CHAPTER 17

The Emergence of Small-Molecule Inhibitors of Capsid Assembly as Potential Antiviral Therapeutics

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Contents

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1. INTRODUCTION

There is a large degree of variation between viruses that cause human disease, but the general life cycle of viruses share common features that are potential targets for antiviral drugs. These are viral attachment, viral entry, uncoating, replication and release. Each of these key processes represents an opportunity to disrupt the viral life cycle and prevent the virus from replicating [1]. Thus, in order for antiviral treatments to be effective, they must either block the entry of the virus into the cell or be active within the infected cell. Because viruses use the host cell's own systems to reproduce, therapeutic approaches can target not only the viral life cycle but also the cellular proteins that are hijacked and manipulated by the virus. Enzyme inhibitors have the unique ability to affect processes within infected cells. They bind to enzymes in a way that disrupts their function, preventing a step in the infectious process from occurring. The classes of enzymes currently targeted by antiviral therapy include reverse transcriptase, protease, integrase, replicase, and neuraminidase [2]. Other approaches that are employed to disrupt the viral life cycle include entry inhibitors, fusion inhibitors, and integrase inhibitors. More recently, attention has focused on the later stages of viral replication and the capsid assembly pathway [3,4].

2. CAPSID ASSEMBLY INHIBITORS

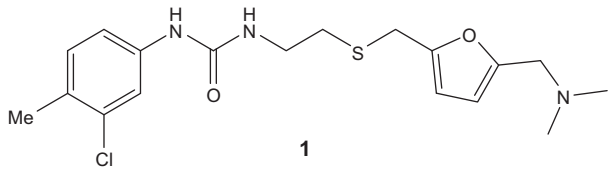
The viral capsid is a structural protein that encloses and protects the genetic material of the virus during the viral replication process. It is a unique protein synthesized by specific genes in the nucleic acid of the virus [5]. Within the viral life cycle, the capsid protein is the most conserved of all the viral structural proteins. Mutations in the sequence and structure of the capsid protein have led to alterations in the binding mode, which prevented dimerization, processing and ultimately diminished viral infectivity [6,7]. Capsid assembly (CA) stands out as a step common to viral replication and infectivity, yet it has not been the subject of extensive drug discovery programs until now. In the past few years, a body of data has opened new opportunities for antiviral research to focus on the modulation of CA processes as a novel approach to the inhibition of viral infections [8]. Most of the early efforts have focused on disrupting the normal interactions of the capsid protein, key protein–protein interactions in the

capsid binding region, and modulating the processing of the CA protein N-terminal and C-terminal domains (CA_{NTD}–CA_{CTD}).

2.1. Inhibitors that bind directly to HIV-1 CA

Early validation of this approach was obtained through point mutations in the CA which resulted in diminished levels of processing, dimerization, and infectivity during the viral replication cycle [9,10]. Mutations on CA severely disrupt the cell-cycle independence of HIV. The loss of cell-cycle independence can be cell-type specific, which suggests that a cellular factor affects the ability of HIV to infect nondividing cells. The identification of small molecules capable of disrupting these same interactions could lead to novel approaches to supplement current therapies to combat viral infections.

2.1.1. HIV-1 CA_{NTD} inhibitors



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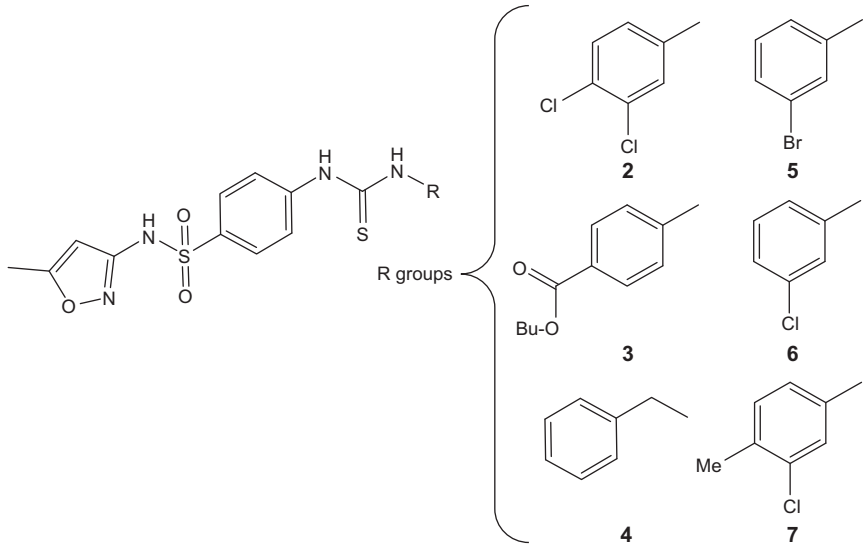
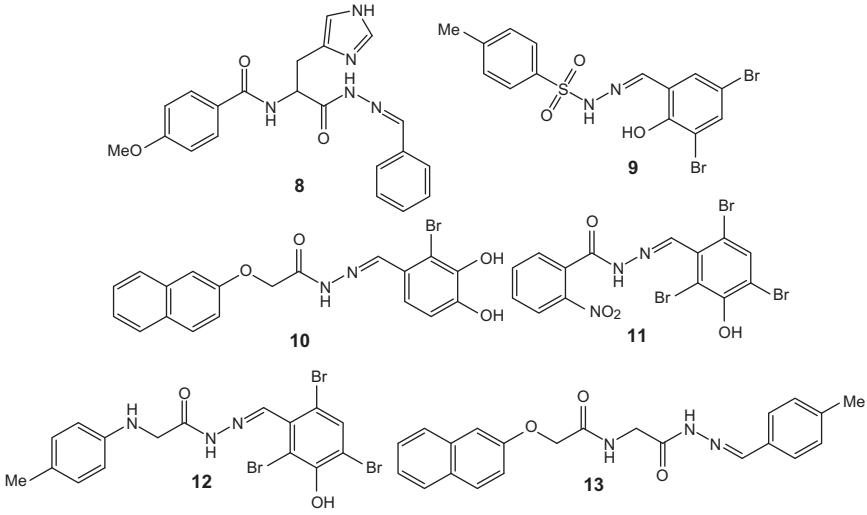
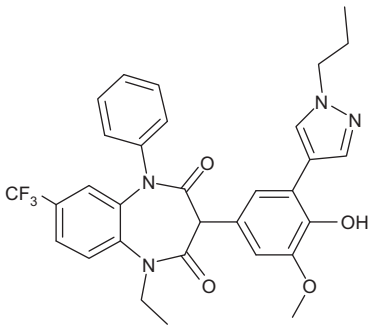
Gag is the major structural protein for the virus particle and is responsible for viral budding. CA is synthesized as a domain within the 55-kDa Gag precursor polyprotein and as thousands of copies of Gag assemble near the plasma membrane, they bud to form an immature, noninfectious viral particle. CA is cleaved through proteolysis, and a conformational shift leads to the formation of the capsid particles. Urea **1** (also known as 1-(3-chloro-4-methylphenyl)-3-(2-((5-((dimethylamino)methyl)furan-2-yl)methylthio)ethyl)urea or CAP-1) was one of the earliest compounds shown to bind to the HIV-1 capsid (CA) protein using nuclear magnetic resonance (NMR) spectroscopy [11,12]. Compound **1** does not inhibit cell growth or virus production, but the particles produced are poorly infectious. The phenotype of aberrant capsid formation was shown to be the direct result of the inhibitor binding to the CA_{NTD} through displacement of Phe 32 residue for the CAP-1 aromatic residues, which disrupts capsid maturation and interferes with Gag–Gag interactions during assembly of immature particles [13].



A series of thioureas **2**, **3**, and **4** were shown to act as dual inhibitors of CA and Cyclophilin A (Cyp A) and inhibit HIV-1 in the low micromolar range as measured in an optical density assay for turbidity during the CA process [14]. These compounds also displayed low toxicity for cell proliferation against uninfected CEM cells (TC_{50}) with values greater than 100 μM [14]. Additional analogs **5**, **6**, and **7** were also found to cause 50% inhibition in the submicromolar range [15]. With Cyp A also inhibited by this novel class of thioureas, both capsid disassembly and CA are blocked in the viral replication life cycle [16].



Several groups of acyl hydrazones were also identified as potent inhibitors of Simian Immunodeficiency Virus (SIV) and have been shown to bind to CA_{NTD} in a similar manner as compound **1** [17,18]. Compounds **8** through **12** inhibit SIV with EC_{50} values in the low micromolar range. Compound **13** inhibited SIV with an EC_{50} of 0.47 μM while it was not toxic against uninfected cells at concentrations above 100 μM.



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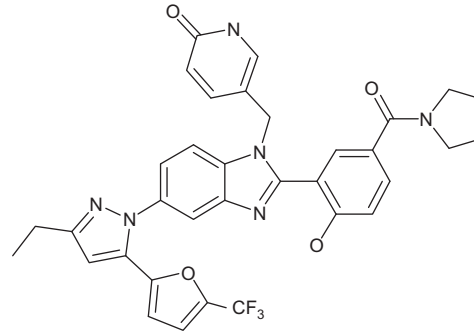

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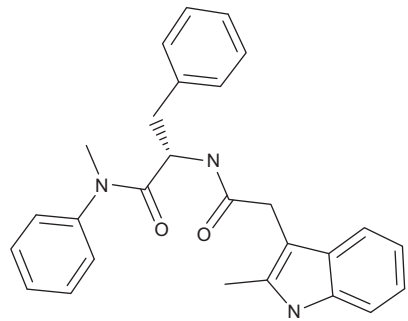
Two chemical series were identified as binding to CA_{NTD} and inhibiting HIV-1 replication through NMR spectroscopy [19,20]. Compound **14** is a benzodiazepine with $EC_{50} = 70$ nM and low toxicity for a 50% reduction of cell proliferation against uninfected cells (CC_{50}) of 28 μM. Compound **15** is a benzimidazole with $EC_{50} = 62$ nM and a comparable cellular toxicity profile, with CC_{50} greater than 20 μM. The mechanism of action for these compounds was similar to the other CA inhibitors in that they were shown to bind directly to the CA_{NTD} and these compounds do not show cross-resistance with other antiviral mechanisms. Resistance mutants within the CA inhibitor binding pocket map to the CA_{NTD} and these mutations offset the binding and function of the inhibitors. The site of interaction was confirmed by electron microscopy (EM), NMR, and co-crystallization (Co-Xtal) data [19]. Lead optimization for both lead series was stopped due to the inability to reconcile the shift in potency due to protein binding and the development of a complex resistance profile.



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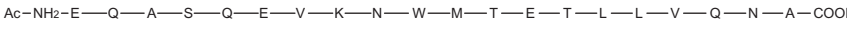
Compound **16**, PF74, targets the CA and is active following HIV-1 envelope mediated entry and before reverse transcription [21,22]. It also binds directly to the HIV CA and blocks the formation of infectious virion by triggering premature uncoating of the capsid in target cells. Compound **16** also binds to CA with micromolar affinity and inhibits a wide range of HIV isolates. Selection for drug resistance in culture identified a series of five mutations in CA (Q67H, K70R, H87P, T107N, and L111I) that prevented compound binding, collectively conferring resistance to the molecule.



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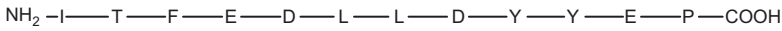
A 20-mer peptide, **17**, CAC1, was designed to mimic the structural and energetic interactions of the HIV CA_{CTD} [23]. The amino acid sequence corresponds to residues 175–194 of α-helix 2 of the CA_{CTD} of HIV, and **17** interacts specifically with this region. The affinity of **17** for the CA_{CTD} region is approximately 50 μM and may represent an excellent opportunity to initiate the development of peptidomimetic CA inhibitors.



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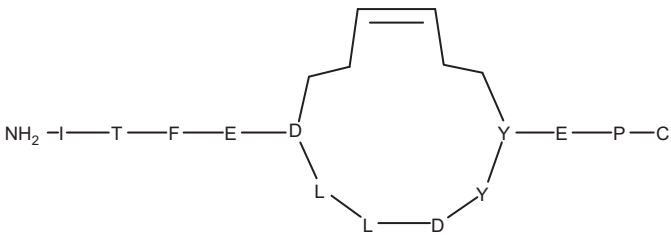
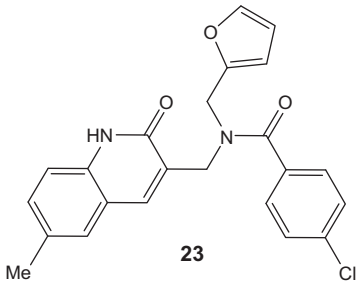


19

A 12-mer peptide, **18**, CAI, that binds the CA domain of Gag was identified through a phage display screen [24–27]. Peptide **18** inhibits assembly of immature and mature HIV capsid particles *in vitro* but not in cell culture due to poor cell penetration. The next generation of these peptide inhibitors, peptide **19**, NYAD-1, displayed activity in cell culture and had 10-fold better potency over CAI [28,29]. Theses peptide inhibitors of HIV-1 block the CA_{NTD}–CA_{CTD} interaction by competing for the natural binding region in the CA_{NTD}. Altering the CA_{NTD}–CA_{CTD} dimer interface that is crucial for connecting the hexameric rings in the CA lattice would weaken the hexamers, impair assembly, and destabilize the assembly cores.

GPG-NH2,20 ALG-NH2,21 RQG-NH2,22

Recently, several tripeptides were identified as inhibitors of HIV-1 CA, with an 80% reduction of HIV-1 replication [30–32]. Tripeptides **20**, **21**, and **22** were shown to interact with CA with EC_{50} values in the low- to mid-micromolar range and resulted in the formation of a range of aberrant capsids. The tripepetides were effective against HIV-1 drug-resistant strains, and **22** did not display resistance mutations after 30 passages of the HIV-1 virus. The tripeptides represent a promising new approach to combat HIV starting with molecules of low molecular weight.



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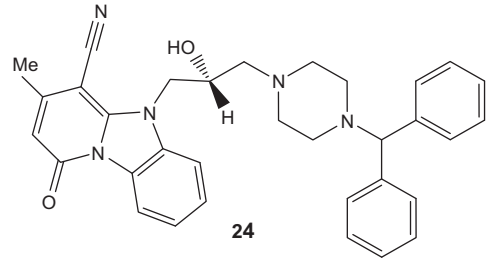
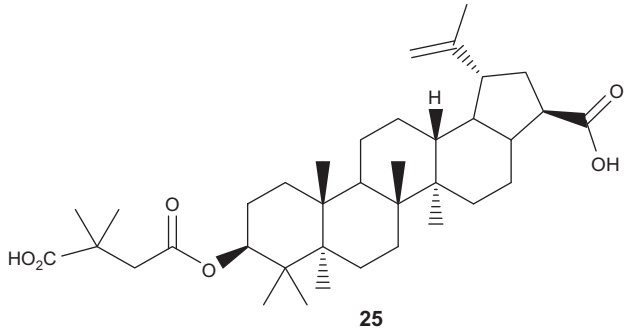


24

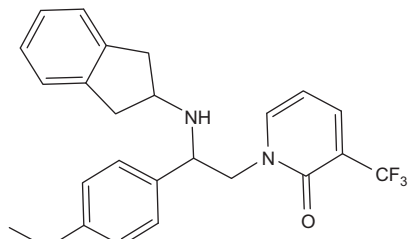
A docking-based virtual screen of the HIV-1 CA_{CTD} binding pocket occupied by the tripeptide-based inhibitors above leads to the identification of small molecules capable of occupying the same hydrophobic cavity. Compounds **23** and **24** are potential HIV-1 inhibitors with EC_{50} values equal to 1.1 and 1.8 μM, respectively [33]. These compounds are capable of inhibiting the formation of mature viral particles, which was verified with EM studies. Both chemical series show comparable activity against a wide range of HIV-1 laboratory-adapted isolates.

2.2. Maturation inhibitors: Antivirals that block cleavage of HIV-1 CA-SP1



25

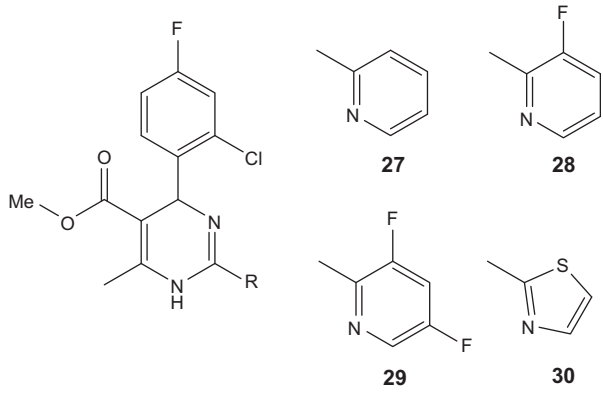
Maturation inhibitors are distinct from protease inhibitors (PIs). PIs block Gag and the precursor protein (Gag–Pol) cleavage by aspartyl proteases to the structural capsid proteins, matrix antigen (MA p17), capsid antigen (CA p24), nucleocapsid (NC p7, NC p1), transcriptase (p66/p51), integrase (p32), and other functional proteins (p11/p11) [2]. Maturation inhibitors block the cleavage of capsid precursor (CA-SP1) to mature capsid protein p24 (or CA), as exemplified by compound **25** (BVM, a triterpene derived from betulinic acid) with an $EC_{50} = 1.3$ nM, $CC_{50} = 43$ μM [34–40]. By preventing the cleavage of SP1 from the C-terminal of the CA, there is an accumulation of the CA-SP1 intermediate which results in defective core condensation. The viral particles released were noninfectious. Oral administration of **25** to SCID-hu Thy/Liv mice reduced viral RNA by a factor of 100. A dose-dependent inhibition of capsid-SP1 cleavage in HIV-1-infected human thymocytes obtained from mice was also observed. Maturation inhibitor **25** was advanced to clinical trials for further evaluation of efficacy and drug resistance. The activity of **25** in human patients was found to be dependent on a specific Gag polymorphism. Patients without the mutations Q369, V370, or T371 were more likely to respond. Resistance was also found to be common in treatment-naïve patients as well as patients with previous exposure to an HIV PI. The development of **25** was stopped after completion of a Phase 2b clinical study as the patient population became less responsive.



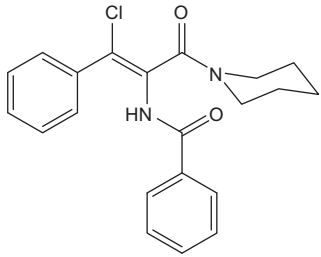
26

An additional compound **26**, PF-46396, was discovered through a high throughput screen (HTS) for inhibitors of HIV and, subsequently, found to affect viral uncoating and assembly [41]. Compound **26** blocks the processing of HIV-1 in manner similar to BVM and was effective against multiple HIV-1 laboratory strains. Viral resistance to **26** was raised after routine serial passage of the NL4-3 strain of HIV-1. A single amino acid mutation (I201V) conferred resistance to **26** and restored the regular HIV-1 CA-SP1 cleavage, even in the presence of the inhibitor.

2.3. HBV CA inhibitors



The heteroaryldihydropyrimidines (DHPs) **27**, **28**, **29,** and **30** represent a class of compounds capable of inhibiting replication of the hepatitis B virus (HBV) in HepG2.2.15 cells and thus suppress the production of viral DNA [42–44]. These compounds have been shown to bind to either HBV capsid or newly synthesized core protein. When the inhibitors were bound to the capsid proteins, the core protein cannot assemble properly and was easily degraded. The inhibition of HBV results from disruption of CA by binding of the compounds to dimers and oligomers of core protein. The mechanism of action was established through extensive co-crystal studies with HAP-1. Other direct binding inhibitors of HBV capsid formation are compounds **31** and **32** [44]. Both bind to the C-terminal region of the HBV.



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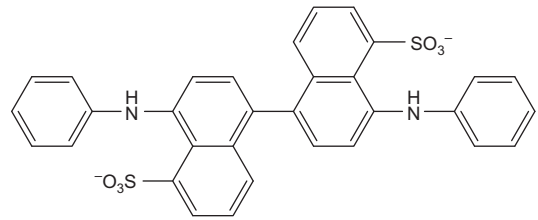

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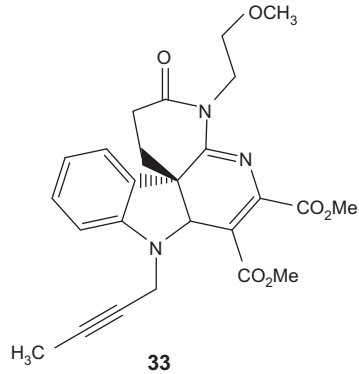
2.4. HCV CA inhibitors



33

Compound **33** was recently identified from an automated screen of indoline alkaloids as a potential inhibitor of HCV [45]. The screen selected for inhibitors of the capsid protein core of the virus, ultimately resulting in the production of a noninfectious virion. The EC_{50} of compound **33** was 2.0 μM with a CC_{50} of greater than 320 μM.

3. CA INHIBITION *VIA* HOST FACTOR MODULATION

Protein–protein interactions are involved in every biological process responsible for cellular function, including the propagation of viral replication. The viral replication process is also subjected to extensive protein–protein interaction during the replication process. In general for viral families, one of the most conserved proteins produced during the replication process is the capsid protein. The capsid protein shares no homology with cellular host proteins even though transcription occurs using host factors and other cellular components. Capitalizing on these differences provides the basis for a novel approach to viral inhibition by focusing on the cellular host factors to disrupt the assembly of the viral capsid.

3.1. Pathway-wide screen using the cell-free screening system

Protein–protein interactions occurring in higher animals have been effectively modeled by homologous interactions occurring in more biochemically tractable systems derived from non-metazoans. The *in vitro* assembly for the *de novo synthesis* of the capsid protein on ribosomes in a cell-free (but cell-derived) extract under physiological conditions was used to direct newly synthesized capsid protein to form structures that

were indistinguishable from authentic viral capsids by biochemical and biophysical criteria, and electron microscopic appearance [46]. The assay is one of cell-free translation programmed by viral capsid mRNA in 384-well plates containing small molecules. The compounds that inhibit CA are identified through an antibody detection system.

3.2. Modulation of CA pathway leads to novel antivirals

Compounds identified as potential inhibitors of the CA pathway were evaluated for dose-dependent inhibition and for cellular toxicity against relevant cell lines. Two distinct phenotypes expressed by inhibition of the CA pathway were (1) the blocked release of virus, with accumulation of an intracellular assembly intermediate, presumably the site of action of the host protein drug target [47]; (2) the release of aberrant capsids [48,49], consistent with the demonstration that viral particles are produced upon drug treatment of virus infected cells, leading to the production of noninfectious particles. This novel approach was presented at the 23rd Annual International Conference on Antiviral Research (ICAR) [48–56]. Figure 1 represents a summary of the number of distinct chemical series identified as hits (or pre-leads) for each virus screened with the aforementioned screening format. The EC_{50}s for these antiviral pre-lead hits ranged from the low micromolar to the low nanomolar range. Although numerous distinct chemical classes were identified and described at ICAR (Figure 1), only one chemical class of compounds was presented, shown below as the generic structure **34**. In general, compounds active against one member of a viral family were also found to be comparably active against the other members (and strains) of the same viral family. There was also a fair degree of interfamily antiviral activity, as some compounds

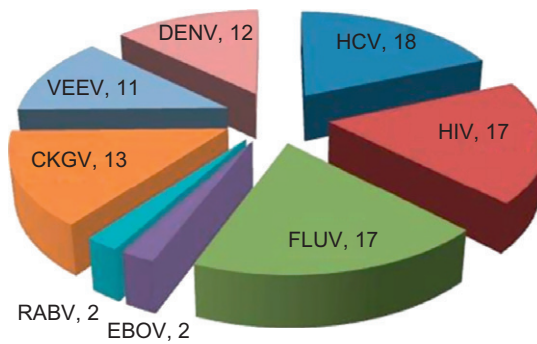
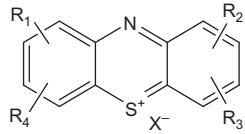


Figure 1 The number of distinct pre-lead chemical series identified from the cell-free screening system. All pre-leads display antiviral activity in the cell culture live virus assays.

demonstrated activity against multiple viral families. Preliminary results were presented for an animal efficacy study, where mice challenged with the Ebola virus were treated once daily (for 10 days) with a 5-mg/Kg dose intraperitoneal with a compound related to **34** resulting in 100% survival of the animals [56]. Lead optimization is underway for several therapeutic programs along with elucidation of the mechanism of action.



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4. CONCLUSION

Over the past three decades, great strides have been made to advance antiviral research and to develop new antiviral drugs, with the majority of these approaches focusing on different stages of the viral life cycle. With the frequent development of resistance and toxicity from drug treatment, interest has increased for research on novel approaches to antiviral therapies. Research that targets the later stages of viral replication, such as the CA process, offers a new opportunity to develop therapeutic approaches to treat viral infections. Antivirals that interfere with the normal assembly or processing of the viral capsid are gaining momentum as potential therapies because the integrity of the viral capsid protein is crucial for infectivity and the replication of the virus. The early success of BVM, which demonstrated efficacy in the HIV-1 mouse model and progressed to Phase II in the clinic, provides encouragement that future endeavors along these lines of research may achieve comparable success. Antiviral programs which feature capsid assembly inhibitors will need to address the issues of drug resistance and toxicity from drug treatment in order to be successful.

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