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Leveraging APOBEC3 proteins to alter the HIV mutation rate and combat AIDS

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Abstract

At least two human APOBEC3 proteins – APOBEC3F and APOBEC3G – are capable of inhibiting HIV-1 replication by mutation of the viral cDNA. HIV-1 averts lethal restriction through its accessory protein Vif, which targets these APOBEC3 proteins for proteasomal degradation. The life-or-death interaction between human APOBEC3 proteins and HIV-1 Vif has stimulated much interest in developing novel therapeutics aimed at altering the deaminase activity of the APOBEC3s, thus changing the virus' mutation rate to either lethal or suboptimal levels. The current state of mechanistic information is reviewed and the possible risks and benefits of increasing (via hypermutation) or decreasing (via hypomutation) the HIV-1 mutation rate through APOBEC3 proteins are discussed.

Keywords

AIDS; APOBEC3F; APOBEC3G; Coffin's razor; DNA cytidine deamination; HIV; host-pathogen interaction; hypermutation; hypomutation; retrovirus restriction; Vif

Basic concepts in antiviral therapy

The most straightforward and traditional approach to blocking viral replication is through the use of compounds that are engineered to be highly specific inhibitors of viral proteins. Such inhibitors are pervasive among therapeutics today, and are often used as standard treatments for chronic viral infections. The treatment of HIV, for example, generally includes one or more compounds that inhibit essential HIV-1 proteins, such as non-nucleoside reverse-transcriptase

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inhibitors, protease inhibitors and integrase inhibitors. In general, these compounds have few off-target effects and avoid most problems with cytotoxicity. Despite the numerous successes of this approach, a major problem with virus-specific compounds is the evolution of drug resistance. Rapidly evolving viruses, such as HIV-1, invariably mutate to alter their amino acid composition and resist the drug. The invariant correlate that all effective drugs eventually select drug-resistant viruses is known as Coffin's razor.

A second general strategy takes advantage of the fact that viruses are dependent on host proteins for replication and pathogenesis. If inessential to the host, such proteins are good targets for antiviral compounds. Cellular proteins are many magnitudes more stable (less mutable) than viral proteins and, therefore, much less likely to contribute to the evolution of drug resistance. While this may increase the long-term efficacy of the drug, a potential drawback to this approach lies in the risk that disrupting a cellular process may have unintended consequences or off-target effects. Thus, a thorough knowledge of the human interactome within the realm of the targeted protein and extensive preclinical studies are required for any therapeutic compound in order to utilize this strategy. As with viral protein inhibitors, administration requires careful calculation and consideration of the cost-benefit ratio.

A third and relatively new antiviral strategy has been realized with the discovery of cellular proteins that function to inhibit viral replication. These 'restriction factors' include dominant-acting cellular proteins that block a specific stage of the retroviral life cycle, are under selective pressure and so more diverse than most other cellular proteins, are susceptible to neutralization (or evasion) by viral counter measures and often induced by interferon or by viral infection itself (i.e., as part of the innate immune system). Examples include APOBEC3G [1–5], TRIM5 α [6–10] and BST2/TETHERIN [11,12]. The discovery of these antiviral factors has opened the door to novel therapeutic approaches intended to facilitate, supplement or improve the endogenous restriction strategies that are already in place. This article will focus on the APOBEC3 family, the impact of these proteins on HIV-1 biology and their potential influence on the future of HIV/AIDS treatments.

Attacking the viral genome: the APOBEC3 protein family

APOBEC3G is the archetype of the APOBEC3 subfamily of ssDNA cytidine deaminases. This seven-member group of DNA mutators – APOBEC3A, B, C, DE, F, G and H – plays a central role in innate immunity, defending the genome against mutation induced by the invasion of exogenous pathogens, such as retroviruses [4,13–24] and the movement of endogenous retroelements, such as human endogenous retrovirus, long interspersed repetitive element and Alu (Table 1) [25–34]. APOBEC3G was originally identified as a potent ssDNA mutator [1] with significant homology to the RNA0-editing enzyme and family namesake APOBEC1 [35]. Concurrently, APOBEC3G was identified as a dominant inhibitor of Vif-deficient HIV-1 replication, whose effects could be completely overcome by the neutralizing activity of the Vif accessory protein [2]. APOBEC3G's potent DNA deaminase activity and its ability to restrict HIV-1 replication were quickly found to be linked [1,3–5]. The mutation and subsequent restriction of Vif-deficient HIV-1 by APOBEC3G has been extensively studied and has helped set the paradigm for continuing mechanistic studies on the role of DNA deamination in retroviral restriction and innate immunity.

In the absence of Vif, APOBEC3G is encapsidated into the core of budding virions in a manner dependent on an interaction with both RNA and nucleocapsid protein (Figure 1) [36–42]. Once the virion fuses to a target cell and deposits its core, the availability of deoxyribonucleotide triphosphates enables reverse transcription. Within the deposited core, APOBEC3G is hypothesized to bind the viral gRNA and this action alone is thought to be sufficient to partially inhibit accumulation of reverse transcripts through a deaminase-independent mechanism(s)

[43–50]. During first- (or minus-) strand synthesis, RNaseH degrades the gRNA to allow for second-strand synthesis, not only liberating APOBEC3G, but also exposing its ssDNA substrate [51]. APOBEC3G binds and deaminates cytidines to uridines on the exposed minus strand [3–5,13,15,17,52]. This heavily edited cDNA is highly susceptible to degradation, although the identity of the responsible factors remains unclear [45,53–56]. Edited viral cDNA copies that escape degradation template second-strand synthesis. The uridines code for plus-strand adenosines, thus, ultimately manifesting as G–A mutations [3–5,13]. The generation of frequent nonsense and missense mutations renders the resulting provirus incapable of further replication and infection.

The APOBEC family is characterized by a conserved zinc(Z)-binding motif, H-x-E-x_{25–31}-C-x_{2–4}-C, required for deaminase activity [1,13,35,57–59]. These domains are organized in a modular fashion at the APOBEC3 locus to give rise to a series of single or double Z-domain proteins. The human APOBEC3 locus encodes four double Z-domain proteins (APOBEC3B, APOBEC3DE, APOBEC3F and APOBEC3G) and three single Z-domain proteins (APOBEC3A, APOBEC3C and APOBEC3H) from seven genes located in tandem on chromosome 22 (Table 1) [35,57]. These loci vary dramatically across species with for example, the sheep and cattle loci encoding three single Z-domain proteins and one double Z-domain protein from only three genes [59], or the cat locus encoding four single Z-domain proteins and one double Z-domain protein from four genes [60]. The Z-domains fall into three phylogenetic clusters based on conserved amino acid variations within the zinc-binding motif and are designated type Z1, Z2 or Z3 [58,59]. While no functional difference has yet been described to the different domain types, the Z-domain nomenclature has been useful for making cross-species comparisons and modeling the evolutionary history of the family [58,59].

The human, double-domain APOBEC3s have several properties that distinguish them from their single-domain family members, which may offer clues to their specific physiological functions. First, the double-domain APOBEC3s tend to display a separation of function between domains; one determines ssDNA cytidine deaminase activity and sequence specificity, while the other is responsible for subcellular localization, RNA-binding and encapsidation. For example, the N-terminal catalytic domain of APOBEC3G is responsible for viral RNA-binding and is required for encapsidation, but it lacks DNA deaminase activity [44,46,61,62]. By contrast, the C-terminal catalytic domain confers both DNA deaminase activity and sequence specificity (APOBEC3G prefers to deaminate cytidines in a 5'-CC context), but is unable to encapsidate on its own. APOBEC3F has a similar functional distribution, although it prefers cytidines in a 5'-TC context [15,57,61,63]. APOBEC3B mostly follows this rule, with the C-terminal domain being catalytically dominant and determining preference for cytidines in a 5'-TC context [64], but the N-terminal domain still elicits activity capable of mutating the HIV-1 genome [42]. The N-terminal domain of these three proteins also determines subcellular localization, nuclear for APOBEC3B and cytoplasmic for APOBEC3F and APOBEC3G [29,33,34,65–68]. It is worth noting that the active determinants of subcellular localization overlap with those that determine RNA-binding capacity, suggesting the two may go hand-in-hand, although protein–protein interactions have also been implicated in APOBEC3G trafficking and localization [67–69].

Second, the double-domain APOBEC3s are distinguishable from their single-domain family members in their capacity to homo and hetero-oligomerize to form higher order multimers in cells. APOBEC3G has long been known to be capable of homo-oligomerization, an interaction greatly facilitated by RNA [35,41,62,69,70]. It is also capable of assembling into much larger, heterogeneous high molecular mass (HMM) ribonucleoprotein complexes [71]. These HMM ribonucleoproteins lack definitive identification as either specific RNA granules, such as Staufen-containing RNA granules, or nonspecific stress granules and processing bodies [31, 65,72,73]. Either way, APOBEC3G in HMM complexes has been shown to be catalytically

inactive and incapable of HIV-1 restriction [71]. Similar to APOBEC3G, APOBEC3F is also known to self-oligomerize and assemble into HMM complexes [63,73,74]. The relevance of these higher order complexes is not yet known, although several reports provide clues that they may be functionally important to the restriction of retroelements, such as L1 and Alu [31,75]. Furthermore, APOBEC3G is not restricted to homo-oligomers, but has been shown to hetero-oligomerize with APOBEC3B, APOBEC3DE and APOBEC3F [23,63,76]. It is unknown whether or not these hetero-oligomers are incorporated into HMM complexes and what their physiological relevance may be.

Finally, and perhaps most notably, all of the double-domain APOBEC3s are capable of restricting Vif-deficient HIV-1 while the single-domain APOBEC3s appear to only do so weakly, if at all Table 1) [77,78]. Double-domain APOBEC3G and APOBEC3F have consistently demonstrated an impressive ability to restrict Vif-deficient HIV-1 in both single-cycle assays and spreading infections experiments performed by numerous laboratories [2–5, 13,15,17,57,63,79]. Likewise, APOBEC3B and APOBEC3DE show strong inhibition by single-cycle assays, although there are a few conflicting reports [15,20,23,28,64,76]. By contrast, single-domain APOBEC3C, while highly efficient at restricting SIV, has only been reported to weakly inhibit HIV-1 [15,23,28,29,63,64,76,80]. Of the four APOBEC3H variants, only haplotype II appears capable of HIV-1 restriction, although to a lesser extent than either APOBEC3F or APOBEC3G [81–83]. APOBEC3A is also unable to restrict Vif-deficient HIV-1 [15,20,23,28–30,45,63,64]. While it is still unknown which APOBEC3s are relevant to HIV restriction *in vivo*, several APOBEC3s have been implicated. Analysis of proviral sequences obtained from clinical samples of HIV-1-infected patients demonstrates a mutational spectrum consisting of not only GG→AG mutations indicative of APOBEC3G, but also of GA→AA and GC→AC mutations indicative of APOBEC3F/APOBEC3B and APOBEC3DE, respectively [23,57,84,85]. While perhaps not an absolute correlation, the ability of the double-domain APOBEC3s to restrict HIV is striking and may be linked with one of the unique physical attributes delineated previously.

Besides HIV-1, the APOBEC3 subfamily has been shown to act on ssDNA-replication intermediates of other retroviruses, such as SIV, murine leukemia virus (MLV), human T-cell leukemia virus (HTLV), porcine endogenous retrovirus and foamy virus [4,13–16,18–24,64, 86] and retroelements, such as human endogenous retro-virus, long interspersed repetitive element and Alu [25–34,56,87–89]. While working to promote the overall genomic integrity of the cell, it is unclear what specifically drove the maintenance and rapid expansion of the APOBEC3 locus from one to several unique genes. The single APOBEC3 homolog present in mice was found to be inessential, although the knockout mice were found to be more susceptible to mouse mammary tumor virus and MLV infection [90–92]. Did selective pressure from exogenous retrovirus transmission sculpt the locus and/or was the pressure derived from some endogenous retroelement replicating unrestrained in the genome? Regardless of factors responsible for shaping the APOBEC3 locus, the current APOBEC3 repertoire provides a potent, endogenous means by which to defend the host genome from both endogenous and exogenous parasitic elements.

Defending the viral genome: Vif & other inhibitors

Perhaps nothing speaks to the significance of the APOBEC3 family in viral restriction more clearly than the fact that nearly every relevant retroviral pathogen has evolved to be able to neutralize, bypass or otherwise overcome the APOBEC3 replication block. As any effective antiviral drug obeys Coffin's razor and selects for resistance over time, so too do the APOBEC3s. The APOBEC3s have pressured viruses to evolve a variety of resistance mechanisms that include avoidance, sequestration and degradation (Figure 2). The emerging theme is that all of these mechanisms appear to work by preventing the encapsidation of

APOBEC3s and, thereby, protecting the viral nucleic acid. The main paradigm for APOBEC3 antagonism, which has been set by the HIV-1 accessory protein Vif, is focused on here. All lentiviruses, except for equine infectious anemia virus, encode a Vif accessory protein that, while divergent in sequence, has a conserved function and a largely conserved mechanism of action.

HIV-1 Vif is a highly basic, 23-kD accessory protein that, while not strictly required for viral replication, is essential for efficient pathogenesis on certain cell types, including primary CD4⁺ T cells and macrophages [93–95]. For over two decades, the field has known that a Vif-deficient virus could sustain a spreading infection on certain ‘permissive’ cell lines, but could not in other ‘nonpermissive’ cell lines [93–95]. While Vif-deficient virions produced on permissive cells could infect nonpermissive cells, Vif-deficient virions produced on nonpermissive cells lacked the ability to efficiently infect any target cell [93,95]. This indicated that either Vif was required to overcome some viral restriction factor expressed by nonpermissive cells or that the absence of Vif required supplementation by a positive factor in permissive cells. Hybrids formed by the fusion of permissive and nonpermissive cells were unable to support Vif-deficient virion replication, arguing for the presence of a dominant negative factor in nonpermissive cells [96,97]. Subtractive hybridization between a parental nonpermissive line and a nearly isogenic, but permissive, daughter line identified APOBEC3G as this dominant restriction factor [2]. However, the ability of APOBEC3G to restrict required the absence of Vif. A Vif-proficient virus can overcome APOBEC3-mediated restriction and replicate almost equally well on either type of cell line.

Vif counters APOBEC3G-mediated restriction by primarily decreasing the steady-state level of APOBEC3G protein in an infected cell (Figure 2). Vif acts as an adaptor molecule, linking APOBEC3G to an ELONGINB/C–CULLIN5–RBX1 E3 ubiquitin ligase complex [98–100]. Bound APOBEC3G is then polyubiquitinated and degraded by the 26S proteasome [98,99, 101–104]. In the presence of Vif, the half-life of APOBEC3G has been reported to fall from more than 8 h to anywhere between 5 min and 4 h [98,101,102,104]. While degradation is clearly a main contributor towards the exclusion of APOBEC3G from viral particles, several auxiliary mechanisms have been proposed based on reports demonstrating that the ability of Vif to degrade APOBEC3G does not necessarily correlate with its ability to restore infectivity or inhibit deaminase activity [105–107]. While the degradation-independent mechanism of APOBEC3G inhibition remains unclear, several hypotheses have been posited, including direct inhibition of enzymatic activity, indirect inhibition by promoting the incorporation of APOBEC3G into HMM complexes, steric hindrance of the interactions required for encapsidation and inhibition of mRNA translation [14,104,106–109].

Structural data on the HIV-1 Vif protein remain largely elusive owing to the difficulty of expressing high levels of the soluble, recombinant protein. Nevertheless, comparative studies between SIV, HIV-1 and -2 have identified several conserved interaction domains that subsequent mutagenesis studies proved essential for coordinating APOBEC3G degradation (reviewed in [110]). Two of the more conserved motifs serve to recruit the E3 ligase complex: a HCCH Z-coordinating motif and a SOCS-box motif. The HCCH motif consists of broadly conserved His/Cys pairs and several flanked hydrophobic residues of highly conserved spacing. Z-binding by the HCCH residues is thought to maintain a structural conformation that aligns the hydrophobic residues forming the CULLIN5 binding surface [111–114]. The SOCS-box (or BC-box) motif includes a highly conserved ₁₄₄SLQ(Y/F)LA₁₄₉ sequence responsible for the binding of ELONGINB [100,115–117]. Substitution of the SLQ residues with alanines results in a dramatic loss of HIV-1 infectivity on nonpermissive cell lines owing to the inability of this Vif variant to recruit the E3 ligase complex and degrade APOBEC3G [99,118]. *In silico* modeling of this conserved amino acid sequence, based on the structures of analogous BC-box motifs, predict an α -helical form with the conserved hydrophobic residues clustered,

allowing for binding in the hydrophobic pocket of ELONGIN C [100]. This prediction was confirmed by recent crystallographic studies showing the HIV-1 Vif BC-box peptide bound to CULLIN5 [119].

The N-terminal domain of HIV-1 Vif is largely responsible for binding APOBEC3s [102, 120–125]. The Vif residues involved are arranged in a nonlinear fashion, indicating the involvement of multiple surfaces. For instance, APOBEC3G binding is dependent on a hydrophilic patch that includes the conserved ₂₃SLVK₂₆ and ₄₀YRHHY₄₄ motifs, as well as on a hydrophobic patch that includes four tryptophans at the very N-terminus and a ₆₉YWxL₇₂ cluster [120,121,123–125]. Thus, the binding of APOBEC3G by Vif requires the accurate arrangement of hydrophilic and hydrophobic residues on multiple surfaces. This complex binding scheme probably exists to ensure partial APOBEC3G binding and neutralization even if Vif has incurred one or more mutations in a binding motif. Vif also can bind and neutralize several other APOBEC3 family members, including APOBEC3C, APOBEC3DE and APOBEC3F (Table 1) [23,63,64,79,80,126]. The N-terminal domain of Vif is similarly responsible for the binding of these APOBEC3s, although through other residues. APOBEC3F binding, for example, is mediated by residues ₁₄DRMR₁₇ and an exclusive set of two tryptophans at the N-terminus [124,125,127].

Several other Vif domains are also crucial for HIV infectivity in nonpermissive cells. The central, hydrophilic ₈₈EWRRKR₉₃ motif is essential for protein stability, a mutation that causes a dramatic drop in Vif steady-state levels [128]. The ₁₆₁PPLP₁₆₄ proline-rich domain is required for Vif homomultimerization. Disruption of multimerization by either mutation of the domain or by expression of a peptide antagonist ablates the ability of Vif to prevent APOBEC3G encapsidation [129–131]. Finally, an RNA-binding domain exists at the N-terminus that mediates Vif interaction with viral gRNA [132–134]. Mutations of key residues within this domain also render HIV-1 incapable of replication on nonpermissive cells [134]. This RNA interaction is also required for efficient Vif incorporation into virions, although the role of Vif within virions is unclear [133,135–137]. It may be that Vif acts in the particle to inhibit APOBEC3G in a degradation-independent manner (as discussed earlier) or that Vif is required to fulfill some other role in the particle required for replication. For example, Vif is thought to play several roles that are similar to an RNA chaperone and it may function during genome folding and processing [138,139].

In contrast to APOBEC3G, which can effectively inhibit a broad array of retroviruses from other species, including SIV, equine infectious anemia virus, HTLV, porcine endogenous retrovirus and MLV [4,13–16,64,86], the potency of Vif and Vif-like molecules appears somewhat species specific. For example, HIV-1 Vif can neutralize human APOBEC3G, but not African green monkey APOBEC3. Similarly, SIV_{agm} Vif can neutralize African green monkey APOBEC3G, but not human APOBEC3G [14,140]. This specificity has been traced to a single amino acid in human APOBEC3G at position 128, which as a D allows for the binding of HIV-1 Vif and as a K allows for the binding of SIV_{agm} Vif [141–144]. This tendency towards species specificity probably reflects the strong positive selection that Vif is under to neutralize the particular APOBEC3 repertoire of its host species [14]. This is contrasted by the selective pressure exerted on the APOBEC3s to restrict a diverse set of targets, including not only human lentiviruses, but other viruses and retroelements that are both human and nonhuman. This mutual selection for broad antiviral activity by the APOBEC repertoire and for species specificity by the viral APOBEC antagonist may help explain why retroviral zoonotic transmissions are relatively rare [59,77,86].

While Vif-directed proteasomal degradation has provided a useful paradigm, several other mechanisms exist by which APOBEC3-mediated restriction is successfully avoided (Figure 2). For example, the foamy viruses are a family of complex viruses that infect a variety of

mammals and whose replication can be inhibited by APOBEC3 proteins [19,21,24]. The primate foamy virus (PFV) accessory protein, Bet, functions similarly to Vif and can rescue infectivity of Vif-deficient HIV-1. Similarly, PFV Bet appears to function in a somewhat species-specific manner as it can bind to both human APOBEC3G and African green monkey APOBEC3G, but not to mouse APOBEC3 [21]. However, neither PFV Bet nor the related feline foamy virus Bet has been shown to decrease steady-state levels of their target APOBEC3s. Instead, it is hypothesized that Bet functions to sequester the APOBEC3s in the cell, thereby preventing encapsidation and restriction [19,21].

The ability of a virus to neutralize the APOBEC3s is not always dependent on the presence of a specialized accessory protein, such as Vif or Bet. For example, MLV is a simpler virus that lacks accessory genes. It has been found to be resistant to its host APOBEC3 (mouse APOBEC3), although not to human APOBEC3G [18]. This is owing to the failure of mouse APOBEC3 to encapsidate, probably due to its failure to efficiently bind MLV cap-sid protein (Figure 2). It has been hypothesized that MLV Gag has evolved to avoid mouse APOBEC3 binding, and that this may be aided by an inhibitory effect that the viral RNA has on this interaction [18,22]. This strategy appears similar to that used by HTLV-1 to evade human APOBEC3G. HTLV-1 similarly lacks a Vif- or Bet-like accessory protein, but rather, uses a novel motif at the C-terminus of the nucleocapsid to prevent APOBEC3G encapsidation [145]. APOBEC3G packaging is dependent on RNA and on a direct or indirect association with viral nucleocapsid [36,39,41,69,146]. It is hypothesized that this C-terminal motif precludes or disrupts the crucial APOBEC3G/nucleocapsid association [145].

The prevailing trend is that every APOBEC3-susceptible retroelement has evolved a means to escape restriction. In addition to the mechanisms reviewed here – degradation, neutralization and avoidance – it is likely that viruses, as a whole, possess many other novel strategies for evading the APOBEC3 proteins. For example, recent data from our laboratory demonstrate that HIV-1 is capable of overcoming APOBEC3-mediated restriction by yet another means, tolerance [147]. Passaged continuously on CEM-SS cells stably expressing APOBEC3G, Vif-deficient virus can evolve APOBEC3G resistance by accumulating both a pyrimidine at position 200 and a null mutation in the accessory gene *Vpr*. While the role of the *Vpr* mutation is unknown, the A200C/T mutation was shown to dramatically increase viral titer by increasing translational efficiency [148]. This increase in viral titer serves to effectively titrate out available APOBEC3G, dropping mutational load back to tolerable levels [147]. As our knowledge of host–virus interactions continues to expand, other APOBEC3 evasion mechanisms are certain to emerge.

Modulating the APOBEC3s to facilitate virus eradication

The remarkable success of HIV as a pathogen can largely be attributed to an optimal mutation rate that allows for stable transmission to successive generations while seeding sufficient diversity to allow for quick evolution in response to selective pressure (Figure 3A). Therapeutically, alteration of the viral mutation rate provides two conceivable means by which to restrict the virus *in vivo*. Either the mutation rate must be increased to a level that prohibits stable transmission (hypermutation), or the mutation rate must be decreased to inhibit the evolution of resistance and, thus, foster susceptibility to existent selective pressure (hypomutation). The APOBEC3G–Vif interaction provides us with a putative means by which to control the mutation rate and investigate both of these novel drug-design strategies.

Hypermutation

APOBEC3G functions to increase the viral mutation rate and preclude stable transmission, while Vif functions to rein in APOBEC3G and restore the mutation rate to sublethal levels (middle panel, Figure 3B). Thus, if the APOBEC3G–Vif interaction could be disrupted or if

Vif function could be disabled, the viral mutation rate would be predicted to soar to lethal levels (top panel, Figure 3B). Hypermutated viral cDNA would be subject to degradation or would encode frequent nonsense and missense mutations rendering the virus incapable of replication as seen *ex vivo* and detailed earlier. In other words, APOBEC3G-mediated hypermutagenesis would push the genome beyond the threshold for genomic stability, causing catastrophic error and replication failure. Recently, a screen to identify small-molecule inhibitors of the APOBEC3G–Vif interaction discovered a number of promising compounds [149]. One inhibitor, RN-18, functions to alter targeting of the APOBEC3G–Vif–E3 ligase complex to polyubiquitinylation Vif instead of APOBEC3G. This results in Vif degradation, increased encapsidation of APOBEC3G and inhibition of viral replication.

While an attractive strategy, the use of hyper-mutagenesis to destroy the virus is inherently risky. First, very little is known about the mechanisms controlling APOBEC3G's discrimination of self from non-self DNA. APOBEC3G can act as a genomic DNA mutator in heterologous systems and poses an intrinsic hazard if not properly regulated [1,27]. While APOBEC3G is cyto-plasmic in human cells and sequestered from the genome, several redundant mechanisms most likely exist to ensure proper discrimination and must exist for those APOBEC3s that reside in the nucleus. Despite these systems, there is evidence that the nuclear APOBEC3s can pose problems for the host with maintenance dependent upon the cost–benefit ratio. For example, some human populations lack one or both copies of the *APOBEC3B* gene [150]. While there are no clear clinical manifestations of this event, it is possible that maintenance of the deletion is being driven by unintended, off-target deamination events whose costs outweigh the benefits of normal APOBEC3B function. Were any therapeutic to disrupt the enigmatic processes regulating APOBEC3 discrimination of self from non-self, the results could be catastrophic with the resultant genomic G-to-A hypermutation leading to massive cell death and/or to cancer.

Second, hypermutagenesis is also a risky strategy because it depends on reaching a lethal level of mutation in the viral genome sufficient to induce collapse of the population (Figure 3A). Failure to reach that threshold would promote further genetic diversification and could enhance the virus' ability to become resistant to immune responses and/or therapeutics. If APOBEC3G was not completely neutralized by the virus, for example, it could be used to alter the mutation rate in the face of selective pressure and could contribute to the creation of a new resistant population. In this respect, Vif may act as a regulator of viral mutation rate and serve to modulate APOBEC3 levels rather than eliminate them (middle panel, Figure 3B) [151]. In other words, the optimal mutation rate of the virus may be dependent on the exploitation of the host's APOBEC3 proteins to seed genetic diversity and, therefore, the APOBEC3 proteins may be beneficial to the virus in instances of sublethal mutation. Thus, any therapeutic designed to enhance or restore APOBEC3 function would have to do so fully and without altering its normal regulatory mechanisms.

Finally, the APOBEC3G–Vif interaction may not be an ideal target for disruption based on the genetic flexibility of the *Vif* gene itself. While other therapeutics inhibit the action of essential viral proteins with limited mutagenic potential, such as integrase, protease or reverse transcriptase, Vif is an accessory protein whose primary function is to overcome APOBEC3-mediated restriction. Thus, it is unclear how readily HIV-1 Vif will mutate to overcome small-molecule inhibition of the interaction. Moreover, as previously discussed, Vif-deficient HIV-1 has been shown to evolve in order to resist APOBEC3G in a Vif-independent manner [147]. Based on the wide range of mechanisms employed by various viruses to overcome the APOBEC3s (Figure 2), much more research is required on the ability of HIV-1 to evolve resistance before therapeutics can be developed and are clinically tested.

Hypomutation

As opposed to hypermutagenesis, which comes with a fair number of caveats, it is tempting to consider what would happen if one could render HIV-1 less mutable (Figure 3A). HIV-1 depends on an optimal mutation rate to ensure genetic variability in the face of an unending series of selective pressures. When selective pressure is exerted on the population in the form of an adaptive immune response or drug, there typically exist a number of resistant clones that survive and serve to repopulate the host. Hence, Coffin's razor is dependent on a basal level of genetic variability in the population. By dropping the mutation rate of the virus, it has been hypothesized that the genetic variation of the virus could be sufficiently diminished such that it is no longer able to evade the host immune system and antiviral compounds (bottom panel, Figure 3B) [151].

One contributing factor to the genetic variation of an HIV-1 population is likely to be the APOBEC3 family itself [152–155]. The main function of Vif is to rein in APOBEC3-mediated hypermutation and restore the mutation rate to sublethal levels. However, it is known that Vif does not always neutralize APOBEC3G completely. For example, early in infection, APOBEC3G levels have not yet been depleted and Vif levels are still on the increase resulting in a window where neutralization is incomplete [147]. In addition, defective *Vif* alleles, which would lead to incomplete neutralization of APOBEC3G, often arise *in vivo* and are detected in HIV-1 isolates from infected patients [156]. Furthermore, in clinical samples from patients infected with HIV-1, some proviral sequences show evidence of G-to-A hypermutation, demonstrating a failure by Vif to completely neutralize the APOBEC3 proteins [84]. While it is unclear to what extent the viruses depend on the APOBEC3s as sources of 'beneficial' mutations, there is some evidence that these mutations can lead to drug resistance. In a tabulation of all mutations associated with HIV-1 drug resistance, Berkhout and coworkers found G-to-A mutations are the most frequent [152]. While reverse transcriptase itself is responsible for a number of G-to-A mutations, even a minor dependency on the APOBEC3s for variation can be exploited by designing specific APOBEC3 inhibitors as therapeutics [151]. Even if APOBEC3 inhibitors do not considerably diminish the genetic variability of the virus to allow the adaptive immune system to clear infection by itself, they may still be useful as adjuvants that can enhance the long-term efficacy of other currently available therapies.

This approach is also not without its risks. Again, the normal physiological function of the APOBEC3s in the absence of viral infection is unknown and it is unclear what effects complete inhibition may have on the cell. Current data are consistent with an exclusive role in retroelement restriction, but more studies are needed to bolster this important point. For instance, while mouse APOBEC3 is inessential [90–92] and human APOBEC3B-null populations lack a definitive clinical symptom [150], unintended effects cannot be ruled out at this time. Furthermore, secondary viral infections in the absence of APOBEC3-mediated restriction may be significantly exacerbated. Such a therapy must also be careful to preserve the function of activation induced deaminase, an APOBEC3-related deaminase responsible for antibody gene diversification (reviewed in [157]). All therapeutics and antiviral drugs come with their own benefits and risks and so all require extensive preclinical research and rigorous clinical testing. However, the use of small compounds to alter the mutation rate of HIV-1 by leveraging APOBEC3 function is likely to provide, either alone or in conjunction with other antiviral drugs, a viable therapeutic option in the future.

Conclusion

At least two human APOBEC3 proteins, APOBEC3F and APOBEC3G, have the capacity to inhibit the infection of a broad number of retroelements, including the AIDS virus HIV-1. DNA cytidine deamination is a key part of most mechanisms of retrovirus restriction. Viruses circumvent this powerful innate defense in many different ways. HIV-1 encodes an accessory

protein, Vif, which binds APOBEC3F and APOBEC3G, triggers their degradation and prevents their encapsidation. Numerous strategies are being explored currently to harness the therapeutic potential of these amazing proteins.

Future perspective

The discovery of APOBEC3G and the realization that retrovirus restriction factors can block the infectivity of many retroviruses, including HIV-1, has paved the way for new therapeutic ideas. We anticipate that one or more of these ideas will reach fruition within the next decade, most likely first for HIV/AIDS. Primarily, this will include the development of molecules, such as RN-18, which render viruses nonfunctional by hypermutation. Next, and perhaps even more innovative and paradigm shifting, will be therapies that work by diminishing viral mutation rates and rendering viruses susceptible to normal immune responses. This approach can be summarized as therapy by hypomutation. Also within the next decade, many other viruses will be proven susceptible to APOBEC3-dependent restriction. Probable examples already include HBV and human papillomavirus. Mechanistic information derived from studying the conflict between the human APOBEC3s and HIV-1 will undoubtedly facilitate efforts to combat these and other viruses as well. Overall, we expect antiviral therapies that work through the modulation of host-restriction factors will move from theory to reality and prove successful in the near future.

Executive summary

Basic concepts in antiviral therapy

- Antiviral therapies typically target viral proteins.
- Host proteins are also viable targets for antiviral therapy.

Attacking the viral genome: the APOBEC3 protein family

- APOBEC3 proteins are DNA cytidine deaminases.
- APOBEC3 proteins block the replication of a broad number of retroviruses and retrotransposons.
- HIV is a prominent pathogen that is susceptible to APOBEC3-mediated restriction.

Defending the viral genome: Vif & other inhibitors

- Viruses have evolved numerous mechanisms to prevent restriction.
- HIV-1 prevents APOBEC3F and APOBEC3G encapsidation by triggering their proteosomal degradation.

Modulating the APOBEC3s to facilitate virus eradication

- Strategies to enable the APOBEC3s to hypermutate retroviral substrates are therapeutically feasible, but may inadvertently facilitate viral evolution.
- Strategies to block APOBEC3 function may increase viral genetic stability and, thereby, render the virus more susceptible to adaptive immune clearance and less likely to become drug resistant.
- More research is required in order to discover and evaluate therapies that work through hypermutation or hypomutation

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Figure 1. Mechanism of APOBEC3G-mediated HIV-1 restriction

The integrated provirus in the producer cell is transcribed and either packaged as gRNA or processed and translated to produce essential viral proteins, such as capsid, RT and integrase. In the absence of Vif (upper left), APOBEC3G can be encapsidated within the nucleic acid-containing core of the budding virus dependent on an interaction with both gRNA and nucleocapsid protein. This virion will be rendered replication deficient by three APOBEC3G-dependent mechanisms that occur after the viral core is deposited in the target cell. **(1)** APOBEC3G binds the gRNA-inhibiting RT in a deaminase-independent manner. **(2)** Closely following RT, the gRNA is degraded by RNaseH, both freeing APOBEC3G from its RNA-bound state and exposing the ssDNA of the minus strand. APOBEC3G deaminates available cytidines to uridines resulting in hypermutation. These mutations are fixed as G→A transitions upon second-strand synthesis. This heavily edited viral cDNA is highly susceptible to degradation by yet unidentified factors. **(3)** If the cDNA escapes degradation and is integrated, the frequent nonsense and missense mutations within the provirus prevent further replication. In the presence of Vif (lower left), APOBEC3G is polyubiquitinated and degraded by the 26S proteasome. Thus, the budding virions are fully infectious. RT Reverse transcriptase.

Figure 2. Mechanisms of natural APOBEC3 resistance

Every relevant retroviral pathogen has evolved a mechanism to overcome APOBEC3-mediated restriction. For example, the HIV-1 accessory protein Vif acts as an adaptor between several human APOBEC3s and an ELOB/C–CUL5–RBX1 E3 ubiquitin ligase complex that polyubiquitinylates APOBEC3, targeting it for degradation by the 26S proteasome. This effectively decreases the steady-state levels of APOBEC3 in the cell, thus preventing sufficient encapsidation for viral inhibition. Vif is also hypothesized to have secondary, degradation-independent mechanisms to overcome the APOBEC3 replication block. These include translational inhibition and promotion of APOBEC3 accumulation in inactive HMM complexes. All of these mechanisms serve to inhibit the encapsidation of APOBEC3 and allow for the production of infectious viruses. The foamy viruses, such as PFV, similarly encode an accessory protein, Bet, that binds APOBEC3G and prevents it from encapsidating. However, Bet does not promote APOBEC3 degradation, but rather, is hypothesized to sequester APOBEC3 away from the sites of virion budding. MLV and HTLV do not require a specific accessory protein to overcome APOBEC3-mediated restriction. They both encode specialized nucleocapsid proteins that preclude APOBEC3 binding and encapsidation.

HMM: High molecular mass; HTLV: Human T-cell lymphotropic virus; MLV: Murine leukemia virus; NC: Nucleocapsid; NC*: Specialized nucleocapsid; PFV: Primate foamy virus.

Figure 3. Leveraging the activity of the APOBEC3s to alter the HIV-1 mutation rate

(A) HIV-1 depends on an optimal mutation rate that allows for stable transmission to successive generations while seeding sufficient diversity to allow for quick evolution in response to selective pressure. Large increases in the mutation rate (hypermutagenesis) will prevent stable transmission of the genetic material to successive generations, decreasing viral fitness to lethal levels. By contrast, decreases in the mutation rate (hypomutagenesis) will impede the virus' ability to evolve resistance to other sources of selective pressure and, hence, can be equally detrimental to viral fitness. (B) The optimal mutation rate of HIV-1 is modulated, at least partially, by the APOBEC3 mutators and their viral inhibitor, Vif, making them attractive targets for therapeutic compounds (middle panel). If Vif function could be inhibited or APOBEC3 activity augmented, high levels of APOBEC3 would be packaged into budding virions (top panel). This would increase the viral mutation rate and the resulting hypermutated

viral cDNAs would either be degraded or code nonfunctional proteins. By contrast, if APOBEC3 activity could be abolished or Vif function enhanced, virtually no APOBEC3 would be encapsidated (bottom panel). This would decrease the viral mutation rate and inhibit accumulation of genetic diversity, impairing the virus' ability to evolve resistance to the adaptive immune system and other antiviral compounds. Adapted from [151].

Table 1

The human APOBEC3 repertoire.

	A3A	A3B	A3C	A3DE	A3F	A3G	A3H
Domains	Z1	Z2Z1	Z2	Z2Z2	Z2Z2	Z2Z1	Z3
Subcellular localization	Cell wide	Nuclear	Cell wide	Cytoplasmic	Cytoplasmic	Cytoplasmic	Cell wide
ΔVif HIV restriction	No	Yes	Weak	Yes	Strong	Strong	Some variants
HIV Vif interaction	No	No	Yes	Yes	Yes	Yes	Yes
Expression	PBLs, spleen, bone marrow and lung	PBLs, bone marrow, lung and stem cells	PBLs, thymus, spleen, lymph node, testis, ovary, small intestine, colon, liver, pancreas, heart, lung and adipose	PBLs, thymus, spleen, lymph node, ovary, lung, liver and adipose	PBLs, thymus, spleen, lymph node, testis, ovary, uterus, brain, lung, small intestine, colon, liver, kidney and pancreas	PBLs, thymus, spleen, lymph node, testis, ovary, uterus, brain, lung, small intestine, colon, liver, kidney and pancreas	PBLs, thymus, testis, ovary, brain, small intestine, colon and skin

PBL: Peripheral blood leukocyte.

See text for references. Expression data compiled from [Refsland ER, Stenglein MD, Brown WL, Harris RS: Quantitative profiling of the APOBEC3 mRNA repertoire in human lymphocytes and tissues. Manuscript in Preparation 2009] [1,35,57,158].