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REVIEW

Antiviral Stratagems Against HIV-1 Using RNA Interference (RNAi) Technology

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Abstract: The versatility of human immunodeficiency virus (HIV)-1 and its evolutionary potential to elude antiretroviral agents by mutating may be its most invincible weapon. Viruses, including HIV, in order to adapt and survive in their environment evolve at extremely fast rates. Given that conventional approaches which have been applied against HIV have failed, novel and more promising approaches must be employed. Recent studies advocate RNA interference (RNAi) as a promising therapeutic tool against HIV. In this regard, targeting multiple HIV sites in the context of a combinatorial RNAi-based approach may efficiently stop viral propagation at an early stage. Moreover, large high-throughput RNAi screens are widely used in the fields of drug development and reverse genetics. Computer-based algorithms, bioinformatics, and biostatistical approaches have been employed in traditional medicinal chemistry discovery protocols for low molecular weight compounds. However, the diversity and complexity of RNAi screens cannot be efficiently addressed by these outdated approaches. Herein, a series of novel workflows for both wet- and dry-lab strategies are presented in an effort to provide an updated review of state-of-the-art RNAi technologies, which may enable adequate progress in the fight against the HIV-1 virus.

Keywords: HIV-1, RNA interference (RNAi), antiviral therapy, RNAi screens, miRNA

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An Update on HIV

Human immunodeficiency virus (HIV) affects an estimated 34 million people worldwide, with 3.4 million of those affected below the age of 18. In 2010 alone, 1.8 million people died from AIDS.[1] Since 1996, when highly active antiretroviral therapy (HAART) was introduced, the life expectancy of HIV-positive individuals has greatly improved. However, HAART is unlikely to ever be curative and is known to cause numerous side effects, ranging from dizziness and migraines to lipodystrophy and hepatitis, severely affecting patients' quality of life.[2] In addition, the production of antiretroviral drugs is very costly, impeding their use in developing countries. Additionally, antiviral-resistant strains are constantly emerging, hampering the progress made in tackling the virus.[3]

HIV is a lentivirus that can be divided into two major subtypes: HIV-1 and -2.

The latter is considered less effective in causing disease; AIDS is most commonly associated with the former.[4,5] The HIV-1 genome consists of the structural genes *gag*, *pol*, *env*, the regulatory genes *rev* and *tat*, and the accessory genes *nef*, *vpu* (or *vpx*), *vif*, and *vpr* (Fig. 1).[6] The dimerization initiation site (DIS) is involved in the dimerization of the HIV-1 genomic RNA.[7,8] Briefly, the virus infects T lymphocytes and macrophages by binding to $CD4^+$ cell surface receptors, C-X-C chemokine receptor type 4 (CXCR4), and cysteine-cysteine chemokine receptor 5 (CCR5). The viral single-stranded (ss)RNA is reverse-transcribed into double-stranded (ds)DNA, which is then integrated into the host DNA, known as the 'provirus'. Infection can be latent, but once the virus is reactivated, it hijacks the host's machinery to replicate itself, leading to syncytium formation (ie, cell to cell fusion) and cell lysis.[6]

Several treatments against HIV have been proposed to date, including the use of ribozymes and RNA decoys. Ribozymes can be engineered to cleave specific RNA sequences, thereby targeting a gene of interest,[9] whereas RNA decoys can bind to HIV regulatory proteins, blocking their functions.[10] Attempts have also focused on engineering an anti-HIV vaccine; three HIV vaccine Phase III clinical trials have been completed, while 37 are on-going and are in various phases.[11] An alternative and potentially safer method is be RNA interference (RNAi). It is known that RNAi can be mediated through different types of interfering RNA molecules, including siRNA, shRNA, and miRNA, as well as long synthetic antisense sequences.

Recent efforts have focused on identifying host factors required by HIV-1. Brass et al performed a large-scale interfering RNA screen and identified 250 HIV dependency factors, such as Golgi transport proteins (Rab6 and Vps53), a karyopherin (TNPO3), and the mediator complex (Med28) as genes highly expressed in immune cells.[12]

In another similar study by Zhou et al, 311 host factors were identified, including members of the specificity protein 1 (SP1)/mediator complex and the nuclear factor (NF)-κB signaling pathway and other uncharacterized host factors for HIV such as AKT1, PRKAA1, CD97, NEIL3, BMP2K, and SERPINB6.[13] These factors may participate in cellular functions involved in the viral life cycle and may represent potential targets for therapy.

siRNA, shRNA, miRNA, and Antisense Oligonucleotides

Synthetic RNA has been designed to manipulate gene expression. When this exogenous dsRNA enters the cell, a ribonuclease III enzyme named Dicer cleaves it into short RNA duplexes to generate short interfering RNAs (siRNAs) approximately 21–25 nucleotides (nt) long. These siRNAs are subsequently loaded onto a multiprotein complex known as the RNA-induced silencing complex (RISC). During RISC assembly, the siRNA molecule is unwound and only a single strand, the guide strand, is retained, whereas the other, the passenger strand, is removed and likely degraded.[14] The single-stranded siRNA bound to

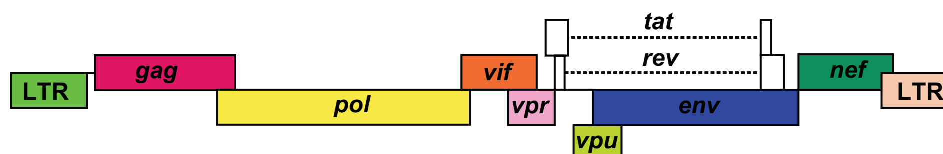

Figure 1. Schematic presentation of the HIV-1 proviral genome.

the complex then base-pairs with target mRNAs, inducing their cleavage by a RISC-associated RNase H enzyme belonging to the Argonaute protein family.[15] In effect, these exogenously administered siRNAs are typically transfected into cells to achieve transient gene knockdown. A peak in siRNA activity is typically observed between 24–72 h post-transfection depending on the number of cell divisions.[16] Transgenes encoding short hairpin RNAs (shRNAs) processed by Dicer into siRNAs, which in turn cleave perfectly complementary mRNA targets, which can be also delivered to cells.[17,18]

MicroRNAs or miRNAs are naturally occurring non-coding RNAs that control endogenous post-transcriptional gene expression. miRNAs are approximately 18–25 nt long and are initially transcribed as pri-miRNAs and cleaved into pre-miRNAs and mature miRNAs. miRNAs bind to miRNA-response elements (MREs) in mRNA transcripts. However, in contrast to other types of RNA, miRNAs do not always share perfect complementarity with their MREs and they can inhibit the translation of a target mRNA by base-pairing with their seed region (base 2–8) to multiple mismatched targets in the 3′ untranslated region (UTR) (reviewed by Siomi and Siomi[19]). Notably, HIV-1[20,21] infection can change a cell's miRNA profile (123, 124).

In addition, antisense oligonucleotides (AOs) are artificial single-stranded RNA or DNA molecules (up to 1 kb in length) that target complementary mRNA sequences for degradation or translational repression.[22]

Reciprocal Interactions between Viral and Cellular RNA

HIV-1 infection of human cells results in intricate interactions between viral and cellular interfering RNAs, which effect HIV-1 biogenesis, pathogenesis, and host cell's immune response.[23] Four types of viral-cellular interfering RNAs interactions have been identified.

Viral interfering RNAs versus viral mRNA

HIV-1 encodes miRNAs which play a role in HIV-1 replication and maintenance of viral latency (121).[24] Klase et al[25] and Quellet et al[26] identified transactivator of transcription-derived (Tat) Trans-activation response (TAR) element in HIV-1-infected CD4+ T cells and primary lymphocytes. These miRNAs were suggested to recruit chromatin remodeling components such as histone deacetylase-1 to the HIV-1 long terminal repeat (LTR), leading to transcriptional inactivation.[25] Notably, TAR miRNAs were also found to possess anti-apoptotic activity.[27] Another group demonstrated that the HIV-1 encoded miRNA miR-N367 was able to suppress *nef* expression and downregulate LTR-induced transcription.[28,29]

Cellular interfering RNAs versus viral mRNA

Host cells encode certain interfering RNAs that are thought to restrict HIV-1 infection. Five human miRNAs are suggested to target the *nef*, *vpr*, *vif*, and *vpu* accessory genes of HIV-1.[30] In addition, cellular miR-29a was shown to target a conserved site in the 3′ UTR of HIV-1 mRNA and suppress its replication.[28] miR-29a was also found to direct HIV-1 mRNA to P bodies,[31] which are distinct cytoplasmic foci involved in mRNA turnover.[32] Cellular miRNAs have also been proposed to contribute to HIV-1 latency. In particular, Huang et al demonstrated that cellular miRNAs miR-28, miR-125b, miR-150, miR-223, and miR-382 target the 3′ UTR region of HIV-1 mRNA in resting primary CD4+ T lymphocytes.[33] Transfection of quiescent T cells from HIV-1-infected donors or patients on HAART with antagonists against these five miRNAs increased viral production.[33]

Viral interfering RNAs versus cellular mRNA

HIV-1 counteracts the host's antiviral immune response by encoding viral miRNAs and RNA silencing suppressors (RSS). Bennasser et al showed that HIV-1 RNA elements are processed by Dicer into five miRNAs which target several cellular mRNAs.[34]

Tat has been identified as a bona fide RSS protein which interferes with Dicer.[35–37] Moreover, Tat has recently been shown to behave as an RSS through a charged interaction similarly to Tombavirus P19.[38] A viral Tat-derived shRNA, TAR, was found to possess RSS activity.[39] TAR RNA sequesters the activity of the host protein TRBP (TAR RNA-binding protein) and thereby attenuates the cellular antiviral RNAi machinery.[39] In addition, HIV-1 has been shown to produce an intrinsic antisense RNA originating from the HIV antisense initiator (HIVaINR) promoter element.

This is thought to be processed into multiple miRNAs, known as HAAmiRNAs. There is strong in silico evidence indicating that these HAAmiRNAs target human interleukin (IL)-15 (IL-15), IL-2 receptor gamma chain (IL-2γC), fragile mental retardation protein (FMRP), and IL-1 receptor-associated kinase 1 (IRAK1) in the host cell.[40] These have yet to be demonstrated in vitro, but it is worth noting that downregulation of IL-15, IL-2γC, and IRAK1 may severely impact the adaptive and innate immune system, while a decrease in FMRP may affect protein synthesis and possibly the cell's RNAi pathway itself.[40]

Cellular interfering RNAs versus cellular mRNA

Host interfering RNAs prevent HIV-1 proliferation by targeting cellular mRNA. Triboulet et al demonstrated that the cellular miRNAs miR-17-p and miR-20a, which are downregulated upon HIV-1 infection, specifically target the mRNA of the histone deacetylase P300/CBP-associated factor (PCAF).[41] PCAF is an endogenous cofactor important for Tat-induced transactivation of the HIV-1 LTR.[42] In addition, the cellular miR-217 is implicated in Tat-driven transactivation of the integrated HIV-1 LTR by targeting the mRNA encoding the deacetylase silent mating type information regulation 2 homolog (SIRT1).[43,44] SIRT1 is a host protein which is recruited by Tat to promote the formation of a transcriptionally repressive chromatin structure in the vicinity of the LTR, thereby inhibiting HIV-gene expression.[43,44]

Notably, inhibition of Dicer and Drosha using specific siRNA in peripheral blood mononuclear cells (PBMCs) from HIV-1-infected individuals was shown to increase HIV-1 replication, suggesting that cellular interfering RNAs have an overall negative impact on HIV-1.[42] Based on these observations, proper manipulation of specific cellular microRNAs may represent a potential therapeutic strategy for preventing HIV-1 replication and production.[45]

Wet Lab RNAi-based Antiviral Approaches

RNAi-mediated antiviral approaches have been successfully applied to efficiently prevent the early and late steps of HIV-1 replication.[46] It has been demonstrated that synthetic siRNAs and plasmid-derived siRNAs target and inhibit transient expression of virtually all HIV-1 genes and LTRs in short-term tissue culturing.[47–52] Furthermore, antagomirs, which are chemically engineered oligonucleotides with perfect complementarity to the target miRNA, have been applied to silence viral miRNAs.[53,54] In lentivirus-mediated-antagomir expression, a lentiviral transgenic plasmid contains an antagomir expression cassette with a H1-RNA promoter located within the U3 region of the 3′ LTR.[55] Klase et al transfected HIV-infected HeLa cells with antagomirs against the TAR miRNA.[27] This resulted in susceptibility of the transfected cells to apoptosis.[27] In addition, polycistronic miRNA constructs may represent a safer approach for suppressing HIV-1 replication, as the expression of miRNA transcripts is both low and controlled, decreasing the risk of toxicity.[57] Furthermore, Song et al demonstrated a cell-type specific, antibody-mediated siRNA delivery to HIV-infected cells. More specifically, a protamine-antibody fusion protein was designed to deliver siRNA against HIV-1 capsid gene gag.[57]

Viral escape mutants

However, RNAi-based inhibition of the genomic HIV-1 RNA is not sufficient to maintain viral inhibition given that after prolonged tissue culturing, RNAi-resistant HIV-1 variants emerged.[58] HIV-1, as a reverse transcriptase-dependent virus, is prone to high mutation rates, leading to the so-called RNAi escape mutants.[59–61] These escape mutants have accumulated deletions or nucleotide substitutions in the RNAi targeted sequence that prevent RNAi-mediated silencing.[59–61] In addition, escape mutants may result from mutations outside of the target RNA that induce a repressive secondary structure of the targeted region, thereby preventing access to the RNAi machinery.[62]

An alternative approach for restricting HIV-1 escape mutants is to target cellular mRNA-encoding proteins required for the HIV-1 life cycle. Anderson and Akkina[63] used a lentiviral vector to deliver multiple shRNAs against the co-receptors CCR5 and CXCR4, resulting in resistance to HIV-1 infection in human macrophages. Similarly, targeted delivery of anti-CCR5 siRNA has been achieved in T cells of humanized mice using specifically designed nanoparticles.[64] Combined targeting of viral and cellular genes by siRNAs can improve silencing activity. In 2008, Kumar et al delivered a combination of

siRNAs against the HIV genes *vif* and *tat* and the CCR5 co-receptor into T cells of humanized mice by intravenously injecting the animals with siRNA complexed to a specific single-chain antibody conjugated to an oligo-9-arginine peptide.[65] This inhibited viral infection and prevented the associated CD4+ T cell loss. In addition, targeting cyclophilin A, a cellular protein that binds to the HIV-1 capsid protein, by antisense U7 snRNAs and siRNA has been shown to reduce HIV-1 multiplication (120).[66]

Another approach for avoiding viral escape is targeting the well-conserved HIV-1 DIS.[7,8] Sugiyama et al introduced a lentiviral vector into a human lymphoid cell line that stably expresses a 19-bp shRNA directed against DIS. Downregulation of DIS resulted in a slower rate of emergence of the RNAi escape mutants.[67]

An optimal strategy for restricting HIV-1 escape and providing effective anti-HIV gene therapy is to use a combinatorial RNAi-mediated approach, in which distinct HIV-1 genome regions are targeted simultaneously using multiple interfering RNAs.[68,69] In a recent study, Knoepfel et al described several criteria for the selection of RNAi-based inhibitors before they were applied in clinical trials in humans.[70] These criteria include targeting of highly conserved viral sequences, as well as selection of inhibitors that exhibit a robust antiviral activity and have no adverse effects on host cell physiology. One group constructed a polycistronic transcript that expressed simultaneously four anti-HIV-1 shRNAs in the miR-17-92 backbone which suppressed viral replication efficiently in human lymphocytes.[71] In another study, three different shRNAs expressed from a single lentiviral vector resulted in a higher level of inhibition of viral replication and delay of virus escape in HIV-1 infected T lymphoid cell line.[72] In a more recent study, an artificial polycistronic transcript consisting of two pre-miR-30a backbones and one pre-miR-155 backbone driven by a cytomegalovirus promoter was shown to inhibit HIV-1 replication efficiently.[73] Furthermore, different types of interfering RNAs can function in a combinatorial manner to effectively suppress viral production by regulating viral gene expression. In particular, it was demonstrated that HIV replication was suppressed in HIV-1-infected CEM T lymphocytes by a combination of anti-HIV-1 siRNAs and nucleolar RNAs (si/sno RNAs).[74]

Furthermore, CD4 aptamer technology has been utilized to specifically suppress gene expression in CD4+ T cells and macrophages in vitro. For this reason, chimeric RNAs are generated, which are composed of an aptamer fused to siRNA for targeted gene knockdown. This methodology has been applied in cervicovaginal tissue samples and in the female genital tract of humanized mice. CD4-aptamer technology aims to specifically knock down HIV genes and inhibit HIV infection in vitro and in tissue explants. In vivo, after intravaginal injection to humanized mice, aptamer technology protected against HIV sexual transmission.[75] Other approaches include the delivery of siRNAs to the desired target tissue at therapeutic doses. Aptamers have been also used as siRNA delivery vehicles, in a form of aptamer-bridge-construct complexed which results in effective delivery of siRNAs in vivo. Such an approach may result in effective knockdown of target mRNAs and inhibition of HIV-1 replication in vivo.[76] In other studies, siRNA nanoparticles consisting combination of dicer substrate siRNAs (dsiRNAs) targeting both viral and cellular transcripts have been systematically delivered to suppress HIV-1 infection and protect against CD4+ T-cell depletion.[77]

Clinical applications of RNAi-mediated anti-HIV-1 therapies

Treatments based on shRNA and antisense oligonucleotides have already reached the clinic. Enzo Biochem has developed an antisense vector expressing antisense *tar* (an element critical for LTR transactivation) as well as antisense against *tat*/*rev* (transactivators that phosphorylate specific cellular proteins and prevent mRNA splicing before nuclear export, respectively) and commenced clinical trials.[78] In addition, DiGiusto et al[79] transduced human hematopoietic progenitor cells from HIV+ patients ex vivo with lentiviral vectors expressing shRNA against a *tat*/*rev* exon, a TAR decoy and a CCR5 ribozyme. However, due to patient security constraints, the amount of transplanted cells was too small to improve their condition, and this study highlighted the safety of this procedure. Vector expression persisted for up to two years, pointing to transcriptional gene silencing and/or cell turnover thereafter.

Similarly, Virxsys Corp (San Carlos, CA, USA) constructed a lentiviral vector expressing antisense *env*. In a phase I clinical trial, ex vivo

transduced autologous CD4+ T cells were intravenously infused back into the patients and were shown to be well-tolerated. Impressively, patients showed high initial engraftment of transplanted cells and stable or decreased HIV titers. In four patients, immune function improved and the vector was detectable even after a 2-year period in two patients.[80]

However, a major drawback of the use of lentiviral vectors for delivering anti-HIV-1 antisense RNA is that the vectors themselves are immunogenic. This prevents the delivery of multiple injections required for the long-term maintenance of a potential transgenic therapy. An alternative approach was used by Amado et al in a phase I clinical trial.[81] Hematopoietic stem cells were isolated from HIV-1-infected patients, allowed to divide and transduced ex vivo with a lentiviral vector encoding an anti-HIV antisense RNA. Mature hematopoietic cells were subsequently reinfused into patients. A sustained output of the introduced cells was observed even in multidrug-resistant patients.

Dry Lab: State of the Art RNAi-Mediated Anti-HIV-1 Approaches

Computational identification of HIV-1 miRNAs and host target genes

Given that mature miRNAs are encoded by pre-miRNAs which form hairpin (stem-loop) structures, the most important criterion for computational prediction of miRNAs is identifying putative hairpins.[82,83] Larger hairpin pre-miRNAs are responsible for processing of miRNAs.[84,85] The RNA-induced silencing complex (RISC) is a ribonucleoprotein containing miRNAs. Joining of a target mRNA 5′ UTR with miRNA may affect gene regulation by downregulation via translation inhibition by slowing degradation of RNA. Recent data suggest that miRNAs may affect and control the regulation of one third of human genes, which consequently determines and controls cellular life. Therefore, it is well-established that miRNAs are key entities controlling a major part of all cellular processes. Some of the most important include differentiation, cell timing, metabolism, proliferation, and apoptosis. It is therefore thought that miRNAs play a very crucial role in cancers and a series of other as important human diseases.[86] Existing widely used computational methods, such as MiRscan,[87,88] miRAlign,[89] Srnaloop,[90] and miRseeker[91] for miRNA[87,88] prediction in animals and plants rely heavily on sequence and/or structural phylogenetic conservation. However, as opposed to animal and plant miRNAs, viral miRNAs lack conservation as they evolve rapidly, and by using these methods a large number of non-conserved pre-miRNAs is omitted.[92] Therefore, computational methods that are not dependent on comparative genomics would be more suitable for predicting novel HIV precursor miRNAs.[93]

The computational tool Triplet-SVM[94] incorporates a machine learning approach for the ab initio prediction of miRNA precursors. Specifically, a set of properties based on sequence and structural information derived from 3 consecutive nucleotides (“triplets”) located in known (real) pre-miRNAs was defined. Collectively, 32 different triplets were counted in each hairpin; a 32-dimensional vector was generated which was subsequently used to train a support vector machine (SVM) to separate real pre-miRNAs from hairpins unlikely to encode miRNA (pseudo pre-miRNAs). In a similar manner, the program Virgo[95] employs SVM to discriminate between real viral pre-miRNAs and pseudo pre-miRNAs. The computational algorithm VirMir[96] was specifically developed to predict virally encoded miRNAs in small genomes. A window of 100 nucleotides was tiled over the entire viral genomes in both orientations. The RNA secondary structures corresponding to each window were submitted to the RNAfold algorithm,[97] and individual hairpin structures were obtained. Hairpins were scored based on their structural features and the resulting score was multiplied by the free energy. The highest-scoring hairpin was plotted as the cumulative score for each window.

Given that virally encoded miRNAs can regulate the expression of cellular genes, identification of potential host genes targeted by HIV miRNAs would enhance our understanding regarding HIV-host association. In this way, novel targets regulated by viral miRNAs can be detected, which may lead to the construction of more effective vaccines including injections of virus containing antagomiRs or shRNAs against their targets. In both cases, inactivated viral vectors can be utilized as carriers. Computational methods for miRNA target prediction depend on three major properties: (a) sequence complementarity between the miRNA and the 3′ UTR of the targeted gene, with greater focus on the seed region (bases

2–8) of the miRNA, (b) thermodynamic stability of the miRNA-mRNA hybrid, and (c) evolutionary conservation of target sequences across species.[98,99] Some state-of-the-art computational tools for the prediction of putative viral miRNA targets include MiRanda,[100,101] TargetScan,[102] PicTar,[103,104] RNAhybrid,[105,106] and PITA.[107] The outputs of these programs can be combined to strengthen our predictions. All software described above is summarized in Table 1.

Biostatistical and computational analysis of RNAi screens

Statistical techniques used to analyze the RNAi screens aim to explore genome-scale RNAi assays produced using high-throughput screens (HTS) technologies.[101,108] Results from genome-scale RNAi HTS offer a wealth of existing new data which provides insight into the complexity of biological systems, while introducing new challenges in the statistical interpretation of the data and forcing for innovative approaches for understanding functional networks in cells.

Specialized software programs and statistical methodologies are available for most screening formats, offering plate-based or screen-based normalization options, sensitization scores and multiple testing correction methods.[109,110] Examples include Applied Biosystems, Biotek, Cellomics, POwerMV, siRna, and some special libraries embedded in popular programming languages such as R software and Matlab. RNAither,[111] a recently developed R library, generates lists of relevant genes and pathways from raw experimental data. However, given the increasing amount of information produced and their increasing complexity, customization may be necessary for to enhance interpretation of the data. As with other cases of biomedical data, the need for data reporting standards is also important for assuring transparency of hit detection analysis methods and facilitating data integration.[112] Some efforts towards this goal are presented by the Minimum Information about an RNAi Experiment (MIARE), and Probe, PubChem NCBI databases.[112]

With HTS RNAi data, a large number of single measurements of samples are often considered, primarily focusing in identifying good quality hits that significantly differ from the negative controls. In doing so, *P*-values derived from quality control measures are considered, such as the z-score, the signal to noise ratio, or the Student *t*-test for estimating mean differences.[113] The z-score is widely applied, although it can only be applied to screens without replicates. Compared to the *P*-value, the strictly standardized mean difference (SSMD) directly measures the magnitude of the difference between two compared groups. SSMD is robust for both measurement units and strength of positive controls.[113] There are other more sophisticated approaches, such as the Haystack computational methodology,[114] which either confirms hits via an orthogonal metric or identifies genes originally missed due to library composition. Depending on the question of interest and the nature of the data (eg, case-controls), standard statistical methodologies can be applied. Naïve Bayes, decision trees, and k-nearest neighbor along with support vector machine (SVM) approaches are some of the methodologies often applied in cheminformatics, whereas one of the main goals of analysis is to estimate RNAi antiviral pathways. Typically, classification models are trained using positive and negative controls while accounting for over-fitting, and are subsequently used to predict the class of the remaining wells. Similar results can

Table 1. Summary of the RNAi-related software prediction tools and their websites.

Prediction tools	Website
Hairpins prediction for miRNA	
miRscan	http://genes.mit.edu/mirscan/
miRAlign	http://bioinfo.au.tsinghua.edu.cn/miralign/
Srnaloop	http://arep.med.harvard.edu/miRNA/pgmlicense.html
miRseeker	http://tinyurl.com/mirseeker
Triplet-SVM	http://bioinfo.au.tsinghua.edu.cn/software/mirnasvm/
Viral miRNA prediction servers	
Virgo	http://miracle.igib.res.in/virgo/
VirMir	http://140.109.42.4/
Secondary structures of single stranded RNA or DNA sequences	
RNAfold	http://rna.tbi.univie.ac.at
Target prediction	
miRanda	http://www.microrna.org/microrna/home.do
TargetScan	http://www.targetscan.org/
PicTar	http://pictar.mdc-berlin.de/
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/
PITA	http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html

be produced through unsupervised clustering or partitioning algorithms. Other procedures include GO enrichment (eg, from GeneGo), often using network techniques. Han et al[115] selected biologically interesting compounds by employing decision tree models based on structural fingerprints of compounds from PubChem HTS data. An optimum model was estimated by pruning the decision tree to minimize classification errors in a 10-fold cross-validation setting. Decision trees were also employed by Klekota and Roth[116] to validate the 'privileged' substructure concept to estimate substructures predisposed to bioactivity. SVM, in combination with a maximum-common sub-graph kernel, were employed by Mohr et al[117] to predict the genotoxicity of a compound. Linear SVM were employed by Rosenbaum et al,[118] who reported a high performance on large-scale data sets and assigned a particular color to each atom or bond of a compound based on the weights of the linear discriminant function.

A very interesting study concerning the analysis of time-lapse experiments was recently published by Walter et al,[119,120] where genome-wide data were partitioned based on the automatic recognition of chromosome morphologies, to distinguish primary from secondary phenotypes and group genes according to their phenotypic kinetics. Interaction networks and co-clustering methodology was employed at RNAi-based functional screens in Gonzales and Zimmer;[119] clustering was performed by simultaneously considering two types of data, such that genes which are both near each other in the interaction network and at the same time and showing strong links to the phenotype of interest (ie, RNAi data) were generally clustered together.

Overall, the results of HTS RNAi data appear to be sensitive to the quality of reagents, the assay design, or sampling issues, and are subject to appropriate adjustment of multiple hypothesis testing.[119] A widely accepted approach for addressing this problem is a multiple-testing correction, which adjusts the statistical confidence measures based on the number of tests performed.[121] Among commonly applied corrections include the conservative Bonferroni adjustment as well as the Bonferroni-Holm and the less strict Benjamini-Hochberg corrections.[121] Birmingham et al[108] thoroughly presented a number practical techniques for RNAi HTS analysis, optimized for low false-positive rates along with normalization strategies, such as improvements on median values calculations, median absolute deviations, quartile-based selections, or Bayesian models. Additionally, Zhang et al[122,123] suggested two methods for addressing multiple hypothesis testing issues by controlling the false discovery rate (FDR). In their first approach, they control for FDR through posterior probability assuming normality under a Bayesian framework,[116] whereas in their second approach, an error control method is employed based on strictly standardized mean differences, to effectively control both false-negative and false-positive rates.[123]

Nevertheless, new methods should be developed towards large-scale verification and validation screening. A very promising future direction involves integration with other "omics" approaches, such as microarray, genomic, and protein-protein interaction data, which already produce interesting results[119,124] towards a system-wide understanding of gene networks involved in various processes, events, and behaviors.

Conclusions

Collectively, RNAi technology can be employed to moderate the activity of genes within living cells. However, since HIV-1 mutates at extremely high rates, targeting for inactivation is difficult using traditional techniques based on gene conservation, as is the case in mammals. Consequently, the efforts of RNAi antiviral research focus on a combinatorial RNAi-based approach, where multiple HIV-1 sites are targeted. Wetlab approaches evolve quickly, and data accumulation is becoming a major issue within the field. Therefore, one of the greatest challenges in antiviral RNAi science is to appropriately handle, mine, and analyze huge amounts of genomic data that have recently become available through an expansion in RNAi database sizes. En masse, some glimmers of hope in the battle against the elusive HIV-1 virus come from the field of RNAi technology, where wetlab, bioinformatics, and biostatistical methodologies are combined in an effort that will inevitably yield invaluable results.

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Author Contributions

Conceived and designed the experiments: DV, SK. Analysed the data: DV, GT, AP, MGR, SCT, SK. Wrote the first draft of the manuscript: DV, SK. Contributed to the writing of the manuscript: DV, SK. Agree with manuscript results and conclusions: DV, GT, AP, MGR, SCT, SK. Jointly developed the structure and arguments for the paper: DV, SK. Made critical revisions and approved final version: DV, GT, AP, MGR, SCT, SK. All authors reviewed and approved of the final manuscript.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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