhibitors (HIV-1 11808), NRTIs (HIV-1 7406 and 7404), NNRTIs (HIV-1 12227 and 12235), and integrase inhibitors (HIV-1 11845). Data were taken from ref. 243–245.

strategy that combined the key structures of both molecules. Selective DDX3 helicase inhibitors of this new family of compounds were inactive against the related human DDX1 and the ATPase activity of DDX3. Six of the 21 compounds described by Brai *et al.*[245] showed promising antiviral activity against West Nile virus without significant cytotoxicity. Among them, compound **44** was the most potent candidate, showing an EC_{50} of 2.3 μM. These studies confirmed that DDX3 helicase inhibitors might reveal an Achilles' heel in the virus, while demonstrating that human proteins can be successfully targeted to combat new emerging viral threats.[245]

4.3 Combination therapies targeting viral and host factors simultaneously

Another interesting possibility would be to use combination therapies targeting the virus and host cell functions relevant for infection. This approach was assumed by older therapies against chronic hepatitis B and C when interferons were used to stimulate host antiviral defenses, usually in combination with nucleoside analogues with antiviral activity. However, there are few clinical studies showing the effects of combining bona fide antiviral compounds with drugs acting on host factors required for viral replication. Xiao and colleagues also showed that a combination of host-directed agents (erlotinib, and dasatinib), host-directed antibodies (anti-CLDN1, anti-CD81, and anti-SR-BI), and virus-directed agents (*e.g.* combinations including HCV protease inhibitors, daclatasvir or sofosbuvir) were highly effective against HCV.[246] In addition, Schloer *et al.* have shown that the combination of the influenza A neuraminidase-targeting drug oseltamivir and itraconazole, a licensed antifungal that blocks influenza virus endosomal escape, had a synergistic effect compared with the administration of oseltamivir alone.[247]

Comprehensive studies and high-throughput analysis carried out in cell culture with many approved drugs have revealed synergistic effects of combinations involving virus-directed agents and host-directed antiviral drugs.[248] Examples found in these analyses include the combination of sofosbuvir (an FDA-approved anti-HCV drug) with brequinar (an investigational anti-cancer agent) and niclosamide (an approved anthelminthic agent); or in the case of HIV, monensin (a veterinary antibiotic) with lamivudine and tenofovir (both approved anti-HIV agents). All these combinations were shown to boost the antiviral activity of the individual drugs.[248] More recently, the combination of remdesivir (an approved inhibitor of the viral RNA polymerase) and baricitinib, a Janus kinase (JAK) inhibitor with the chemical name [1-(ethylsulfonyl)-3-(4-(7*H*-pyrrolo(2,3-d)pyrimidin-4-yl)-1H-pyrazol-1-yl)azetidin-3-yl]acetonitrile has been approved by the FDA for emergency use for hospitalized people requiring supplemental oxygen, invasive mechanical ventilation, or extracorporeal membrane oxygenation. In this case, baricitinib acts as an immune modulator that suppresses lung macrophage production of cytokines and chemokines responsible for inflammation and neutrophil recruitment.[249]

5. Summary and discussion

5.1 Medicinal chemistry strategies

Antiviral drugs based on new targets and mechanisms are important to combat drug resistance.[250,251] Viral replicative cycles involve many proteins and protein-mediated processes that can be inhibited with antiviral drugs. These can be directed against host factors, viral proteins or both.[229] Host proteins have recently received widespread attention for their unique advantages as targets of antiviral intervention. However, most of the antiviral research projects have been devoted to the exploitation of viral proteins as targets of specific drugs. In addition, unlike single chemical entities, multi-target drugs can achieve the effects of two or more types of inhibitors, while avoiding limitations of drug combination therapies.[138,151]

If the conformation of the drug molecule in the binding pocket can be adapted to the presence of an amino acid substitution resulting from a resistance mutation, then it is very likely that the drug would be effective against the resistant viral strain. Multiple conformations, torsional flexibility, torsion elasticity, repositioning and reorientation are attributes of compounds with potential activity against resistant viral strains.[165] On the other hand, drug candidates can adopt an appropriate bioactive conformation, reducing the energy required for binding, which may compensate to some extent the affinity decrease due to the presence of the amino acid substitution resulting from the resistance mutation.[166]

Relatively conserved regions selected for rational structure-based drug design can reduce the risk of emergence and selection of mutations leading to drug resistance. Targeting new interaction sites within the binding pocket can reduce the binding affinity-dependence of the drug and the mutated site.[193] Substrate envelope-based drug design strategies require

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only the interaction with the necessary residues in the substrate envelope to avoid the effects of mutations occurring at surrounding accessory residues.[112]

In order to compensate for the decreased affinity observed between drug and mutated residue, additional interactions can be increased or enhanced. Forming or strengthening the hydrogen bond network[188,193] or increasing covalent bonding,[198] halogen bonds,[200] and van der Waals forces[204] between the drug molecule and the target protein can also improve drug resistance. For proteins with multiple symmetric binding sites, the development of multivalent binding drugs may offer more unique advantages than monomeric inhibitors, such as compensation for the decrease in affinity caused by mutations and better activity against resistant strains.[205]

In addition to focusing on the process of identification and binding, some mechanisms in the host cell can be used to eliminate the virus. For example, the newly developed protein degradation targeting chimera technology (PROTAC) uses the ubiquitin system for a protein degradation mechanism in cells to process viral target proteins.[212]

5.2 Drug discovery based on new targets and new mechanisms

Antiviral drugs based on new targets and new mechanisms will remain prevalent in the future. Basic research can help discover new targets. For example, studies found that conservative nucleotide changes in the viral genome can influence HIV-1 RNA packaging,[252] while recent studies have shown that HIV-1 transcripts that differ by as few as one or two 5′-guanosines adopt distinct structures that modulate RNA function and fate.[253] Viral RNA (or DNA) could become new targets for antiviral therapy, supporting the development of novel strategies leading to the development of therapies effective against drug-resistant strains.

On the other hand, many of the new targets for antiviral therapy will be host factors. Drugs targeting host proteins or biochemical processes might be more advantageous than those targeting viral molecules. These drugs can overcome the existing resistance caused by conventional inhibitors of viral function and avoid complex therapeutic combinations used in common antiviral therapies. Whether it is a new virus or a new viral phenotype, as long as the role and the relationship between the virus and host is clarified explicitly, many host-targeted drugs can be used in the clinic directly, particularly when considering those previously tested as therapies against other pathologies. This is particularly useful when considering that their safety has been tested previously in clinical trials. In particular, broad-spectrum antiviral activity can be of enormous importance in large-scale outbreaks of emerging viruses such as SARS-CoV-2. However, we should not ignore limitations of host-targeted drugs, such as side effects affecting the immune system, their potential cytotoxicity, and inconsistent activity *in vivo* and *in vitro*. With the development of uniform screening methods for *in vitro* and *in vivo* activities, research may still need to concentrate on the discovery and identification of lead compounds with reduced effects on the immune system and low cytotoxicity.[31]

5.3 Structural biology studies help to elucidate resistance mechanisms and conduct precise drug design

With the development of structural biology, bioinformatics, and computer science, approaches focused on solving the drug resistance problems have become precise, automatic, and interdisciplinary. Comparison of crystal structures of proteins found in drug-resistant and wild-type viral strains are helpful to understand the mechanism of drug resistance. Precise drug design based on structure can play a pivotal role in the design of antiviral drugs, effective against resistant viruses, particularly when structural information is available for drug-resistant mutants. Furthermore, crystallographic and other structural data can be very useful in the implementation of virtual screening technology applied to high-throughput screening of large libraries of compounds.[254]

5.4 Screening platforms expedite drug discovery

The combination of virtual screening and biological/chemical screening platforms has greatly accelerated the discovery of small molecules effective against drug-resistant strains. Structure-based virtual screening has the potential to improve the efficacy of screening campaigns aimed at identifying valuable hit compounds.[255] Virtual docking and medicinal chemistry strategies can then be used for optimization and modification of the lead compounds. Finally, phenotypic screening determines the antiviral effect of the selected compounds, while established structure–activity relationships guide their further optimization.

Conflicts of interest

The authors declare no conflicts of interest.

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