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Maturation Inhibitors: a New Therapeutic Class Targets the Virus Structure

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Abstract

The current standard of care for HIV/AIDS in the developed world is HAART therapy, usually a combination of two reverse transcriptase inhibitors and a protease inhibitor. Despite the success of this regimen, there is a continuing need for new drug options to overcome problems with tolerability and the emergence of viral resistance. In this review we discuss the discovery of a potential new class of antiretroviral therapeutics, known as maturation inhibitors, and the development of the first-in-class compound, bevirimat. Bevirimat is distinguished from the currently available antiretrovirals by its unique target and mode of action. While the specific interactions responsible for activity have yet to be fully characterized, it is clear that the target for bevirimat is the Gag polyprotein precursor, the main structural protein responsible for assembly and budding of virion particles. As basic research continues on the precise mechanism of action of bevirimat, clinical development is progressing, with demonstration of both safety and efficacy in early-stage trials. These encouraging results, coupled with the discovery and development of future generations of maturation inhibitors, suggest that maturation inhibitors may be added to the growing set of tools available to control HIV/AIDS. **(AIDS Rev. 2007;9:162-72)**

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Key words

HIV. Inhibitor. Virus assembly. Gag protein. Drug development.

Introduction

Virtually all currently available therapeutic inhibitors of HIV replication target the enzymatic components of the virion. Reverse transcriptase inhibitors, whether chain-terminating nucleoside/nucleotide analogs or nonnucleoside allosteric inhibitors, target the viral replicase, while protease inhibitors target the enzyme responsible for cleavage and functional release of both the enzymatic and structural components of the infectious virion. Integrase inhibitors, which are in various stages of clinical development, target the enzyme responsible for insertion of the retroviral genome into the cellular DNA. Currently, the one notable exception to these enzyme inhibitors is a class of drugs known as entry inhibitors that target either the viral envelope glycoprotein (Env) or one of three receptor proteins on the cell surface. The receptors can be the primary receptor, CD4, or one of two main coreceptors – either the CCR5 or CXCR4 chemokine receptor. Entry inhibitors work either as traditional ligand antagonists, or in the case of the approved drug enfuvirtide, as a steric inhibitor of the viral Env protein conformational change necessary for fusion of the viral and cellular membranes. A new class of inhibitors is now emerging that targets the internal structural precursor protein, Gag, and its function in final assembly of the mature, infectious virion; these are the maturation inhibitors. This new class is typified by the compound 3-O-(3',3'-dimethylsuccinyl)-betulinic acid, known alternatively as bevirimat[1], PA-457[2], DSB[3,4], or YK-FH312[5].

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Discovery

Bevirimat emerged out of an effort to screen natural products for antiviral activity. From bioactivity-directed fractionation of an extract of *Syzigium claviflorum,* the

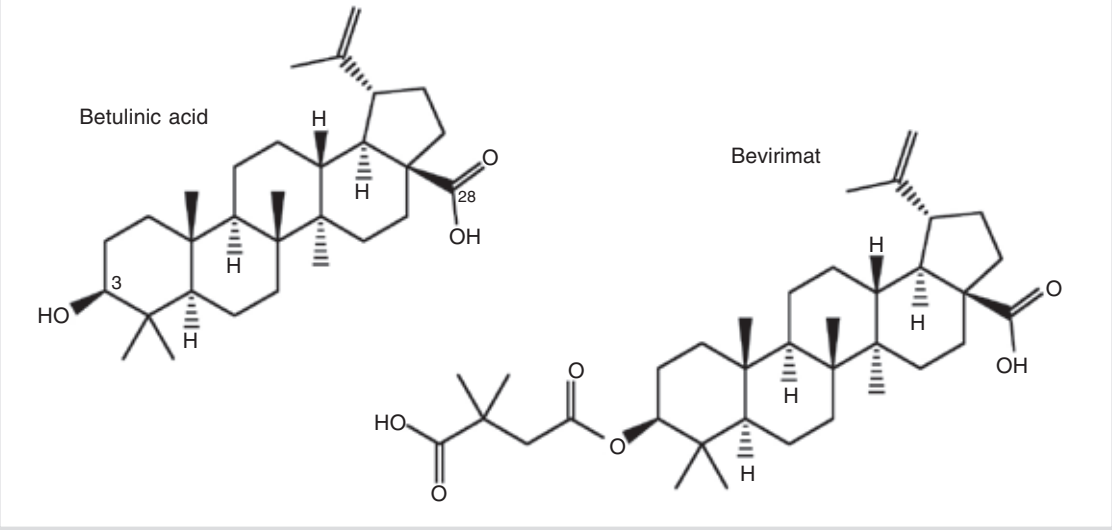


Figure 1. *Structures of betulinic acid and bevirimat. The triterpenoid with modest anti-HIV activity, betulinic acid, was originally identified from an extract of* Syzigium claviflorum, *a traditional Taiwanese medicinal plant. Betulinic acid can also be easily converted from betulin, which is a major component of the bark from the white birch tree, genus* Betula, *and thus is relatively abundant. Betulinic acid is shown with the C-3 and C-28 carbons marked. Bevirimat is the 3-O-(3',3'-dimethylsuccinyl) derivative of betulinic acid.*

lupane-type triterpenoid, betulinic acid (Fig. 1) was identified as an anti-HIV principle in viral replication assays[6]. As the employed screen was non-deterministic, the mechanism of betulinic acid inhibition was not immediately clear. Interestingly, betulinic acid had previously been identified as a potential cancer chemotherapeutic, with significant activity against melanoma in both cultured cells and in a tumor growth model in athymic mice[7]. Subsequent analysis revealed activity against a broader spectrum of tumor types (reviewed[8]). Structure-activity analysis of betulinic acid for HIV inhibition led to a focus on acyl derivatives of the C3 hydroxyl (Fig. 1) yielding the compound 3-*O*-(3',3'-dimethylsuccinyl) betulinic acid, now termed bevirimat, with an *in vitro* therapeutic index of greater than 20,000[9]. Structurally related molecules such as 3-*O*-(3',3'-dimethylsuccinyl) betulin, with a hydroxymethylene instead of a carboxylic acid at position 28 or 3-*O*-(3',3'-dimethylglutaryl) betulinic acid showed comparable activity, with the betulin derivative also yielding a therapeutic index greater than 20,000[10]. These compounds could serve as leads to subsequent generations of HIV therapeutic inhibitors (see Future Directions).

Separate studies had identified triterpenes as inhibitors of HIV-1 protease by potentially blocking active dimer formation[11]. Unlike the cellular aspartic proteases, which are single polypeptide chains, retroviral proteases are composed of two identical subunits and only the homodimer is the active form[12]. However, enzymatic tests against protease indicated the activity of betulinic acid in the µM range, far above the reported *in vitro* effective concentration for bevirimat of less than 1 nM[10]. Further mechanistic analysis failed to reveal a mode of action, but ruled out activity against reverse transcriptase or other early events in the infectious cycle such as fusion or integration[9,10,13], leaving the late events of assembly or maturation as possible targets.

Assembly of HIV particles

A full understanding of the bevirimat mechanism of action first requires a detailed knowledge of its target, Gag. Gag is the fundamental structural protein of the virus, which undergoes a series of complex interactions both with itself and with other cellular and viral factors to achieve the assembly of infectious virions[14]. HIV assembly is a two-stage process involving an intermediate immature capsid that, upon budding from the plasma membrane, undergoes a morphologically dramatic maturation step to yield the infectious particle (Fig. 2). This transformation is mediated by the viral protease, which multiply cleaves the Gag polyprotein precursor, allowing the liberated components to reassemble to form the core of the mature virion (reviewed[15]). The structure of the resulting conical HIV core has been the subject of intensive scrutiny. Not only has the core of budded particles been analyzed in detail by electron[16] and cryoelectron microscopy[17-19], but several *in vitro* assembly systems have been developed that imitate cone formation by the assembly of tubes[20-23] and even

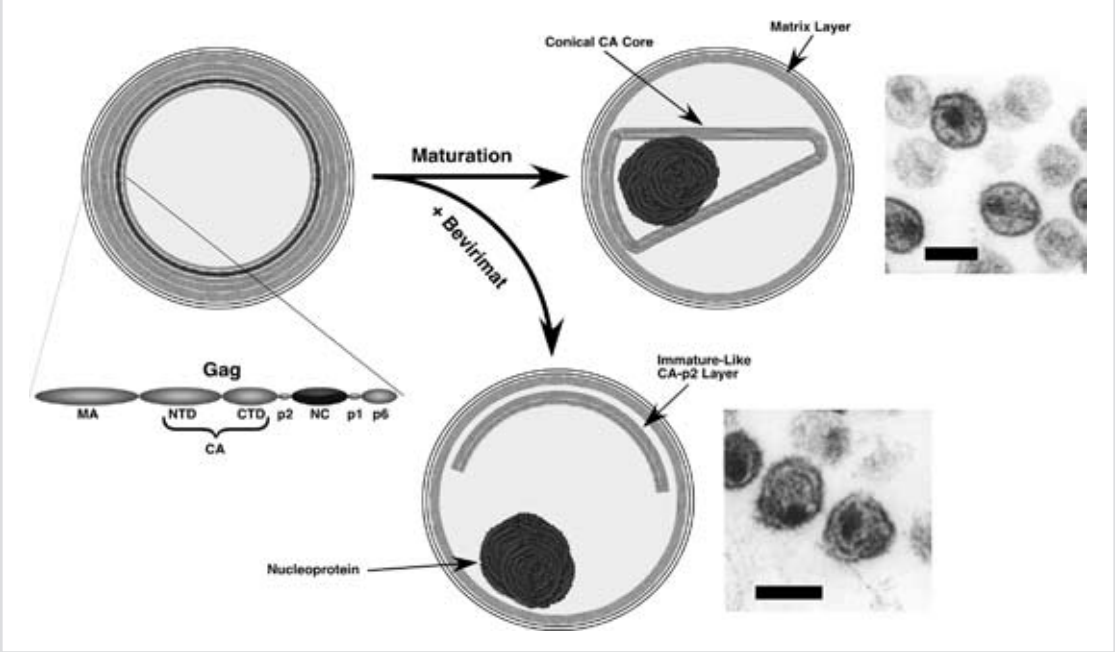


Figure 2. *Morphological consequences of maturation inhibitor action. The immature HIV particle (top left) is composed in the majority of radially arranged Gag precursor proteins with their N-terminal matrix (MA) domains in close association with the inner leaflet of the viral membrane and the remaining C-terminal domains forming concentric rings toward the center of the particle. The nucleocapsid (NC) domain, which is responsible for RNA binding and packaging, is depicted as the darker ring. An enlarged diagram of Gag is provided with each domain labeled. The CA domain is depicted in its two components, the N-terminal domain (NTD) and C-terminal domain (CTD). Timely processing of the Gag cleavage sites results in the liberation and reassembly of the Gag domains into a mature infectious particle (top right), containing a dense nucleoprotein composed of NC and RNA surrounded by a cone-shaped core assembled from CA. The MA remains as a matrix layer associated with the viral membrane. In the presence of the maturation inhibitor, bevirimat, processing to produce fully matured CA is delayed or blocked, resulting in the formation of an aberrant, noninfectious particle, displaying partial immature morphology (bottom). In this interpretation, the concentric partial ring of density seen in electron micrographs of such particles is depicted as being composed of p25 (CA-p2). Thin section electron micrographs of particles produced in the absence or presence of bevirimat are provided to the right of the respective diagrams. Bars equal approximately 100 nM.* (Micrographs are reproduced from reference[2]; Copyright 2003, National Academy of Sciences, USA.)

morphologically authentic cones[24]. Analysis by cryoelectron microscopy of these structures produced *in vitro* has led to the development of a fullerene model for the HIV core[17,25,26].

The immature virion is composed primarily of polyprotein precursors and appears in thin-section electron microscopy as a particle composed of concentric rings of varying density surrounding an electron lucent center (reviewed[27]). These immature particles, like their mature counterparts, can vary greatly in mass and diameter, reflecting variability in the number of structural precursor molecules[28-30]. The pleomorphic nature of immature retrovirus particles has made traditional crystallographic atomic-level structural analysis impossible. However, cryoelectron microscopy has revealed a radial arrangement of densities within the primary structural protein Gag[28,30]. The order of densities from exterior to interior corresponds with the Gag domains in their N to C terminal positions within the precursor protein: N– matrix (MA), the two domains of capsid (CA), and nucleocapsid (NC) –C. The correspondence of primary elements in immature particles to the Gag polyprotein precursor is consistent with the widely confirmed result that Gag is the only viral gene product necessary for such particles to assemble and bud from the cell (reviewed[15,31]). Systems using purified fragments of Gag[32-37] or Gag synthesized *in vitro*[38,39] to form immature particle-like structures have further confirmed Gag as the only essential protein of the assembled immature particle.

During or following budding the activated viral protease cleaves the polyprotein precursors, Gag and Gag-Pro-Pol into their constituent domains. These now-matured proteins are then free to reassemble to form the structures of the infectious virion. Maturation of the precursor proteins begins by intramolecular cleavage of Gag-Pro-Pol[40] and continues by a process of ordered cleavages governed by differences in the inher-

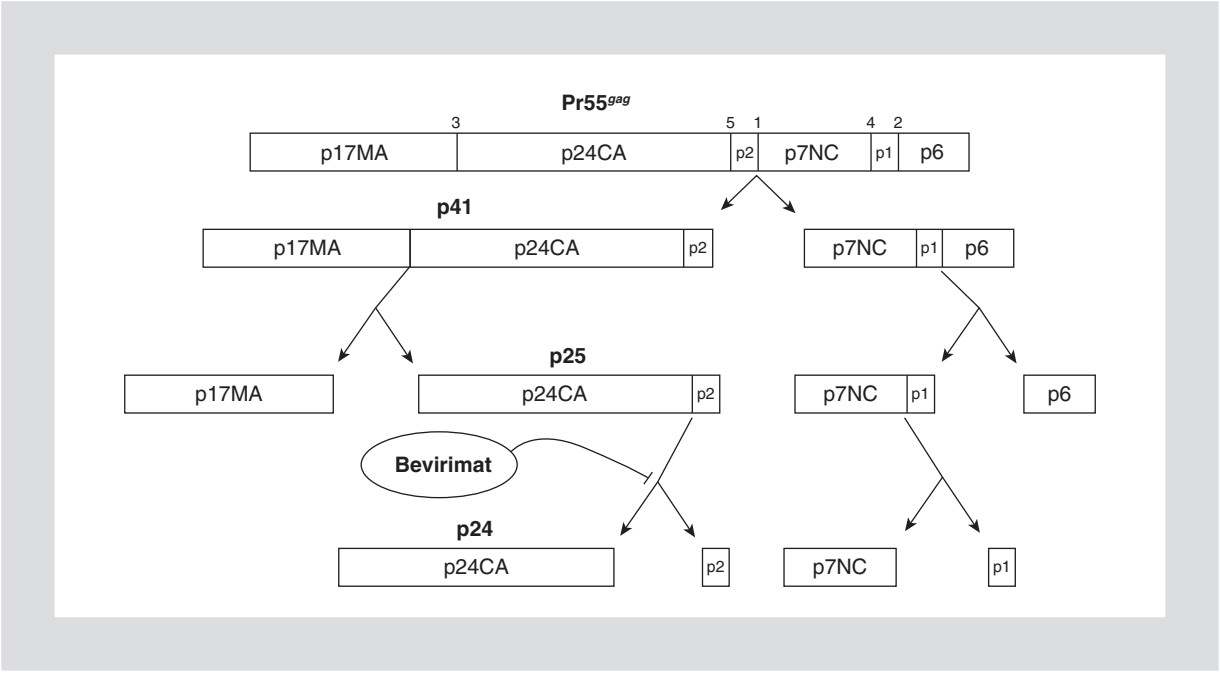


Figure 3. *Maturation inhibitor mechanism of action. The processing of the Gag polyprotein precursor proceeds via ordered cleavages by the viral protease. The order, indicted at the top of the figure by the numbers over the cleavage sites, is determined partly by the intrinsic processing rate of each cleavage recognition sequence and partly by preceding cleavages at neighboring sites. The ultimate release of fully matured capsid protein (CA) is critical to proper formation of infectious particles and proceeds via a series of three cleavages: first of Pr55 to yield p41, next by the generation of p25, and finally by the release of p2 to form p24CA, as shown. It is the last of these cleavages that is blocked by the maturation inhibitor, bevirimat.*

ent processing rates at each cleavage site (Fig. 3)[41-45], which for HIV Gag can vary up to 400-fold[43,44]. The ordered processing suggests that a regulated cleavage cascade is necessary for proper virion maturation and, therefore, infectivity. Indeed, mutations that either abrogate or alter the rate of cleavage of HIV Gag lead to the formation of aberrant, noninfectious particles[44,46].

Bevirimat mechanism of action

Initial reports on the mechanism of action for bevirimat indicated that the inhibitor acts at the stage of virion assembly and/or budding, but these results were observed at a concentration approximately 100-times the *in vitro* IC_{50}[5]. As the compound moved toward clinical trials the necessity for a detailed mechanistic understanding grew more urgent and additional *in vitro* culture analyses were undertaken to identify the target. Specifically, the steps of virus assembly, release, and maturation were examined in detail, revealing a subtle but dose-dependent change in the viral protein profile in both cells and released particles treated with bevirimat[2,3]. The amount of the fully mature CA protein, p24, was reduced, while its immediate precursor, p25, was increased. As discussed above, the processing of Gag is a cascade of cleavages regulated by the residues flanking the scissile bond. The slowest and thus last of these cleavages is the one within p25 between CA and the spacer peptide, p2 (also known as SP1; Fig. 3). While the effect of the compound seemed modest by analysis of the Gag protein, in terms of morphology the consequences were dramatic (Fig. 2). Rather than the expected mature particles with conical cores surrounding the electron-dense nucleoprotein, thin-section electron microscopy of bevirimat-treated cultures revealed aberrant structures with partly immature-like rings of density and an acentric nucleoprotein[2]. This morphology was highly reminiscent of particles produced by a mutant containing a cleavage refractive sequence between CA and p2[45].

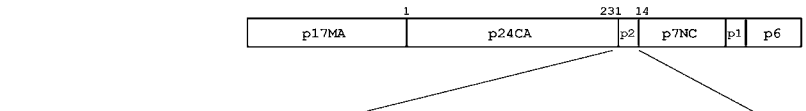
The importance of the CA-p2 cleavage site was further demonstrated by the *in vitro* selection of the first bevirimat-resistant mutants. Serial passage of HIV-1_{NL4-3} in the presence of the inhibitor yielded a virus containing a single residue substitution of valine for alanine at the N-terminal residue of p2 (A1V)[2], or phenylalanine for leucine at the C-terminal residue of CA (L231F)[3]. Complete sequencing of Gag and protease, and the demonstration of comparable virus resistance to bevirimat after introduction of these single mutations into the wild-type background strongly suggested that the target was Gag rather than protease and ruled out the involvement of other viral proteins. Yet, it could not be excluded that the inhibitor somehow altered protease

function such that only the last and slowest cleavage site (CA-p2) was affected. Even the development of the resistance mutation A1V could be interpreted as evasion of altered protease function, since this mutation increases the efficiency of cleavage[1,47].

Typically, at this stage of an investigation into an inhibitor's mechanism of action, crystallographic or at least *in silico* analysis of the compound bound to its target is undertaken to understand the molecular interactions responsible for activity, with an eye towards rational improvement in the chemical structure. Unfortunately with this new class of HIV therapeutics, the necessary structural data for the target is unavailable. Although atomic-level structural information for a substantial proportion of the Gag precursor has been obtained, either by crystallography or nuclear magnetic resonance (NMR), little such information is available for the region flanking the CA-p2 cleavage site; the very C-terminal part of CA is disordered in crystals[48,49]. However, modeling studies predicted an α-helical structure for the 13 amino acids spanning the cleavage site (Fig. 4)[50], which is consistent with subsequent NMR analysis of a Gag fragment containing the CA C-terminal domain (CTD), p2, and NC, where p2 and the flanking 13 residues in both CA and NC were found to be flexible with a small propensity to form an α-helix[51]. Further NMR analysis of a Gag fragment, CA-L211 to NC-V13, dissolved in 30% trifluoroethanol yielded a stable amphipathic helix for the region encompassing p2, from CA-G225 to NC-M1, that could be modeled into a trimer[52]. This helix could be extended further into NC by the binding of stem loop three (SL3) of the viral RNA packaging signal, suggesting a potential role for RNA in promoting the Gag structure recognized by bevirimat. A competing model has recently been proposed with p2 as a bundle of six helices fit into the structure of Gag derived from a cryotomographic analysis of immature virions[53]. Although the resolution was insufficient to make the fit precisely, the six-helix bundle could be logically placed within an envelope of density directly below the center of the CA hexamer ring.

While the stoichiometry of the helices, possibly a trimer or a hexamer, is not yet known, the requirement for a higher-order oligomeric Gag structure of some type for bevirimat activity has been established. An initial failed attempt to replicate inhibitor activity with purified recombinant Gag suggested that an oligomeric or assembled state of Gag is required for inhibition of CA-p2 processing[2]. Consistent with that result, bevirimat could be efficiently incorporated into HIV-1 pseudovirions, but not into mutant pseudovirions carrying amino acid substitutions at positions P1 and P2 of the CA-p2 cleavage site mimicking the sequence of SIV, which is resistant to bevirimat[54]. Confirmation that an immature particle-like assembled state is necessary for maturation inhibitor activity came from studies utilizing *in vitro* synthesized chimeric Gag molecules capable of self-assembly[47]. Dose-dependent inhibition of CA-p2 processing by bevirimat was evident when assembled Gag was the substrate in processing experiments, but not when unassembled Gag was the substrate. Thus, a higher-order assembled Gag structure is likely recognized and bound by bevirimat, but the precise nature of this oligomeric Gag is yet to be determined.

Further evidence for a direct interaction between bevirimat and Gag comes from several analyses of mutants. Both site-directed mutagenesis and the selection and mapping of resistance mutations have identified the residues flanking the CA-p2 cleavage site as critically important for inhibitor activity (summarized in Fig. 4). Both residues immediately adjacent to the scissile bond were found altered in the earliest reports of resistant mutants grown in culture. For both of these mutants, L231F and A1V, introduction of the single residue change into a wild-type background resulted in resistant phenotypes, indicating that no other changes in the virus, either in protease or in the remainder of Gag, were required for resistance[2,3]. These first reports also highlighted the specificity of bevirimat for HIV-1, since HIV-2 and SIV were both resistant. However, a three-residue substitution in SIVmac239 that essentially converted the cleavage site into that of HIV-1 was sufficient to render the virus susceptible to bevirimat[55]. Further site-directed mutational analysis of the P1 and P1' positions revealed that virtually all substitutions tolerated by the virus, meaning that infectious progeny could be produced, yielded a resistant phenotype[56]. A less deterministic approach of exhaustively screening for mutations that developed in culture yielded only four critical residue positions for resistance: CA-H226 and -L231, and p2-A1 and -A3 (Fig. 4)[1]. The spectrum of changes permissible in these positions was limited, with only single substitutions to tyrosine and valine allowed at positions CA-226 and p2-1, respectively, and only two changes each permitted at positions CA-231 and p2-3: to phenylalanine and methionine, and to threonine and valine, respectively. The limited positions occupied by resistance mutations could be interpreted as the sites of critical molecular contacts with the inhibitor, while the specific substitutions may represent the functional limitations for this region of Gag. Of significance to the potential development of resistance in bevirimat-treated patients, these four positions are highly conserved in HIV-1, implying that any changes may carry a fitness cost for the virus[1]. Indeed, mutations A3T and A3V imparted a partial replication defect on the virus that in turn was compensated by a second site mutation, G225S. Without the second site change, mutant virus was found to be largely morphologically immature. Interestingly, the A3 mutants displayed im-



Mutant	Created	CA G H K A R V L	p2 A E A M S Q V T N P A T I M	Resistance	Reference
HIV wt(NL4-3)		- - - - - - -	- - - - - - - - - - - - - -	-	
ΔG225	E	Δ - - - - - -	- - - - - - - - - - - - - -	n	57
ΔH226	E	- Δ - - - - -	- - - - - - - - - - - - - -	n	57
H226Y	R	- Y - - - - -	- - - - - - - - - - - - - -	+	1,56
ΔK227	E	- - Δ - - - -	- - - - - - - - - - - - - -	n	57
ΔA228	E	- - - Δ - - -	- - - - - - - - - - - - - -	n	57
ΔR229	E	- - - - Δ - -	- - - - - - - - - - - - - -	n	57
ΔV230	E	- - - - - Δ -	- - - - - - - - - - - - - -	n	57
ΔL231	E	- - - - - - Δ	- - - - - - - - - - - - - -	n	57
L231F	R	- - - - - - F	- - - - - - - - - - - - - -	+	1,3,55,56
L231M	R	- - - - - - M	- - - - - - - - - - - - - -	+	1,56
L231C	E	- - - - - - C	- - - - - - - - - - - - - -	+[a]	47
L231N	E	- - - - - - N	- - - - - - - - - - - - - -	n	56
L231Y	E	- - - - - - Y	- - - - - - - - - - - - - -	+	56
V230L/L231M	E	- - - - - L M	- - - - - - - - - - - - - -	+	55,56
ΔA1	E	- - - - - - -	Δ - - - - - - - - - - - - -	n	57
A1V	R	- - - - - - -	V - - - - - - - - - - - - -	+	1,2,57,56
A1F	E	- - - - - - -	F - - - - - - - - - - - - -	+	56
A1I	E	- - - - - - -	I - - - - - - - - - - - - -	+	56
A1L	E	- - - - - - -	L - - - - - - - - - - - - -	+	56
A1M	E	- - - - - - -	M - - - - - - - - - - - - -	+	56
A1Y	E	- - - - - - -	Y - - - - - - - - - - - - -	n	56
ΔE2	E	- - - - - - -	- Δ - - - - - - - - - - - -	+	57
ΔA3	E	- - - - - - -	- - Δ - - - - - - - - - - -	+	57
A3V	R	- - - - - - -	- - V - - - - - - - - - - -	+[b]	1
G225S/A3V[c]	R	S - - - - - -	- - V - - - - - - - - - - -	+	1
A3T	R	- - - - - - -	- - T - - - - - - - - - - -	+[b]	1
G225S/A3T[c]	E	S - - - - - -	- - T - - - - - - - - - - -	+	1
ΔM4	E	- - - - - - -	- - - Δ - - - - - - - - - -	+	57
ΔS5	E	- - - - - - -	- - - - Δ - - - - - - - - -	+	57
ΔQ6	E	- - - - - - -	- - - - - Δ - - - - - - - -	+	57
ΔV7	E	- - - - - - -	- - - - - - Δ - - - - - - -	+	57
ΔT8	E	- - - - - - -	- - - - - - - Δ - - - - - -	+	47,57
ΔN9	E	- - - - - - -	- - - - - - - - Δ - - - - -	-	57
ΔP10	E	- - - - - - -	- - - - - - - - - Δ - - - -	-	57
ΔA11	E	- - - - - - -	- - - - - - - - - - Δ - - -	-	57
ΔT12	E	- - - - - - -	- - - - - - - - - - - Δ - -	-	57
ΔI13	E	- - - - - - -	- - - - - - - - - - - - Δ -	+	47,57
ΔM14	E	- - - - - - -	- - - - - - - - - - - - - Δ	+	47,57
SIV wt(mac239)		- Q - - - L M	- - - L K E A L A - V P - P	+	3,55
SIVm2	E	- Q - - - - -	- - - L K E A L A - V P - P	+	55
SIVm3	E	- Q - - - - -	- - - - K E A L A - V P - P	-	55,54

Figure 4. *Summary of mutational analysis of the CA-p2 cleavage region. All identified resistance and engineered mutations are shown below a schematic for Gag, with cleavage site residue numbers indicated for CA and p2. Below the amino acid sequence of the cleavage region the putative cleavage-site-spanning α-helix is indicated in* ***underlined bold.*** *Also shown at bottom are the versions of simian immunodeficiency virus (SIV) cleavage regions examined. Columns are as marked: Mutant, mutation designation; Created, R for originally developed through resistance in culture and E for engineered; Resistance, – for sensitive, n for not determined due to lack of particle production, and + for some degree of resistance noted either in culture or in* in vitro *cleavage assays; and Reference, provides the reference numbers for studies describing each mutant. [a]Mutant L231C is particle production defective and was therefore only analyzed in the* in vitro *assembly system. [b]These mutants are partly dependent on bevirimat. [c]This second-site mutation, G22S, partly compensates for the impaired replication of the single A3V and A3T mutants.*

proved replication kinetics in the presence of inhibitor and thus partial dependence, suggesting an accommodation for the bevirimat molecule within the complex structure of assembled Gag[1].

As might be expected for mutants that remain morphologically immature, the A3 position resistant mutants were processed more slowly than wild-type virus. This is in contrast to the initially described resistance mutations, L231F and A1V, which resulted in more rapid or complete cleavage of the CA-p2 junction[2,3], but is consistent with data from the site-directed L231C mutant[47]. Changing the P1 residue to cysteine was

previously shown to slow CA-p2 processing[43]. Since this mutation also rendered the virus defective for particle release, it could only be assessed *in vitro,* where it was indeed processed more slowly, but was also partly bevirimat resistant[47]. Resistance to bevirimat is therefore not strictly correlated with the rate of cleavage and more likely results from loss of direct inhibitor interaction with Gag.

How then does bevirimat block processing of the CA-p2 cleavage site? The simplest mechanism would be occlusion of the scissile bond: bevirimat blocks access of protease to the cleavage site. Alternatively, bevirimat could induce or stabilize a regional conformation that is refractive to cleavage by protease. The most obvious candidate for this structure would be the proposed α-helix. Whether trimeric or hexameric, stabilization of the helix would be expected to reduce the accessibility of the scissile bond to the protease active site, which requires an extended conformation. This idea is consistent with a previous proposal that the p2 region acts as a regulator of CA-p2 cleavage and thus of virion maturation[44]. Evidence to support this more complex mechanism of action comes from careful analysis of a deletion scan across the CA-p2 region[57]. Single residue deletions were made from CA-G225 to p2-M14 (Fig. 4). The phenotypes of these mutants varied widely. Consistent with previous reports regarding the critical role for the C-terminus of CA in assembly, deletions from G225 to A1 were defective for particle production[50,58]. Deletions from E2 to V7 conferred resistance to bevirimat in processing assays, while deletions of N9 to T12 did not. Deletion of T8 provided partial resistance. The behavior of these mutants can be interpreted as either directly interfering with binding of the inhibitor or interrupting the helical structure necessary for that binding, with deletions C-terminal to T8 being outside the critical α-helix. Interestingly, mutants ΔI13 and ΔM14 also conferred resistance. When analyzed both in culture and *in vitro*, these mutants were processed more rapidly than wild-type[47,57] and also conferred resistance to bevirimat roughly in proportion to the extent of cleavage[47]. Mutations of the p2-NC cleavage site that block processing were previously found to increase the rate of cleavage at CA-p2, which suggested the aforementioned idea that p2 acts as a regulator of CA processing[43]. It is not clear how a lack of processing at p2-NC accelerates cleavage at CA-p2, but it is unlikely to be due to direct effects of side chain alterations on protease recognition, since these deletions are far removed from the CA-p2 cleavage site. The effect of these "action at a distance" mutants is consistent with the idea that a complex structure, likely incorporating an α-helix, is required both for proper control of cleavage and for maturation inhibitor binding and activity.

Preclinical studies

In vitro testing has demonstrated that bevirimat is a potent inhibitor of virus replication, with a mean IC_{50} value of approximately 10 nM against primary HIV-1 isolates[2]. Like many drugs, bevirimat is highly protein bound in the presence of human serum. Serum shift experiments evaluating the effect of human serum on the *in vitro* antiviral activity of bevirimat estimated the protein binding to be approximately 99%, with serum albumin primarily responsible for the binding. From these experiments, the *in vitro* protein-binding-adjusted IC_{90} value was estimated to be approximately 4 μM (unpublished results). Consistent with its novel target, bevirimat retains potent activity against virus isolates that are resistant to currently approved drugs, including reverse transcriptase inhibitors, protease inhibitors, and entry inhibitors[2,59]. Bevirimat is also either additive or synergistic with approved drugs when tested in *in vitro* combination studies[59]. These characteristics make this new class of therapeutics especially attractive for drug development.

Bevirimat has successfully completed a series of nonclinical toxicology studies. These include standard toxicology studies of six-months duration in the rat and nine-months in the marmoset. In these studies there were no findings in either species that would limit the further development of bevirimat (unpublished results). In addition, the standard panel of reproductive toxicology studies has been completed. These include assessment of the potential for developmental toxicity in the rat and rabbit, assessment of fertility in male and female rats, and a peri- and post-natal study in the rat aimed at detecting potential effects of bevirimat on the pregnant and lactating female and on the development of the offspring from the beginning of organogenesis up to sexual maturation. There were no bevirimat-related findings in these reproductive toxicology studies (unpublished results).

The ability of bevirimat to inhibit HIV replication *in vivo* was first tested in the severe combined immunodeficiency (SCID)-hu Thy/Liv BALBc mouse model. In this study, bevirimat was administered orally in doses of 10, 30, and 100 mg/kg/day for 21 days. Bevirimat was found to reduce viral loads for the NL4-3 strain of HIV in a dose-dependent manner by –0.9 and –2.1 logs at the two highest doses (30 and 100 mg, respectively)[60,61]. These results provided the first demonstration of *in vivo* efficacy for bevirimat.

Metabolism

The metabolism of bevirimat has been investigated in the rat model, where it was found to be metabolized into acyl glucuronides excreted through the

bile[62]. The major bevirimat glucuronide was mono-conjugated at C-28, while the minor form was conjugated at the dimethylsuccinic acid side chain. A lower amount of di-conjugated bevirimat was also identified. Follow-up studies in human microsomes revealed the major product from liver microsomes to be the mono- C-28 conjugate, while the predominant product from intestinal microsomes is the dimethylsuccinyl conjugate[63].

Clinical trials: safety

Bevirimat was initially evaluated for safety and pharmacokinetics in a single-dose, randomized, double-blind, placebo-controlled phase Ia clinical study in healthy volunteers[64]. The compound was administered as an oral solution at doses of 25, 50, 100, and 250 mg, with eight subjects per dose level (six received bevirimat and two received placebo). The resulting plasma concentrations were dose-proportional, and the compound was found to be safe and well tolerated, with no dose-limiting toxicities and no serious adverse events reported. Bevirimat was found to have a relatively long half-life in humans of 60-80 hours. These results supported once-daily dosing for bevirimat in subsequent multiple-dose studies.

In a follow-up, 10-day, multiple-dose, randomized, double-blind, placebo-controlled phase Ib dose-escalation study, bevirimat was administered to healthy volunteers once daily as an oral solution in doses of 25, 50, 100, 150, and 200 mg (six subjects receiving bevirimat and two subjects receiving placebo per dose group)[65]. A loading dose of 150 mg followed by daily dosing at 75 mg was also evaluated. The compound was, again, well tolerated, with no related toxicity, and the plasma inhibitor concentrations were dose proportional. Mean trough concentrations at day 10 ranged from 9.87 µM (5.75 µg/ml) at the 25 mg dose to 68.8 µM (40.1 µg/ml) at the 200 mg dose. These concentrations compared favorably with the estimated *in vitro* protein-binding-adjusted IC_{90} value of 4 µM. The half-life of the inhibitor in this study was similar to that observed in the single-dose study, at 56-70 hours. These results supported continued development of bevirimat.

As of early 2007, a cumulative total of over 300 people had been dosed with bevirimat over the course of multiple clinical trials. The compound has proven to be generally safe and well tolerated, with no dose-limiting toxicity identified.

Clinical trials: efficacy

Clinical efficacy was first addressed in a single-dose, double-blind, placebo-controlled phase I/II monotherapy study in HIV-positive patients[66]. Bevirimat was administered as an oral solution in doses of 75, 150, and 250 mg. There were six patients in each dose group as well as in the placebo group. Upon dosing with bevirimat, a statistically significant, dose-dependent reduction in mean viral load was observed. Although this study was conducted with a single dose of bevirimat, it did provide the first proof-of-principle for a maturation inhibitor in HIV-infected patients.

Efficacy of bevirimat was further examined in a multiple-dose, randomized, double-blind, placebo-controlled phase IIa monotherapy study in HIV-positive patients[67]. The compound was given once-daily for 10 days as an oral solution in doses of 25, 50, 100, and 200 mg. Six patients were included in each dose group, with eight patients in the placebo group. Reduction in viral load on day 11 was the primary endpoint of the study. At that time point, a statistically significant reduction in median viral load was observed for the two highest dose groups, with median reductions in viral loads of -0.48 and -1.03 log for the 100 and 200 mg dose groups, respectively. Pharmacodynamic modeling studies based on the data from this trial predicted that more potent responses would likely be achieved by increasing the dose in subsequent studies.

Prior to proceeding with studies in which bevirimat would be added to failing background therapies, two drug-interaction studies were performed in normal volunteers. In these studies, bevirimat was coadministered with either atazanavir[68] or ritonavir[69]. Consistent with previous *in vitro* studies, no significant interactions were observed between bevirimat and atazanavir. Similarly, effects of ritonavir on bevirimat were found to be minor, with a mean reduction in bevirimat plasma concentration, as assessed by area under the curve, of 17-31%. These effects were far less than those observed with many other approved HIV drugs. As a result, no special accommodations were required when adding bevirimat to background regimens in subsequent trials.

A phase IIb functional monotherapy study of bevirimat in a solid tablet form was initiated in 2006 in HIV-positive patients (D. Martin and G. Allaway, unpublished results). In this study, once-daily bevirimat or placebo was added to the patient's existing background regimen that had failed. The primary endpoint for the study was the reduction in viral load on day 15. Upon analysis of plasma inhibitor concentrations at the two-week primary endpoint, it was discovered that much lower concentrations of bevirimat were achieved with the solid tablet formulation than had been predicted based on previous bioequivalence studies. As a result, use of this tablet formulation was discontinued after dosing of the first cohort of patients. Nonetheless, a subset of patients did respond to bevirimat treatment and were allowed to enroll in an extended dosing roll-over protocol in which they continued to receive bevirimat in addition to an optimized background regimen.

The phase IIb study resumed in early 2007, using the original oral liquid formulation, while development of an optimized formulation for phase III studies continued in parallel (D. Martin and G. Allaway, unpublished results). In the revised phase IIb format, now ongoing, the oral solution is being tested in a dose-escalation study, starting at 250 mg with the first cohort of patients. Dosages will be increased in 50 mg increments with each subsequent patient cohort until the top of the dose-response curve is reached. Top-line results from the first cohort, released in June, 2007, indicated no safety or tolerability issues, and plasma inhibitor concentrations and viral load reductions were in line with expectations. It is anticipated that continuing dose escalation in this study will identify an appropriate dose of bevirimat to take forward into later-stage trials. Efforts to identify an improved formulation to support later-stage clinical development are underway.

In vivo resistance?

Although the fact that single point mutations in and around the Gag CA-p2 cleavage site are sufficient to confer resistance *in vitro* might suggest that there will be a low genetic barrier to resistance for this class of inhibitor, it remains to be seen whether these same mutations will arise *in vivo*. The Gag residues implicated in resistance *in vitro* are highly conserved in the HIV sequence database, suggesting that mutations at these positions are selected against *in vivo*[1]. Interestingly, the p2-A3T mutation does exist in one viral sequence (F1.RW.-.VI69_L11796) found in the Los Alamos HIV sequence database[70]. However, it is not known whether this sequence corresponds to a replication-competent isolate. This is important because this same mutation was found to severely impair the replication fitness of a lab-adapted HIV clone in *in vitro* studies[1]. Although several mutations that confer reduced sensitivity to bevirimat *in vitro* seem to be tolerated in *in vitro* replication experiments in immortalized cell lines, it may be that these mutations are associated with a reduction in viral fitness in primary cells or *in vivo*.

A recent survey of the Gag CA-p2 region in protease inhibitor-experienced patients identified one known bevirimat resistance mutation, CA-L231M, in one out of 82 patient virus genotypes examined[71]. This mutation was previously reported to confer an intermediate level of resistance to bevirimat *in vitro* in the context of a lab-adapted virus[1]. However, it is unknown whether, in the context of the patient isolate's complete Gag and protease genes, this mutation would actually confer a reduced level of sensitivity to bevirimat. Population genotyping of patient isolates from the bevirimat phase I/II and phase IIa clinical trials did not reveal any isolates that contained known *in vitro* resistance mutations[72]. It will be interesting to see in later-stage trials at what frequency patients develop resistance to bevirimat over extended dosing periods and which mutations are associated with bevirimat resistance *in vivo*.

Future directions

The demonstration of clinical efficacy for bevirimat serves as proof-of principle for the development of additional classes of HIV maturation inhibitors. The natural product betulinic acid scaffold from which bevirimat was derived, as well as related triterpene derivatives, constitute attractive starting points for the development of additional, second-generation HIV maturation inhibitors. Some structure-activity relationships have been established for analogs of bevirimat[13,73,74]. One analog, termed PA-040, entered phase I clinical trials in normal volunteers in early 2007. This second-in-class maturation inhibitor retained higher levels of potency than bevirimat in the presence of human serum or against the CA-L231M mutant virus in *in vitro* studies[75]. Further modification and testing of this and other betulinic acid derivatives may lead to improved clinical efficacy through improved bioavailability and/or pharmacokinetics.

Analogs based on alternative triterpene scaffolds have also been described that have potent HIV inhibitory activity *in vitro*, similar to that of bevirimat[76,77]. In addition, analogs designed to mimic the structural features of both bevirimat and previously reported betulinic acid-derived HIV entry inhibitors[78] have been described[79,80]. These bifunctional compounds inhibited both HIV maturation and HIV entry *in vitro,* with the dual activities attributed to substitutions at both the C3 position (affecting maturation) and the C28 position (affecting virus entry) of the betulinic acid scaffold. Whether these compounds are viable development candidates or not remains to be seen.

The establishment of proof-of-principle for maturation inhibition will likely also lead to the discovery and development of chemically unrelated, "third-generation" HIV maturation inhibitors. The identification of the CA-p2 cleavage site as a validated drug target opens the door to a realm of possibilities for focused inhibitor screening assays. One such technology has been described which quantitatively detects association of radiolabeled bevirimat with virus-like particles in a scintillation bead proximity assay[56]. Random libraries of compounds could be screened using this approach to identify inhibitors that compete with the radiolabeled compound for binding to Gag. It will be interesting to see if any inhibitors discovered using such an approach have different resistance profiles as compared to betulinic acid-derived analogs.

Conclusions

The breadth of new antiretroviral drugs under development is cause for optimism. However, this optimism is tempered by the likely continued requirement for careful patient monitoring and increasing use of resistance testing to ensure that patients receive current classes of antiretroviral drugs that are active against their individual viral isolates. Clearly, the introduction of any new drug with a novel target is a welcome addition to the physician's armamentarium. The discovery and development of bevirimat, the first representative of a new class of antiretroviral therapeutics, the maturation inhibitors, represents a significant advancement in this direction. The inhibitor was discovered using the classic tools of natural product research, followed by derivatization and structure function analysis. While the mechanism of action has yet to be fully characterized, it is clear that the target, Gag, is an entirely novel one for any antiretroviral. The promise and advantages of targeting viral structural proteins have been recently noted[81,82]. Although bevirimat does not behave precisely as a viral capsid protein polymerization inhibitor, it does provide proof-of-principle that the assembly and maturation processes of viruses can be exquisitely sensitive to perturbation and thus exploited as therapeutic targets. As bevirimat continues in clinical development it seems established that maturation inhibitors will remain an attractive class for development of HIV/AIDS therapeutics.

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