African Journal of Biotechnology Vol. 7 (12), pp. 1791-1796, 17 June, 2008
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB07.516
ISSN 1684–5315 © 2008 Academic Journals

Review

Why bacteria derived R-M nucleic enzymatic peptides are likely efficient therapeutic molecules for use in the design and development of novel HIV inhibitory strategies

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Accepted 19 October, 2007

In the past, we have identified, described and isolated over 200 bacteria derived Restriction Modification (R-M) nucleic enzymatic peptides as efficient therapeutic molecules for use in the development of novel HIV inhibitory strategies. In the issuing months of our publications, 3 questions have been directed to our work; (1) HIV is an RNA virus, thus restriction peptides are impotent as defense peptides. (2) HIV genome is encapsulated in nuclear capsid and viral envelope, making access impossible. (3) Human genome contains several palindromes recognizable by R-M peptides, making safety delineation critical. This paper serves to provide succinct responses to these issues, and highlight critical strategies being employed in ensuring the development of safe Microbides and therapeutic vaccines based on this approach.

Key words: Restriction modification (R-M) systems, restriction enzymes (REases/RNases), methyltranferases (MTases), human immunodeficiency virus (HIV), immune reconstitution, probiotic microbicides.

INTRODUCTION

Many bacteria operate a special nucleic acids enzyme system called the Restriction Modification (R-M) whose main biological role is to protect the bacteria cell from tropism by viruses. Currently, four classes of these enzymes are known according to structure, substrate specificity, catalytic requirements, and reaction end products. The class I consists of three distinct enzymes systems: A Restriction enzyme (REase/RNase, R), a methyl tranferase (MTase, M) and a site specificity protein(S). The class II R-M, the most prolific of all and the one to which most endonucleases belong comprises only two distinct peptides: the R and M. 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). Type IV R-M have only been recently been identified, and comprise of a single polypeptide that serves to both as a methyl tranferase and restriction endonuclease. The model activity of these enzymes is that they recognize a 4 - 8 bp palindromic sequence within the invading viral DNA and cleave within it, or near to it. Protection to the bacteria genome is provided by site methylation effected by the MTase (Murray, 2002; Nelson et al., 1972; Roberts and Macelis, 1991; Janulaitis et al., 1992; Kessler and Manta, 1990; Nelson and McClell, 1991; Radasci and Bickle, 1996; Barcus and Murray 1995; Wayengera 2005)

The class I RMS systems, although the model ancestors in the evolutions of R-M systems, are also more complex, and-using class 1 *Escherichia coli* K-12 strain's as an example, constitute 6 enzymes, whose respective genes are located on the bacteria chromosome in a region called an immigration island: the hsdS gene, hsdR gene, hsdM gen*e*, mcrB/C genes and the mrr gene. Products of the first two genes play the central antiviral defence function-the site specify subunit hsdS product serves to recognise a specific 6 - 8 base pair sequence in the genome of the invading viruses, while the hsdR restriction subunit product cleaves the DNA if this site is unmethylated. The other 4 gene products serve to respectively: The hsdM gene product is a methyltransferase that transfers a methyl group from S-adenosylmethionine (SAM) to the DNA at the indicated A residues; the mcrBC system restricts DNA containing methyl cytosine residues while the mrr system restricts DNA with m^6-methyl Adenine or m^6-methyl cytosine (Murray, 2002; Nelson et al., 1972; Roberts and Macelis, 1991; Janulaitis et al.,

1992) (Figure 3).

In the past, we have identified and isolated over 200 bacteria derived R-M nucleic enzymatic peptides using both computational and *in-vitro* assays as efficient therapeutic molecules for use in the development of novel HIV inhibitory strategies (Wayengera et al., 2007). We have also described strategies for the biochemical construct of HIV Microbicides, and a therapeutic vaccine basing on this model (Wayengera, 2007a-c). In the issuing months of our publications, 3 questions have been directed to our work; (1) HIV is an RNA virus, thus restriction peptides are impotent as defense peptides. (2) HIV genome is encapsulated in nuclear capsid and viral envelope, making access impossible. (3) Human genome contains several palindromes recognizable by R-M peptides, making safety delineation critical. This paper serves to provide succinct responses to these issues, and highlight critical strategies being employed in ensuring the development of safe microbides and therapeutic vaccines based on this approach.

HIV IS AN RNA VIRUS, THUS RESTRICTION PEPTIDES ARE IMPOTENT AS DEFENSE PEPTIDES

Although HIV is an RNA virus, it belongs to the family Retroviridae, genus *Lentiviriade* (*lenti*-Latin for "slow"). Retroviruses are enveloped viruses possessing a RNA genome, and replicate via a DNA intermediate. Retroviruses rely on the enzyme reverse transcriptase to perform the reverse transcription of its genome from RNA into DNA, which can then be integrated into the host's genome with an integrase enzyme. The virus then replicates as part of the cell's DNA (Miura et al., 1990; Schneider and Hunsmann, 1988; Sakuragi et al., 1992 Sakai et al., 1993) (Figures 1 and 2).

Following HIV attachment and entry to target human cells mediated CD4+ and chemokine cell receptors (CCR5/CCRX) (Cochrane et al., 2006; Suh. et al., 2003), viral RNA undergoes reverse transcription mediated by the enzyme reverse transcriptase to proviral DNA. It's this proviral DNA, and not RNA that finally enters the nucleus and gets intergrated into the Human genome (Seguin et al., 1998; Swenarchuk et al., 1999). For human immunodeficiency virus type 1 (HIV-1), it has been proposed that integration may be favored near repetitive elements inclusive of LINE-1 elements or Alu islands (Stevens and Griffith, 1994) or topoisomerase cleavage sites (Stevens and Griffith, 1996). On the other hand, assays of integration in vitro have revealed several effects of proteins bound to target DNA. Simple DNA-binding proteins can block access of integration complexes to target DNA, creating regions refractory for integration (Stevens and Griffith, 1994; Howard and Griffith, 1993; Bor et al., 1995). In contrast, wrapping DNA on nucleosomes can create hot spots for integration at sites of probable DNA distortion (Stevens and Griffith, 1994; Bushman, 1994; Pruss et al., 1994a; Pruss et al., 1994b). Distortion of

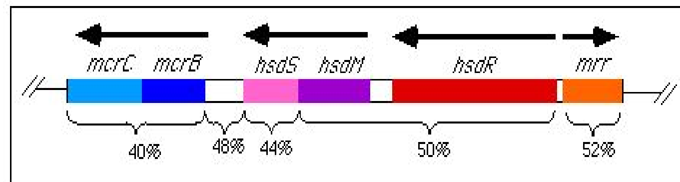

Figure 1. Schematics of *E. coli*-12 immigration Island. http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/genetic-exchange/restriction/Restriction.html

DNA in several other protein-DNA complexes can also favor integration (Pryciak et al., 1992) consistent with the possibility that DNA distortion is involved in the integrase mechanism (Muller and Varmus, 1994; Bushman and Craigie, 1992)

Some studies using HIV have also demonstrated the absence of integration *in vivo* into centromeric haploid repeats, with haploid being absent in integration site sequences but present in controls, and haploid sequences being selectively disfavored in the repeat-specific PCR integration assay; thus providing an demonstration of possibility that certain types of chromatin may obstruct cDNA integration (Scottoline et al., 1997).

HIV GENOME IS ENCAPSULATED IN NUCLEAR CAPSID AND VIRAL ENVELOPE, MAKING ACCESS IMPOSSIBLE

We have previously described two strategies for the *ex vivo* use of bacteria derived R-M nucleic enzymatic peptides as microbicides. The PREX- 1979 Microbicide is a conventional microbicide derived by biochemical integration of R-M peptides cleaving proviral DNA into a proteolytic substrate of specifity to viral envelope and nuclear capsid. In essence, within the vaginal mucosa, the proteolytic substrate serves to digest viral envelope and nuclear capsid, and the R-M nucleic enzymatic peptides cleaving proviral DNA, thus destroying the invading virus (Wayengera, 2007a). On the other hand, x-REPLAB is a live microbicide generated by genetically modifying the predominant vaginal mucosa commensal bacteria to accord it the ability to express both CD4 domain (thus increase viral capture and entry into the recombinant lactobacillus) and R-M nucleic enzymatic peptides cleaving proviral DNA, thus destroying the invading virus (Wayengera, 2007b). Using *Lactobacillus jensenii* xna, cDNA of bacteria R-M peptides cleaving proviral HIV DNA, and pOSEL-651 (OSEL inc. USA), R-M gene of interest was amplified using universal primers by a master cyclero gradient PCR amplification method, cloned into pLUCLaI vector, digested and ligated with BamHI + HindIII cut pET28b vector (Novagen, USA). The resulting plasmid pET28bORF2/3 verified by restriction analysis and sequencing of regions covered in vector inset junction prior to use to transform native *L. jensenii*

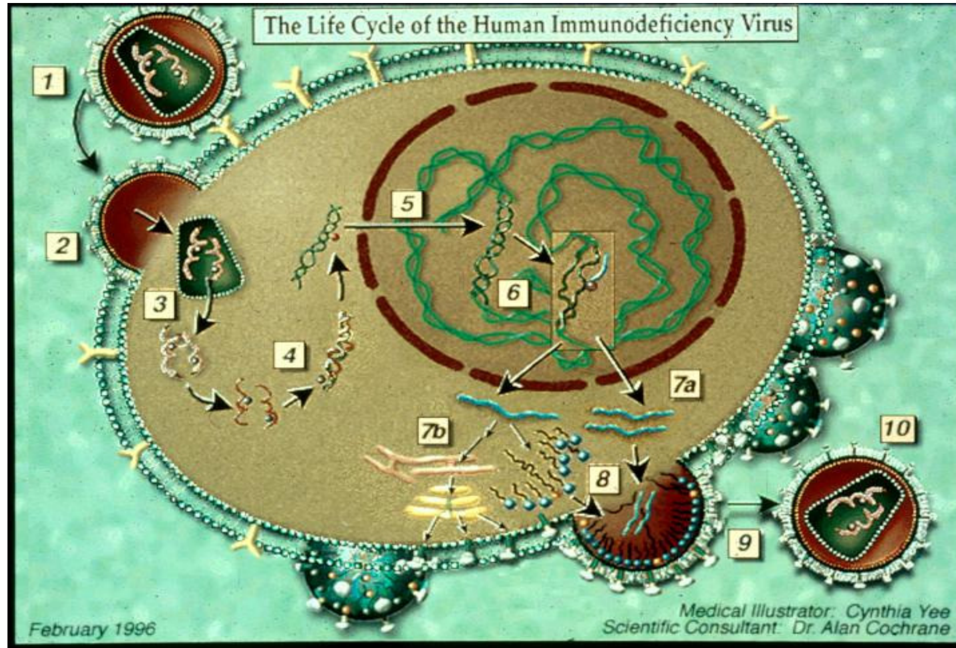

Figure 2. Schematics of the life cycle of human immunodeficiency virus. Adapted from http://www.utoronto.ca/medicalgenetics/PIs/cochranenew.htm, laboratory of Dr Alan Cochrane. (1) Viral attachment, (2) viral entry, (3/4) reverse transcription from RNA to cDNA, (5) entry into nucleus and integration into human genome, (6) synthesis of viral mRNA, (7a/b) viral protein synthesis via transcription, (8) viral assembly, (9) viral budding, (10) new HIV.

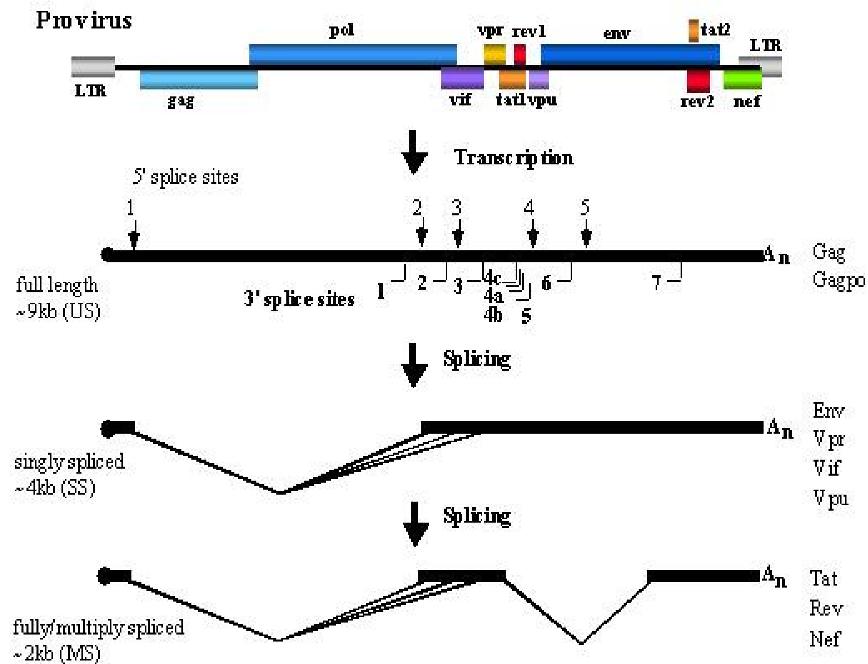

Figure 3. Schematics of processing of HIV 1 RNA from proviral HIV cDNA integrated in the host cell. http://www.utoronto.ca/medicalgenetics/PIs/cochranenew.htm laboratory of Dr Alan Cochrane.

Xna by electroporation. Recombinant *L. jensenii* Xna is grown in Rogosa FL broth (Difco) in presence of kanamycin (50 µmg/ml at 37°C) until OD reached 0.8. Expression of R-M peptide of interest was analyzed at this point by restriction activity following elution by SDS Gel filtration electrophoresis from a clear cell lysate sample after harvest by centrifugation and disruption by French pressure (Aminco,USA), and a two step purification on NiNTA agarose(Qiagen, Germany) and then heparin-supharose(Pharmacia, Sweden) (Lepikhov et al., 2001) The resultant *L. jensenii* Xna(x-REPLAB) strain was transformed by POSEL651 to express GFP labeled functional 2D CD4 as described elsewhere (Wayengera, 2007b; Theresa et al., 2003). Both PREX-1979 and X-REPLAB are currently at phase I preclinical trials stage.

ON HUMAN GENOME CONTAINING SEVERAL PALINDROMES RECOGNIZABLE BY R-M PEPTIDES, MAKING SAFETY DELINEATION CRITICAL

In yet to be published data, we describe the strategies of developing a therapeutic HIV vaccine-VRX-SMR (Restrizymes Corporation Canada, 2007) using the bacteria R-M antiviral model by modifying human cells susceptible to HIV infection. Using a lentiviral vector transduced with cDNA of genes coding for R-M nucleic enzymatic peptides, we intend to transducer human T4 lymphocytes to express these peptides, empowering them to cleave proviral HIV DNA prior to integration into the human genome. The danger within *in vivo* approach is that the human genome, as demonstrated by the Human genome Project (2006), contains several palindromic sequences recognizable by these R-M peptides, thus raising concerns of safety. Many of the safety concerns regarding this approach can however only be answered by phase 1 preclinical trials, which are in lieu. At this point, the safety issues can only be inferred from the available literature.

Type II restriction modification systems (RMSs) have been regarded either as defense tools or as molecular parasites of bacteria. However, an extensively analysis of the evolutionary role from the study of their impact in the complete genomes of 26 bacteria and 35 phages in terms of palindrome avoidance reveals that palindrome avoidance is not universally spread among bacterial species and that it does not correlate with taxonomic proximity. Palindrome avoidance is also not universal among bacteriophage, 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. It is then consistent with a view where type II RMSs is considered as parasites possibly at the verge of mutualism. As a consequence, RMSs constitute a nontrivial third player in the host-parasite relationship between bacteria and phages (Eduardo et al., 2001).

The first issue of particular interest in regard to host genome protection is that within the human genome, with the exception of a few cellular receptors (estrogenic), there seems to be a genomic imprinted, inherited, and tactically balanced methylation of the human genome, with hypo or hyper Methylation of the human genome being associated with carcinogenesis. Actually, inhibition of methylation of newly synthesized human DNA is one of the mechanisms by with the anticancer drug-class of nucleoside analog 5-azacyti-dine (5-aza-CR) function. Genes constitute only a small proportion of the total mammalian genome, and the precise control of their expression in the presence of an overwhelming background of noncoding DNA still presents a substantial problem for their regulation. Noncoding DNA, containing introns, repetitive elements, and potentially active transposable elements, requires effective mechanisms for its long-term silencing. Mam-mals appear to have taken advantage of the possibilities afforded by cytosine methylation to provide a heritable mechanism for altering DNA-protein interactions to assist in such silencing. Genes can be transcribed from methylation-free promoters even though adjacent transcribed and nontranscribed regions are extensively methylated. Gene promoters can be used and regulated while keeping noncoding DNA, including transposable elements, suppressed (Peter and Takai, 2001).

Within the human genome, methylation is also used for long-term epigenetic silencing of X-linked and imprinted genes and can either increase or decrease the level of transcription, depending on whether the methyllation inactivates a positive or negative regulatory element. Most of the 5 -methylcytosine in mammalian DNA resides in transposons, which are specialized intragenomic parasites that represent at least 35% of the genome. Transposon promoters are inactive when methylated and, over time, C-->T transition mutations at methylated sites destroys many transposons. Apart from that subset of genes subject to X inactivation and genomic imprinting, no cellular gene in a non-expressing tissue has been proven to be methylated in a pattern that prevents transcription. It has become increasingly difficult to hold that reversible promoter methylation is commonly involved in developmental gene control; instead, suppression of parasitic sequence elements appears to be the primary function of cytosine methyllation, with crucial secondary roles in allele-specific gene expression as seen in X inactivation and genomic imprinting (Peter and Takai, 2001). With this review of human DNA methyltranferase activity the other big question that arises for further studies to answer is whether the inherit methylation function present within

the human genome can offer protection from the activity of bacteria derived restriction enzymes.

Second and more significant to both human and recombinant *lactobacillus* genome protection strategies is that, a case for genetically modifying human cells (for the *in-vivo* therapeutic vaccine approach) and probiotic *lactobacillus* (for the *ex-vivo* live Microbicide approach) to accord them with bacteria methyltransferase function apart from the restriction proteins can be argued from the fact that while in prokaryotic genomes, some DNA methyltransferase form a restriction-modification gene complex, some others are present by themselves. Dcm gene product, one of these orphan methyltransferases found in *Escherichia coli* and related bacteria, methylates DNA to generate 5' -C^{m}CWGG 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 likely 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. Dcm, therefore, can play the role of a "molecular vaccine" by defending the genome against parasitism by a restriction-modification gene complex (Noriko et al., 2002).

Conclusion

In conclusion, we sustain that bacterium derived R-M nucleic enzymatic peptides are efficient therapeutic molecules for use in the design and development of novel HIV inhibitory strategies

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