



Pre-Integration gene slicing as an alternate or complimentary gene therapy modem to RNA interference

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ABSTRACT

The Human Immunodeficiency Virus (HIV) is one of the current grand global health challenges. Despite the advent of HAART, resistance and treatment failure have increased the need for alternative interventions. Gene therapy is viewed as one alternative, with antisense RNA, RNA decoys, RNA interference, and Ribozymes being exploited. Despite the potential that the inherent antiviral model (the RNA interference or Post transcription gene silencing) offers for HIV gene therapy, off-target gene silencing, immune adverse reactions and resistance build up limit the effective use of the RNAi pathway, underlining the need for alternative or complimentary strategies. This paper reviews the potential of a similar natural inherent antiviral defense model in bacteria (known as the Restriction modification (R-M) System), as a pre-integration gene slicing alternative or complementary gene therapy modem to RNAi, detailing its potential applications in HIV treatment, as well as likely shortcomings.

Keywords: Gene therapy, Human Immunodeficiency Virus, Ribonucleic acids Interference pathway, Post Transcriptional Gene silencing, Pre-integration Gene splicing.

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Introduction

The human immunodeficiency virus (HIV) epidemic poses one of the greatest challenges to human health globally. Despite the discovery of over 26 prototype drugs of the highly active antiretroviral therapy (HAART) category (Mansky & Temin, 1995; Challand *et al.*, 1997), the emergence of resistance and therapy failure has led to the need for alternative strategies for HIV inhibition. Newer products such as chemokine receptor inhibitors and monoclonal antibody entry inhibitors are in final clinical trials (Norris *et al.*, 2005; Anastasopoulou *et al.*, 2006; Davison *et al.*, 2006; Delmedico *et al.*, 2006; Gulick *et al.*, 2006; Markowitz *et al.*, 2006; Mayer *et al.*, 2006; Norris *et al.*, 2006;; Pugach *et al.*, 2006; Sansone *et al.*, 2006).

Gene therapy offers another line of therapeutic intervention in HIV management. As of today, new technologies being developed include the use of antisense and sense RNA (U1snRNA) to inhibit gene expression (Sajic *et al.*, 2006), RNA interference pathway to stop early and late HIV replication through post transcriptional gene slicing (Barichiev *et al.*, 2006; Meshcheryakova *et al.*, 2006), autologous T cell anti-HIV anti-sense Ribonucleic acid (RNA) delivered by the VRX496

lentiviral vector (Rebello *et al.*, 2006), and ribozyme enzymes cleaving HIV RNA (Liu *et al.*, 2006). This review aims to compare two of the gene based therapy models that rely on inherent evolutionary antiviral defenses in other taxa; the RNA interference pathway (also widely known as post transcriptional gene silencing-PTGS), and the recently described pre-integration gene slicing (PTINT_GSX) by way of bacterial restriction modification(R-M) system-derived restriction endonucleases (REases). The principles, gene therapeutic potential and the anticipated challenges are addressed.

Post Transcriptional Gene silencing (PTGS)

RNA interference (RNAi) is the mechanism through which double-stranded RNAs silence cognate genes (Bernstein *et al.*, 2001). Double-stranded RNA (dsRNA) induces sequence-specific post-transcriptional gene silencing in many organisms by a process known as RNA interference (RNAi) (Elbashir *et al.*, 2001). The term RNA interference was coined after the discovery that injection of dsRNA into the nematode *Caenorhabditis elegans* leads to specific silencing of genes that are highly homologous in sequence to the delivered dsRNA (Fire *et al.*, 1998). RNAi was subsequently observed in insects (Kennerdell and Carthew, 1998), frog and other animals including mice (Oelgeschlager *et al.*, 2000; Svoboda *et al.*, 2000), and is likely to also exist in human beings. RNAi is closely linked to the post-transcriptional gene-silencing mechanism of co-suppression in plants and quelling in fungi (Cogoni & Macino, 1998; Catalanotto *et al.*, 2000; Wianny & Zernicka-Goetz, 2000). Some components of the RNAi machinery are also necessary for post-transcriptional silencing by co-suppression (Catalanotto *et al.*, 2000). The natural function of RNAi and co-suppression appears to be protection of the genome against invasion by mobile genetic elements such as transposons and viruses, which produce aberrant RNA or dsRNA in the host cell when they become active (Jensen *et al.*, 1999; Ketting *et al.*, 1999; Ratcliff *et al.*, 1999; Dalmay *et al.*, 2000). Specific mRNA degradation prevents transposon and virus replication, although some viruses are able to overcome or prevent this process by expressing proteins that suppress PTGS (Lucy *et al.*, 2000; Voinnet *et al.*, 2000). In both plants and animals, RNAi is characterized by the presence of RNAs of about 22 nucleotides in length that are homologous to the gene that is being suppressed. These 22-nucleotide sequences serve as guide sequences that instruct a multicomponent nuclease, RISC, to

destroy specific messenger RNAs. Dicer, an enzyme which produces putative guide RNAs for the RISC from shRNA or miRNA, is a member of the RNase III family of nucleases that specifically cleave double-stranded RNAs, and is evolutionarily conserved in worms, flies, plants, fungi and mammals. The enzyme has a distinctive structure, which includes a helicase domain and dual RNase III motifs. Dicer also contains a region of homology to the RDE1/QDE2/ARGONAUTE family that has been genetically linked to RNAi. Because Gene silencing occurs post transcriptionally, the RNAi pathway is sometimes referred to as Posttranscriptional gene silencing (PTGS) (Hamilton & Baulcombe, 1999).

The discovery of RNA interference has been heralded as one of the transforming events in biology in the past decade. RNAi can result in gene silencing or even in the expulsion of sequences from the genome. Harnessed as an experimental tool, RNAi has revolutionized approaches to decoding gene function. It also has the potential for therapeutic exploitation, and clinical trials to test this possibility are already under way (Hannon & Rossi, 2004; Jacque *et al.*, 2002). Perhaps, the area with the greatest potential of RNAi application is in HIV intervention, where PTGS has been explored as therapy by silencing the expression of virus genes inclusive of the TAR, Tat (Coburn & Cullen, 2002; Surabhi & Gaynor, 2002), Rev (Surabhi & Gaynor, 2002), gag, env (Boden *et al.*, 2003), nef (Coburn & Cullen, 2002) and RT. The alternative approach has been that of modulating cellular mediators of HIV infection and pathogenesis such as NF-K, CD4 receptor and the chemokine cell receptors CXCR and CCR5 receptor. These strategies are adopted because of high mutation rates of HIV that would render the RNAi obsolete.

Like any other therapy modern, PTGS has its short comings. These include off-target gene silencing through activation of the enzyme dsRNA-dependent protein kinase (PKR) which leads to destruction of RNA including msRNA, and hence to down regulation of protein synthesis (though this effect is reduced in siRNA relative to shRNA or miRNA) (Doench & Sharp, 2004). Another short coming is occurrence of immune adverse reactions including interferon-regulated responses that serve as antiviral mechanisms. Moreover, by virtue of their multipurpose function within human homeostasis, certain potential targets such as the NF- κ B, CD4, and even the CXCR are not viable for suppression by the RNAi as this would culminate into various adverse reactions. Lastly, in-coming HIV provirus is not a target for the RNAi pathway.

Despite the huge therapeutic potential offered by PTGS (RNAi pathway), the above shortcomings demonstrate a need to continue with the search for alternative or complimentary gene therapy moderns for HIV and other viral pathogens. The following section reviews the gene therapeutic potential of Pre-Integration Gene Slicing (PRINT-GSX) by way of Restriction modification(R-M) derived peptides as an alternative or complementary gene therapy modern to the RNAi pathway.

Pre-integration Gene slicing (PRINT-GSX)

Background: Pre integration Gene slicing refers to the process of splicing foreign or exogenous double stranded Deoxyribonucleic acids (DNA-often incoming as dsDNA viruses or Retrovirus proviral DNA) inherent in several bacteria species through a specialized nucleic acids system called Restriction Modification (R-M) system (Roberts *et al.*, 2007; Radasci & Bickle, 19996; Barcus & Murray, 1996). Just as the main evolutionary role of the PTGS is that of defense against viruses and retroposons (Jensen *et al.*, 1999; Ketting *et al.*, 1999; Ratcliff *et al.*, 1999; Dalmay *et al.*, 2000), the main evolutionary role of the R-M systems is widely believed to be to protect the bacteria cell from tropism by foreign DNA (phages). These systems basically comprise of two or three unit-enzymes: the restriction endonuclease (REase of DNases), a methyl transferase (MTase), and sometimes, a specificity subunit (S). They function essentially by recognizing 4-12 palindromic base

pair nucleotide sequences within the incoming foreign DNA and cleave within or near it. REases have been widely studied as tools in molecular biology where they are used for gene specific cleavage and subcloning (the recognition palindromes sequence of most are well defined) (Roberts *et al.*, 2007). The R-M systems are currently classified into four classes according to structure, substrate specificity, catalytic requirements, and reaction end products (Roberts *et al.*, 2007). Class I consists of three distinct enzymes systems: a restriction enzyme (RNase, R), a methyl transferase (MTase, M), and a site specificity protein (S). The Type III R-M, like type I have 3 functional polypeptides, but the M and S functions are denoted in the same anatomical protein (R, MS) (Nelson *et al.*, 1972; Kessler and Manta, 1990; Nelson & McClelland, 1991; Roberts & Macelis, 1991; Murray, 2000; Eduardo *et al.*, 2001; Lepikhov *et al.*, 2001). Type IV R-M on the other hand have only been recently identified and described, and comprise of a single polypeptide that serves both as a Methyl transferase and a Restriction Endonuclease (Lepikhov *et al.*, 2001; Eduardo *et al.*, 2001). Lastly, the Type II R-M, the most prolific of all and the one to which most endonucleases belong to, comprises of only two distinct peptides: the Restriction endonuclease (REase) and methyltransferase (MTase) (Barcus & Murray, 1996; Dalmay *et al.*, 2000; Jensen *et al.*, 1999; Ketting *et al.*, 1999; Ratcliff *et al.*, 1999; Kolyhalov *et al.*, 1992; Polisson *et al.*, 1992; Purvis *et al.*, 1983; Eduardo *et al.*, 2001).

Type II restriction modification systems (RMSs) have been regarded either as defence tools or as molecular parasites of bacteria. However, an extensive analysis of the evolutionary role from the study of their impact in the complete genomes of 26 bacteria and 35 phages reveals that palindrome avoidance is not universally spread among bacterial species, and that it does not correlate with taxonomic proximity (Eduardo *et al.*, 2001). Palindrome avoidance is also not universal among bacteriophages, even when their hosts code for RMSs, and depends strongly on the genetic material of the phage. Interestingly, palindrome avoidance is intimately correlated with the infective behavior of the phage. It has been observed that the degree of palindrome and restriction site avoidance is significantly and

consistently less important in phages than in their bacterial hosts. This result brings to the fore a larger selective load for palindrome and restriction site avoidance on the bacterial hosts than on their infecting phages (Eduardo *et al.*, 2001), and was a major limitation for the application of R-M systems as safe gene therapy modems since the Human genome, like the bacteria genome, contains several palindromic sequences that may be recognised by REase (International Human Genome Sequencing Consortium Human Genome Project 2006).

More recently, however, it was demonstrated that orphan MTases such as the *E. coli* derived dcm can actually abolish the risk to bacteria chromosomal degradation by REases (Noriko *et al.*, 2002). Dcm gene product, one of these orphan methyltransferases found in *Escherichia coli* and related bacteria, methylates DNA to generate 5' -CmCWGG just as some of its eukaryotic homologues do. Vsr mismatch repair function of an adjacent gene prevents C-to-T mutagenesis enhanced by this methylation but promotes other types of mutation and most likely it has affected genome evolution. EcoRII restriction modification gene complex recognizes the same sequence as Dcm, and its methyltransferase is phylogenetically related to Dcm. Stabilization of maintenance of a plasmid by linkage of EcoRII gene complex, likely through postsegregational cell killing, has been found to be diminished by Dcm function. Disturbance of EcoRII restriction-modification gene complex leads to extensive chromosome degradation and severe loss of cell viability. This cell killing is partially suppressed by chromosomal Dcm and completely abolished by Dcm expressed from a plasmid. This finding, that Dcm can play the role of a "molecular vaccine" by defending the genome against parasitism by a restriction-modification gene complex (Noriko *et al.*, 2002) opens doors to the application of the R-M systems in gene therapy and immune reconstitution through Pre-integration Gene slicing (PRINT GSX) of incoming foreign DNA prior to integration into the Host genome. HIV, though a ssRNA virus of the genus Lentiviridae, family retroviridae, reproduces through a DNA intermediate, and thus remains a target of the PRINT- GSX pathway, and has actually been the major focus of research geared at developing

effective gene strategies using class II REases (Misaki 2007a,b,c; Misaki *et al.*, 2007).

Gene therapy potential of the PRINT-GSX pathway for developing HIV inhibitory strategies

Strategies for designing potential HIV inhibitory strategies based on the PRINT-GSX have been recently described (Misaki 2007a, b, c; Misaki *et al.*, 2007). The first approach is ex-vivo, and involves a recombinant native vaginal commensal bacteria expressing CD4 (to facilitate viral capture within the vaginal mucosa by barring/diverting its primary entry through immature dendritic cells), and REase with potent activity against proviral HIV DNA (Misaki 2007a, b). The later recombinant lactobacillus labelled xREPLAB is a potential probiotic (live) microbicide against HIV, and it is at the proof of principle stage following the same protocols as other similar probiotic microbicides described previously (Noriko *et al.*, 2002). The second approach is in-vivo and involves a therapeutic HIV vaccine (*own unpublished data*; Misaki, 2007c). Since the PRINT-GSX pathway destroys exogenous viral DNA prior to integration, it offers a high chance for both a curative and preventive gene based vaccine. The later, VRX-SMR is a lentiviral vector expressing cDNA of REase with demonstrated potency against proviral DNA; together with dcm as a molecular vaccine against REase degradation of the Human genome (Misaki, 2007c). In addition, an attempt to design antivirals by impregnating proteolytic substrate of specificity to the HIV Envelope and Nuclear membranes aside from Vaginal Squamocolumnar epithelium with REase is also under Proof of Principle trials (Misaki, 2007c, d).

Likely limitations to Pre-integration Gene slicing pathway as a Gene therapy Modem

As with PTGS (Coburn & Cullen, 2002; Jacque *et al.*, 2002; Surabhi & Gaynor, 2002; Boden *et al.*, 2003; Doench & Sharp, 2004; Hannon & Rossi, 2004) and other potential gene therapy modems there exists a number of dark areas for PRINT-GSX. For instance, the danger of host genome-toxicity posed by the REase may not be fully prevented or eliminated by dcm. In such instances, there is a risk of loosing all cells that have effectively been transfected with VRX-SMR.

Further still, because REases, unlike siRNAs are peptides, the danger of eliciting immunologic rejection may be greater in-vivo than for siRNA. All these are issues that are likely to be encountered at preclinical trials stage, which is a step further from the current on-going Proof of Principle trials. In addition, the efficiency of REase expression by both xREPLAB and VRX-SMR can only be determined after the POP trials. In particular, there seems to be a special need for extreme foreign DNA splicing /degradation to ensure that byproducts don't ligate, in which case mutants would be obtained. This is being addressed by using REases with ability to cleave 10 or more times in the HIV proviral DNA. Figure 1 shows the distribution of palindromes and potential splicing Reases within the HIV-1 VPR Gene. Perhaps, the

greater limitation to the application of PRINT-GSX is that its activity is limited to DNA, meaning that only DNA viruses can be targeted by this approach. More recently, however, a number of Ribonucleoprotein complexions in bacteria with both RNA and DNA splicing abilities of mobile class of Group II intron elements origin have been described, with indications that the combination of REase with RNA confers RNA catalytic potential (Gorbalenya, 1994; Shub *et al.*, 1994; Zimmerly *et al.*, 1995). The later is a viable research area for generating recombinant Ribonucleoproteins(RNP) with both RNA and DNA catalytic potential for PRINT-GSX, and its snRNA components have previously been explored as an HIV therapy by catalytic splicing of HIV genomic RNA (Zhou *et al.*, 1994).



Figure 1. Graphic map of the distribution of 6 base pair (bp) Palindromic recognition nucleotide sequences in the HIV-1 VPR gene. Legend: Color key: *catgac* = unrecognised VPR gene sequence; *aagctt* = 6 bps palindrome sequences; *BanI* = potential splicing enzymes. Note the presence of 4 base pair palindromes not referred to here such as the "aatt" at the 219-223 bp; and are susceptible to cleavage by REase recognising 4bp palindrome nts. The Figure was generated by Webcutter version 2 software <http://rna.lundberg.gu.se/cutter2/>. Source citation: Misaki *et al.*, 2007. [Afr. J. Biotechnol.](http://www.biosciences.ilewa.org) 6 (10), 1225-1232 (2007)

Beyond the Horizons of Pre-integration Gene slicing and PTGS

1. PRINT-GSX as a Complimentary Strategy to PTGS in HIV gene therapy

RNA interference through the PTGS occurs after viral integration (precisely, at the viral post transcription/messenger RNA level). PRINT-GSX occurs prior to genomic integration. These two pathways, we predict can be efficiently combined to offer an integrated Gene therapy moderm with both pre HIV proviral DNA-integration splicing and post transcriptional HIV silencing, in which case, any virus escaping intial splicing would have its mRNA silences post-transcriptionally.

2. Exploring PRINT-GSX for double stranded viruses-Human papilloma virus

In yet to be published data (own data) we have identified REases with potent activity against HPV types 6, 11,16 and 18, the epitheliotropic double stranded Deoxyribonucleic acid with no ribonucleic acids stage (dsDNA viruses, no RNA stage) viruses of the order Papillomaviridae; family Alphapapillomavirus, whose life cycle is intimately linked to the stratification and differentiation state of the host epithelial tissues that leads to cervical cancer (Agosti *et al.*, 2007) This is the first step on the road to designing a trial with live (probiotic) microbicide against cervical cancer, as Human papillomaviruses (HPVs) cause virtually all cervical cancers, with HPV types 16 (HPV-16) and 18 (HPV-18) being responsible for approximately 70% of reported cases. Recent reports of a 98% efficacy of quadrivalent HPV-6/11/16/18 L1 virus-like-particle vaccine with amorphous aluminum hydroxyphosphate sulfate (Gardasil, Merck) for the prevention of the primary composite end point in the per-protocol susceptible population and 44% in an intention-to-treat population of all women who had undergone randomization is a major break through as it paves way for the first interventional strategy against both cervical and anogenital cancer (Agosti *et al.*, 2007). However, it is clear from the clinical trial results that the risk is not fully eliminated in the vaccinated groups (Agosti *et al.*, 2007). This emphasizes the need to develop other complimentary strategies to the Gardasil vaccine, and the combination of a probiotic microbicide, such as the one we propose, would completely eliminate the risk of cervical cancer associated with HPV. In conclusion, at this point Pre-integration gene Slicing (PRINT-GSX) by way of R-M peptides can be viewed as a trial alternate or complementary gene therapy moderm with potential applications in the areas of HIV management.

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