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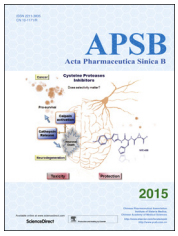

REVIEW

Pharmacological intervention of HIV-1 maturation

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Abstract Despite significant advances in antiretroviral therapy, increasing drug resistance and toxicities observed among many of the current approved human immunodeficiency virus (HIV) drugs indicate a need for discovery and development of potent and safe antivirals with a novel mechanism of action. Maturation inhibitors (MIs) represent one such new class of HIV therapies. MIs inhibit a late step in the HIV-1 Gag processing cascade, causing defective core condensation and the release of non-infectious virus particles from infected cells, thus blocking the spread of the infection to new cells. Clinical proof-of-concept for the MIs was established with betulinic acid derived bevirimat, the prototype HIV-1 MI. Despite the discontinuation of its further clinical development in 2010 due to a lack of uniform patient response caused by naturally occurring drug resistance Gag polymorphisms, several second-generation MIs with improved activity against viruses exhibiting Gag polymorphism mediated resistance have been recently discovered and are under clinical evaluation in HIV/AID patients. In this review, current understanding of HIV-1 MIs is described and recent progress made toward elucidating the mechanism of action, target identification and development of second-generation MIs is reviewed.

Abbreviations: BMS, Bristol-Myers Squibb; CA, capsid; GSK, GlaxoSmithKline; HIV, human immunodeficiency virus; MA, matrix; MI, maturation inhibitor; PR, protease; PI, protease inhibitor; SIV, Simian immunodeficiency virus; SP1, spacer protein 1

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1. Introduction

HIV-1 maturation is the final step of the virus lifecycle. It involves two coupled and highly regulated events: immature virus particle release driven by the viral Gag protein and a proteolytic cleavage cascade directed by the viral protease (PR). Numerous studies have shown that maturation is essential for HIV-1 infectivity because genetic mutations either in Gag or PR that inhibit maturation lead to the production of non-infectious HIV-1 particles[1–7]. Pharmacological intervention in HIV-1 maturation has been successfully explored, resulting in the discovery and development of two classes of HIV-1 inhibitors. One class of inhibitors, the PR inhibitors (PI), target and inhibit the enzymatic activity of the HIV-1 PR. This class has 9 FDA-approved inhibitors that are currently used in treating AIDS patients worldwide. Another class of inhibitors currently under clinical development binds the Gag substrate and specifically blocks PR-mediated Gag cleavage. The compounds that disrupt the Gag cleavage are designated the maturation inhibitors (MIs) in a way to differentiate them from the PI.

The first-in-class MI is bevirimat, also known as 3-*O*-(3′,3′-dimethylsuccinyl) betulinic acid, PA-457, or MPC-4326 (Fig. 1)[8–10]. Bevirimat specifically inhibits a specific step in Gag processing: cleavage of the CA-spacer protein 1 (SP1) intermediate that occurs late in the Gag cleavage cascade (Figs. 2 and 3). Despite promising data in a phase IIa clinical trial, further development of bevirimat was suspended in 2010 due to bevirimat-resistance conferring Gag SP1 polymorphisms present in approximately 50% of HIV-1-infected patients[11].

Considering that a number of review articles on bevirimat, the prototype HIV-1 MI, have been published[12–14], the purpose of this review is to describe what is known about the HIV-1 MIs with particular reference to those advances recently made in the mechanisms of action, target identification and discovery and clinical development of new generation MIs highly effective against bevirimat-resistant viruses.

2. HIV-1 assembly and maturation

In HIV-1 lifecycle, the Gag precursor protein $Pr55^{Gag}$ drives the final stage of viral replication: assembly and maturation. Following synthesis, $Pr55^{Gag}$ is transported to the plasma membrane where virus assembly occurs. Through a complex combination of Gag–lipid, Gag–Gag, and Gag–RNA interactions, a multimeric budding structure forms at the inner leaflet of the plasma membrane. The budding virus particle is ultimately released from the cell surface in a process that is promoted by an interaction between the late domain in the p6 region of Gag and host proteins, most notably the endosomal sorting factor *TSG101* (tumor susceptibility gene 101). As illustrated in Fig. 2, concomitant with particle release, the viral

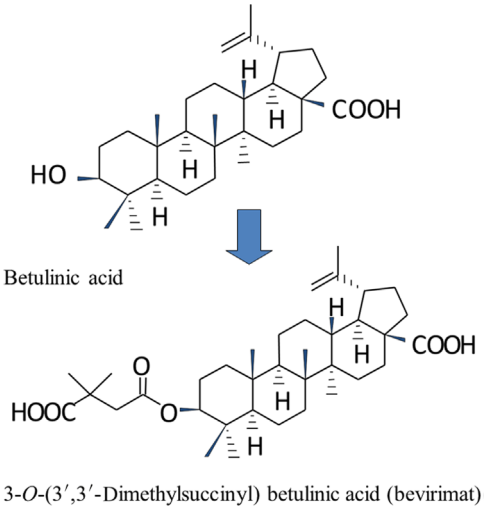

Figure 1 The chemical structures of betulinic acid (top panel) and its derivative bevirimat, 3-*O*-(3′,3′-dimethylsuccinyl) betulinic acid (bottom panel).

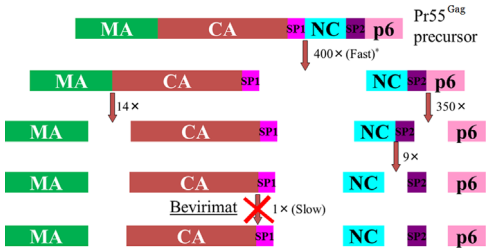

Figure 2 The processing cascade of HIV-1 Gag polyprotein precursor. The proteolytic cleavage of HIV-1 Gag polyprotein precursor *via* the viral protease is a sequential and high-order event. The numbers indicated underneath the various precursors show the cleavage rates of each individual cleavage step relative to that of CA-SP1 precursor cleavage, the final step with the slowest rate of cleavage in the Gag processing cascade. CA-SP1 cleavage is a primary target of the HIV-1 maturation inhibitor bevirimat.

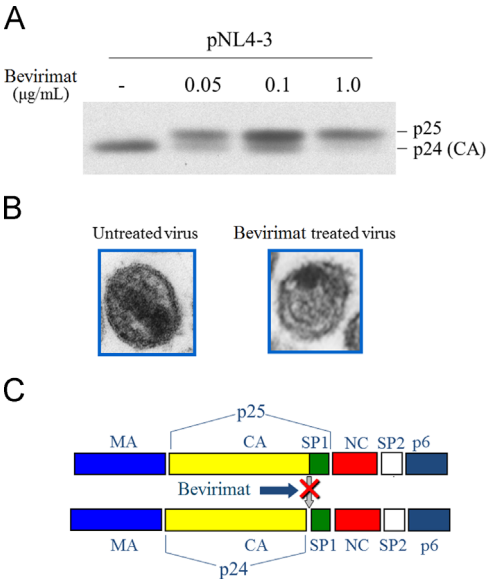

Figure 3 Mechanism of action of HIV-1 maturation inhibitor Bevirimat. In panel A, HeLa cells were transfected with pNL4-3 and cultured in the absence or presence of indicated concentrations of bevirimat. Two days posttransfection, cells were metabolically labeled for 2 h with [^{35}S]Met/Cys. Virus lysates were immunoprecipitated with anti-HIV antibody. The positions of virally encoded proteins p25 and p24 are indicated. Note the accumulation of p25 in the presence of bevirimat. Panel B is the thin section electron microscope analysis of virions produced from bevirimat-treated or -untreated HeLa cells following transfection with pNL4-3 proviral DNA plasmid. Panel C schematically shows that bevirimat disrupts the CA-SP1 cleavage and blocks the release of mature CA protein.

PR cleaves $Pr55^{Gag}$. These processing events generate the mature Gag proteins matrix (MA), capsid (CA), nucleocapsid (NC), p6, and two small Gag spacer peptides (SP1 and SP2). Gag cleavage triggers a structural rearrangement termed maturation, during which the immature particle transits to a mature virion characterized by an electron-dense, conical core. Among the Gag processing cascade, cleavage of SP1 from the C terminus of CA is the final event required for final CA condensation and formation of the conical core of virus particles [4,7,15].

Virion maturation is essential for the released virus particles to become infectious and initiate a new round of infection. The efficiencies with which PR cleaves the Gag sequences vary widely, resulting in a highly ordered Gag processing cascade, so even partial inhibition of Gag processing profoundly impairs virus maturation and infectivity. For example, alterations of the amino acid sequence at the CA protein (*i.e.*, D51A)[16] or the CA-SP1 cleavage site disrupted the CA-SP1 processing, resulting non-infectious virus particles[1,4,7,17]. Phenotypically, these particles defective in the CA-SP1 cleavage manifest themselves in the presence of an extra CA-SP1 band in some biochemical assays such as Western blotting. Although the mechanisms whereby the CA-SP1 processing is inhibited remain largely unknown, these mutant viruses with a defect in CA-SP1 processing are immature and non-infectious. These observations strongly suggest that the proper conversion of the CA-SP1 to mature CA protein, a final event in virus assembly and release, is a key step in the formation of mature and infectious virus particles, making it an attractive target for therapeutic development.

3. Identification of small molecule inhibitors of HIV-1 maturation

Bevirimat, the prototype HIV-1 MI, was identified in a blind drug-screening assay through analyzing natural products coupled with an activity-directed structural modification effort[18]. Bevirimat, also called PA-457 or DSB or MPC-4326, is a derivative of betulinic acid, a weakly active anti-HIV-1 principle in *Syzigium claviforum*, an herb used in traditional Chinese medicine. When modified by addition of a dimethyl-succinyl moiety at the 3′-hydroxy position to yield bevirimat (3-*O*-(3′,3′-dimethylsuccinyl) betulinic acid) (Fig. 1), *in vitro* anti-HIV-1 activity was increased by >1000 fold[8,18]. Bevirimat has potent antiviral activity against multiple wild-type and drug-resistant clinical HIV-1 isolates with an IC_{50} (50% inhibitory concentration) of about 10 nmol/L[8]. Despite potent activity against HIV-1, bevirimat is inactive against HIV-2 and Simian immunodeficiency virus (SIV).

Initial efforts have identified that bevirimat disrupts a late step in Gag processing (Fig. 3) involving conversion of the CA precursor (CA-SP1) to mature CA[8,19]. Virions from bevirimat-treated cultures are non-infectious and exhibit an aberrant particle morphology characterized by a spherical, acentric core and a crescent-shaped, electron-dense shell lying just inside the viral membrane (Fig. 3). Although bevirimat specifically disrupts CA-SP1 cleavage, it has been shown that the compound does not affect the viral PR function[8,19]. Moreover, consistent with the effect on Gag processing, the determinants of bevirimat activity map to amino acid residues flanking the Gag CA-SP1 cleavage site[8–10,19–22]. Bevirimat represents a novel class of anti-HIV compounds termed MIs that exploit a previously unidentified viral target.

In 2009, nearly 10 years after bevirimat was identified, another chemical compound, 1-[2-(4-tert-butylphenyl)-2-(2,3-dihydro-1*H*-inden-2-ylamino)ethyl]-3-(trifluoromethyl)pyridin-2(1*H*)-one, named PF-46396, emerged as a potent anti-HIV-1 drug candidate through a mechanism similar to what is described for bevirimat[23]. It is intriguing to note that bevirimat and PF-46396 are different in chemical structure but both inhibit HIV-1 replication by disruption of CA-SP1 processing. This new addition to the HIV-1 MI strongly indicates that small molecules with diverse chemical classes can inhibit HIV-1 maturation pathway, further demonstrating the feasibility of CA-SP1 processing as a viable target for anti-HIV drug discovery.

4. Mechanism of action of HIV-1 MIs

4.1. *Genetic determinants of anti-HIV-1 activity of bevirimat*

Early stage studies on bevirimat's mechanism of action were focused largely on extensive characterization of bevirimat-resistant mutants that evolved during serial passage of HIV-1 at a suboptimal drug concentration. This method is considered the gold standard for the identification of mutation-mediated resistance to new drugs toward the elucidation of genetic determinants of antiviral activity. Work by several laboratories including our own group identified six amino acid changes (proximal to CA-SP1 cleavage site) (Fig. 4) that independently confer bevirimat resistance in the absence of any change in viral PR and other regions of Gag[8,10,19,20]. Three substitutions were located at the 1st and 3rd residues of SP1 (A1V, A3V and A3T) and other three substitutions were identified at the extreme C-terminus of CA (H226Y, L231M and L231F). Mutations CA-H226Y, CA-L231F, CA-L231M and SP1-A1V do not impose a significant replication defect on HIV-1 in cell culture. In contrast, mutations SP1-A3V and -A3T severely impaired virus replication and inhibited virion core condensation. The replication defect imposed by SP1-A3V was reversed by a second-site compensatory mutation in CA (CA-G225S). Intriguingly, high concentrations of bevirimat enhanced the maturation of SP1 residue 3 mutants as evidenced by the presence of condensed morphology in the majority of released virions[20]. The increased core condensation observed with SP1-A3V at a high concentration of bevirimat is also consistent with the enhancement in virus replication kinetics seen for SP1-A3V in the presence of the compound. Taken together, the clustering of bevirimat-resistance-conferring mutations at the CA-SP1 site further confirms that CA-SP1 region is the major target for bevirimat (Fig. 4). This conclusion is also supported and extended by a comprehensive analysis of a panel of mutant viruses with point deletions spanning the CA-SP1 cleavage region for their susceptibility to bevirimat treatment[9]. This study showed that bevirimat is a specific inhibitor of CA-SP1 cleavage and identified the CA-SP1 region as the primary viral determinant for this novel inhibitor of HIV-1 replication[9].

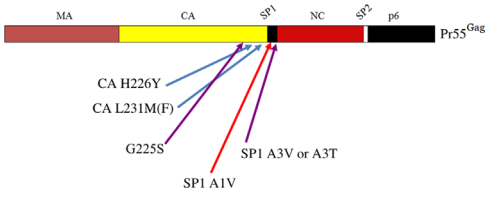

Figure 4 The clustering of bevirimat-resistance-conferring mutation at the CA-SP1 cleavage site.

Cell culture-based drug resistance selection experiments were also performed for PF-46396. Interestingly, a single amino acid substitution in the CA position 201 (CA I201V), distal to the CA-SP1 cleavage site, was found to confer significant resistance to PF-46396[23]. This mutation was also sufficient to confer resistance to bevirimat. On the other hand, bevirimat-resistant SP1-A1V mutation was capable of rendering viruses resistant to PF-46496. Cross-resistance between these two MIs suggests that they act through a similar mechanism, despite that bevirimat and PF-48396 have distinct structures.

4.2. *Antiviral activity by bevirimat is dependent on a high-order oligomeric Gag structure*

Bevirimat exerts its anti-viral effect in cell culture by inhibiting the conversion of CA-SP1 to CA protein. However, the *in vitro* synthetic peptide-based PR cleavage assay, widely recognized as the gold standard screening assay for identifying PR inhibitors, failed to replicate bevirimat-induced defect in CA-SP1 processing. Likewise, an *in vitro* recombinant Gag processing experiment was also incapable of recapitulating the inhibition of CA-SP1 processing by bevirimat. These observations led to a hypothesis by our group and others that bevirimat activity is dependent upon a higher-order Gag structure, which is transiently present during the Gag assembly process but is not formed in either the synthetic peptide or recombinant Gag protein[8,19]. The hypothesis was further supported by other findings[22,24] in that bevirimat-mediated inhibition of CA-SP1 processing could be reproduced in an *in vitro* Gag processing assay if purified HIV-1 immature viral core or assembled HIV-1 Gag chimera containing the Mason-Pfizer monkey virus (M-PMV) p12 domain was provided, respectively, as a substrate. These elegant *in vitro* approaches have played a significant role in better understanding of the mechanism of action of bevirimat.

The distinct phenotypes associated with the mutants described to date imply the existence of multiple mechanisms by which HIV-1 can acquire resistance to bevirimat[20,22,24]. The mutant L231F significantly reduced bevirimat incorporation into immature particles as measured in liquid chromatography–mass spectrometry (LC-MS), indicating that mutations in the CA-SP1 region can confer bevirimat resistance by preventing an interaction between bevirimat and Gag. While this may be a feasible resistance mechanism for some of the mutants isolated, the bevirimat enhancement effect observed for SP1-A3V and -A3T suggests that these mutations do not block bevirimat binding to Gag but rather alter the consequences of binding[20]. The effect of bevirimat binding to these mutants likely promoted, rather than disrupted, virion maturation. An alternative mechanism by which mutations in the CA-SP1 region could confer resistance to bevirimat might be through significantly altering the rate of PR-mediated processing at the CA-SP1 cleavage site to render processing insensitive to bevirimaT[24]. Amino acid substitutions in HIV-1 Gag processing sites have been shown to inhibit or enhance the rate of proteolytic processing. The SP1-A1V mutation caused an increase in the degree of CA-SP1 cleavage relative to wild-type HIV-1 both in cells and in an *in vitro* assembly system, suggesting that the increased CA-SP1 processing rate may contribute to bevirimat resistance development[20,24].

Interestingly, all of the amino acid positions to which bevirimat resistance maps overlap a CA-SP1 region of the Gag protein predicted to exhibit α-helical secondary structure[1,17]. This structure has been visualized as a six-helix bundle within the immature Gag particles[15]. Six-helix bundle of CA-SP1 plays an important role in HIV-1 assembly and budding based on the observation that the introduction of structure-disrupting mutations into this region of Gag results in a defect in virus particle production[1,17]. While the molecular determinant of bevirimat activity maps to this six-helix bundle, the compound exhibits no discernible effect on virus assembly and budding[8,9]. A possible explanation for this observation involves the mechanism(s) by which bevirimat binds the CA-SP1 region in the context of six-helix bundle and disrupts CA-SP1 processing without interfering with the assembly function of the putative structural elements.

4.3. *Molecular target of bevirimat*

The precise mechanism by which bevirimat prevents cleavage of CA-SP1 has not been fully elucidated; however, a large body of experimental data appears to suggest that the CA-SP1 junction of an oligomeric form of Gag within the immature particles is molecular target of bevirimat[8–10,19–22,24,25]. Two working models have been proposed to explain how bevirimat blocks processing of CA-SP1 cleavage site. The first model simply predicts that the compound inhibits CA-SP1 cleavage site by directly blocking access of viral PR to the CA-SP1 cleavage site. The second and perhaps more complex model would be that bevirimat binds to CA-SP1 junction-derived six-helix bundle and alters the conformation of six-helix bundle (stabilizing Gag CA-SP1 structure) so that it becomes refractive to cleavage by viral PR. Two independent lines of evidence seem to support the second model. First, a study of bevirimat-treated HIV-1 viral particles by cryo-electron tomography elegantly demonstrated that the compound likely stabilizes the immature Gag (CA-SP1) lattice through its interaction with Gag[25]. This allosteric effect likely induces a structural transition of CA-SP1 into a stage in which the cleavage of CA-SP1 by viral PR is inhibited. Second, biochemical analysis of bevirimat-treated HIV-1 virions revealed a slow kinetics in the formation of the mature viral core[19].

In addition to unavailable high-resolution structural data for the entire Gag including CA-SP1 junction, another obstacle that has hindered the identification of a molecular target for bevirimat is a lack of a drug–target binding assay. Bevirimat was previously shown to be incorporated into HIV-1 Gag particles but a direct interaction between bevirimat and Gag was not demonstrated[22]. This challenge has not been resolved until an approach with combination of photoactivatable bevirimat-derived analogues and mass spectrometry that was developed in 2011[26]. This approach involves a crosslinking event that covalently attaches photoactivatable bevirimat analogues to immature HIV-1 Gag particles, which is subject to further mass spectrometry analysis. Interestingly, in addition to the CA-SP1 region, the major homology region (MHR) of CA was also identified to interact with bevirimat analogues. The MHR of CA has been recently identified as one of three critical determinants for antiviral activity that is mediated by structurally different PF-46396 that has a similar mode of action to that of bevirimat[27]. Other two determinants are CA amino acid 201 and CA-SP1 junction. Collectively, these results suggest that three regions in the assembled Gag constitute a binding pocket present in a non-linear fashion for the HIV-1 MIs. These data also implicate that bevirimat and PF-46396 interact differentially with this putative pocket[27].

5. Clinical development of bevirimat

The initial results of evaluating bevirimat efficacy in HIV-1/AIDs patients were promising. In multiple phase II clinical trials bevirimat was shown to be both safe and effective with viral load reductions of about 2logs observed in many patients[11,28,29]. These data establish a clinical proof of concept for the HIV-1 MI bevirimat. However, it was also observed in these studies that the antiviral response to bevirimat was not uniform, with some patients experiencing a significant drop in viral load while others showed little or no reduction[11]. Genotypic analyses of these nonresponsive patients' HIV-1 isolates identified three key polymorphic sites in SP1 (Q6, V7 and T8) involved in bevirimat resistance (Fig. 5). These three residues (QVT) are referred to the Gag SP1 polymorphism motif as a whole. Additional bevirimat resistance polymorphism has been identified in CA amino acid 362 in patients without prior exposure to bevirimat[30] (Fig. 5). Intriguingly, these bevirimat resistance-conferring mutations in natural HIV-1 isolates were not identified in cell culture-based drug resistance selection experiments. Further studies have showed that V7 polymorphism is a primary determinant of naturally occurring resistance to bevirimat and is responsible for the majority of instances where a lack of sensitivity to bevirimat was observed[31,32]. Prevalence of V7 polymorphism as high as 50% in HIV-1 isolates particular in non-B subtypes had made $>50\%$ of HIV-1/AIDS patients not responding to bevirimat treatment in clinical trials, even though these patients had sufficient plasma concentrations of the compound. As a result of these observations, clinical development of bevirimat was discontinued in June 2010.

6. Second generation HIV-1 MIs

As a first-in-class MI, bevirimat has several unique features including potent antiviral activity, novel mechanism of action, effectiveness against HIV-1 strains resistant to currently approved antiretroviral drugs, and attractive pharmacological and safety profiles as demonstrated in both animal models and human trials. These desirable attributes have promoted several industry groups to develop second-generation HIV-1 MIs with the primary goal of overcoming the challenge of drug resistance associated with naturally occurring Gag polymorphisms that has halted further clinical development of bevirimat. Through extensive medicinal chemistry efforts, a number of lead compounds in second-generation class of HIV-1 MIs with improved activity against viruses containing SP1 polymorphisms were discovered and are currently under clinical development at various stages.

DFH Pharma has identified a number of second-generation bevirimat-derived HIV-1 MIs (http://www.croiconference.org/). Preliminary data showed that these bevirimat derivatives with modifications at the C-28 position overcame bevirimat resistance conferred by Gag SP1 polymorphisms, especially those occurring at SP1 residue 7. Bristol-Myers Squibb (BMS) recently described a potent second generation inhibitor, dubbed as BMS-955176, which is very active against bevirimat-resistant SP1 polymorphic viruses (http://www.bms.com/). BMS-955176 just completed its antiviral efficacy in a pilot clinical trial with 60 patients enrolled. Despite the chemical structure of this promising inhibitor unavailable, the new drug reduced HIV-1 viral load by almost two orders of magnitude in a 10-day monotherapy trial involving Clade B patients. A clinical trial involving Clade C patients is currently ongoing and is expected for the completion later in 2015. BMS-955176 is very safe without causing any serious consequences to the patients. GlaxoSmithKline (GSK) has also invested extensive effort and time in developing second-generation HIV-1 MIs (http://www.gsk.com/). One leading compound GSK 2828232 has an IC_{50} of 0.8–4.3 nmol/L against a panel of 26 HIV-1 isolates that contain SP1 polymorphisms including those present at SP1 7 position. GSK 2828232 has recently entered multiple-dose Phase I studies for examining its safety and food and drug interactions in healthy volunteers.

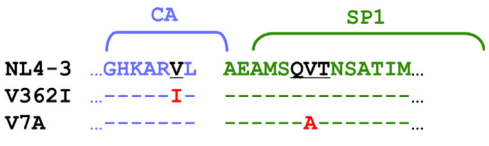

Figure 5 Gag CA-SP1 polymorphisms that are associated with bevirimat resistance in HIV/AIDS patients. Two polymorphism sites found in patients (Gag 362 position in CA and SP1 6-8 positions QVT) are highlighted with the underlined black letters. Among them, SP1 V7A polymorphism is a primary determinant of non-responsiveness in patients to bevirimat treatment in clinical trials.

7. Conclusions and future prospects

After an exhaustive five-year research, the HIV-1 MI program has achieved a significant milestone. Several leading drug candidates in the second-generation MI family have successfully overcome Gag CA-SP1 polymorphisms-associated resistance problems that have long challenged and eventually led the discontinuation of the clinical development of bevirimat. These new MIs are currently at various stages of the clinical development. Clinical profile of safety and pharmacokinetics is very desirable that certainly justifies the further clinical development of these second generation inhibitors. If successfully in future clinical trials, HIV-1 MIs will emerge as a new class of antiretroviral armamentarium for treatment of patients, especially those who harbor viruses resistant to all approved drugs.

Safety and efficacy demonstrated by past and present HIV-1 MIs with diversified chemical structures clearly reinforce that the Gag protein, a primary driving force of virus assembly and maturation, can be further explored as targets for development of HIV/AIDS therapeutics. During the maturation process, multiple cleavage events including CA-SP1 sequentially take place. The identification of the CA-SP1 cleavage site as a validated drug target clearly opens the door to other cleavage sites. Consistent with this prediction, a high-throughput screening assay for the identification of HIV-1 Gag MA-CA cleavage site has been developed and recently used in screening 24,000 compounds[33]. While none of the compounds discovered were active in inhibiting the MA-CA cleavage in the secondary assay, extension of the screening effort to more diverse chemical libraries may enhance a chance to discover potent inhibitor of this cleavage event.

An intricate interplay between Gag and PR is the primary determinant of proper HIV-1 maturation, which is exemplified by documented coevolution of PR and its Gag substrate.

Mutations in PR caused by either low-fidelity viral reverse transcriptase or use of PIs in treating AIDS patients seem to drive the emergence of mutations in Gag cleavage sites or Gag regions distal to cleavage sites. Primary mutations in PR, and corresponding (compensatory) mutations in Gag, work in concert to maintain critical interactions between mutated PR and Gag proteins, ensuring

proper virus maturation and replication fitness, in addition to allowing HIV-1 to evade PI-mediated suppression. Interestingly, extensive characterization of bevirimat-resistant viruses revealed multiple mutations occurring only in the CA-SP1 region of Gag, not in PR. This result is consistent with the observations that Gag mutations alone have little or no substantial effects on susceptibility of HIV-1 to PIs. Nevertheless, the impact of PR mutations on the development of HIV-1 resistance to bevirimat is not clearly understood, which should be investigated in future study.

Identification of residues in Gag that comprises the putative binding pocket of MIs represents a significant advance in the field. As with the traditional approach to elucidate the precise mechanism of action and identify a binding pocket of a drug, a real-time visualized complex consisting of the MI and its target (*i.e.*, assembled Gag), either through the crystallography or high-resolution electron tomography or nuclear magnetic resonance approach, is eventually needed to better define the molecular basis for MI activity as well as to conduct a rational-based design of new MIs with improved potency and breadth of antiviral activity.

Biophysical tools that measure a drug–Gag interaction are largely dependent on mass spectrometry or radiolabeling assay. In either case, it only detects a physical association between drug and Gag in an indirect fashion. Another shortcoming is that two assays are not very robust and have a limitation in the high-throughput potential. Direct binding assays, such as free-label surface plasmon resonance-based biosensors (*i.e.*, Biacore) or isothermal titration calorimetry, should be developed and harnessed to study HIV-1 MIs. These assays can provide real-time measurements for kinetics and thermodynamics of drug-Gag interactions and offer novel insights on the mechanisms of action and resistance for MIs that are currently in clinical trials.

Another immediate need is to further understand resistance mutations and patterns that will evolve in response to treatment of patients by second-generation inhibitors. It has been well established that new inhibitors are active against viruses containing CA-SP1 polymorphisms (Gag 362 and SP1 7 positions) that failed the first generation inhibitor bevirimat. It is still not clear which polymorphisms or mutations in HIV-1 Gag can render viruses non-responsive to second-generation MIs. This issue can be complex because several drug candidates with diverse structures may bind to Gag differently and mutations that will arise in patients may get diversified. Drug resistance information in terms of individual mutations, mutation patterns and at which frequency resistant viruses emerge in patients is perhaps not available until clinical trials that get scaled-up with extended dosing period.

In summary, MIs are a new class of HIV drugs with an attractive clinical development profile. Clinical proof-of-concept demonstrated by the first-in-class MI bevirimat and reappearance of second-generation drug candidates with improved breadth and potency in clinical trials strongly suggest that in the near future multiple MIs can be approved and used for treatment of HIV/AIDS patients.

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