CHAPTER IV
DISCUSSION

The ultimate goal of HIV therapy is to improve 'patients' health and prolong their lives. The key goal of antiretroviral therapy is to slow – or ideally stop – HIV replication and enable recovery of the immune system. The standard of care for anyone on antiretroviral treatment is highly active antiretroviral therapy (HAART). These regimens can dramatically reduce HIV replication by interfering cascade of viral replication and prolonging the life of HIV-infected individuals (Rossi et al., 2007; Arora et al., 2010). However, HAART requires lifelong treatment resulting in cumulative toxicities; some drugs interfere with the normal human cells, causing a variety of side-effects. Moreover, HIV generally develops mutations that make it resistant to drugs and its DNA persists as an integrated- genome in long-lived cellular reservoirs resulting latent infection (Rossi et al., 2007; Arora et al., 2010).

Contemplating on the assembly and maturation inhibitors, novel maturation inhibitors blocking the Gag processing and CA assembly have been recently identified. PA-547 inhibits the Gag processing at the CA-SP1 junction by binding directly to this site. However, this drug does not completely block, but simply delays, the proteolysis of the CA-SP1 junction (Ganser-Pornillos et al., 2008; Adamson and Freed, 2010). The second class of inhibitors such as methylphenylurea compound (CAP-1) and a 12-residues peptide (CA-I) binds within the mature CA_{NTD}-CA_{CTD}

interface, suggesting the inhibition of capsid assembly by disrupting the interaction of CA domain. Nevertheless, CA-I and CAP-1 bind too weakly to become clinically useful, but the discovery of these two inhibitors makes this site as an attractive target for further inhibitor development (Tang et al., 2003; Braun et al., 2008). Given the increasing knowledge of mechanisms that allow control of HIV infection, several researchers are focusing on gene therapy, either as a stand-alone approach or as an adjuvant to pharmacological regimens. Gene therapy offers the promise of preventing progressive HIV infection by sustained interference with viral replication in the absence of chronic chemotherapy (Rossi et al., 2007). In this study, we aimed to discover novel protein-based agents as intracellular inhibitors of viral replication by gene-targeting strategy in the future.

Ankyrin repeat protein was applied as a candidate scaffold for isolating HIV-1 MA-CA specific binding molecules. This motif represents an alternative binding property to conventional antibodies in terms of high binding affinity and specificity. Moreover, it conveys superior attribution over antibodies due to the high level of expression, compact folding in all environment including intra- and extracellular resulting in high stability (Sedgwick and Smerdon, 1999; Mosavi et al., 2004; Stumpp and Amstutz, 2007; Stumpp et al., 2008). This molecular architecture has generated the novel class of protein library name “Design Ankyrin Repeat Proteins (DARPins) library (Binz et al., 2003) and isolated many specific binders to various targets molecules (Binz et al., 2004; Christian Zahnd et al., 2007; Huber et al., 2007; Schweizer et al., 2007).

However, the construction of DARPins library restricted the number of internal repeats to 2 (N2C) or 3 (N3C) repeats. The amino acids in the random positions were designed to use mixture of degenerated codons which code the same proportion of all amino acid except cysteine, glycine and proline (Binz et al., 2003). According to natural ankyrin repeat protein sequences, the number of repeats varies ranging from one to thirty three with an enrich fraction at two to six modules. This suggests that elongated domains with a continuous target-binding surface which is variable in size as well as number of repeats can be varied (Sedgwick and Smerdon, 1999; Mosavi et al., 2004). For such open structures, there is no theoretical limit on repeat number since incremental addition of repeats is not sterically hindered. These rod-like or super helical structures present an extensive solvent-accessible surface that is well suited to bind large substrates such as proteins and nucleic acids (Andrade et al., 2001). Nevertheless, the fixed number of internal domain as presented in DARPins library might limit the binding surface by their size.

Therefore, artificial ankyrin repeat protein library constructed in this context was designed to resolve the DARPins's limitation by varying number of repeat (**Figure 3.6A**) and the proportion of amino acid in random positions was stimulated to mimic the natural sequences (**Table 3.1 and Figure 3.4**). The consensus sequence was defined with some modifications in conserved residues to generate the recognition site (*Bsm* BI) for the polymerization process (**Figure 2.1 and 3.1**) without disturbing the protein structure (**Figure 3.2**). The chosen variable positions were localized at the first α-helices and oriented towards the binding sites (**Figure 3.3**). We applied Phi 29 DNA polymerase to magnify the oligonucleotide fragments. This enzyme was an attractive

polymerase for cloning because it copies DNA with high fidelity, has a proofreading activity, and is highly processive (Hutchison et al., 2005). Our procedure was based on the rolling-circle amplification (RCA) reaction (**Figure 2.2**) carried out isothermally by this polymerase enzyme at 30ºC (Dean et al., 2001; Gill and Ghaemi, 2008). Moreover, the strand displacement property of this enzyme produces large amount of amplified product from circular template. Mixture of single-stranded circularized DNA fragments was converted to long homopolymers of double-stranded products and subsequently dissociated by treating with *Bsm* BI. This step resulted in a very large amount of fragment representing a single repeat (**Figure 3.5**). In order to variegate the repeat number, the library was constructed by performing the direct polymerization of DNA fragments into the vector (**Figure 2.3**). The library size created in this study was not as large as DARPins but was efficient enough to generate phage display libraries.

Protein expression of naïve library was assessed by CoFi blot analysis (**Figure 3.6B**). The population of coding sequence was in concordance with sequencing result. These unexpected non-coding sequences could result from oligonucleotides synthesis, or from amplification process using random hexa primers providing in the kit. The solutions to solve this problem would be using sets of designed specific primers. Herein, the coding populations were enriched up to 60% after two round of phage pre-selection (**Figure 3.7**). However, inactive clones containing non-coding sequences still survive in the filtrate library. This could be explained if a fraction of non-displaying phage particles remain associated to the displaying particle during PEG precipitation and therefore could later be selected resulting in high selection

background. To evaluate the effect of PEG on the non-specific binding, the first round of phage panning was done by using supernatant containing phages and PEG-precipitated phage. The background of precipitated phage was 10 times higher than the other referring by number of phage output from un-coated wells. Therefore, supernatant containing phages of both naïve and filtrated phage library were used for the following round of selection. Focusing on H_6MA-CA binders, 30% (10 of 33), and 13% (4 of 32) of positive cones were detected from filtrate and naïve library after two rounds of selection, respectively (**Figure 3.10 upper panel**). Regarding this result, the pre-selection technique was efficiently increased the population of coding clones and the probability of isolating binders. Interestingly, many specific binders to two individual targets (H_6MA-CA and A3) could be fetched out (**Figure 3.10**). However, the success of regaining binding molecules against A3 molecule was proved by many positive clones after selection round using naïve library indicating the efficiency of this constructed library.

From restriction and sequence analysis of H_6MA-CA binders, there are two major groups classified by the number of repeat (**Figure 3.11**). Candidate clones containing three repeats were firstly chosen to express as soluble protein for further characterization the binding activity by various methods. The specific binding activity was verified by competitive ELISA against irrelevant target molecule (A3) and other ankyrin candidates (**Figure 3.17**). The competition effect was observed while using H_6MA-CA binders (1D4, 1B8, and 6B4) as inhibitors but not with 2E3 representing the specific reaction of selected binders. The specific recognition site of binders was located at CA domain as identified by Western immunoblotting (**Figure 3.18A**) and

confirmed by ELISA using recombinant CA domain (**Figure 3.18B**). Epitope mapping of all binders and antibodies against CA domain was evaluated by ELISA using the biotinylated-molecules. Positive binding signals were observed while capturing H_6MA-CA on the wells *via* antibodies (**Figure 3.18C**). This result suggested the distinct epitopes to the examined antibodies. The candidate clone, 1D4 demonstrated the best binding activity as compared to the other chosen clones by Dot blot and ELISA (**Figure 3.16**). Hence, it was taken to evaluate the binding affinity and specificity by ITC experiment. This method could be directly applied to evaluate the interaction of molecule and its cognate molecule by measuring the binding constant (K_d) and stoichiometry (N) insolution (Karsten and Cook, 2006; Dam et al., 2008; Freyer and Lewis, 2008; Parker and Stalcup, 2008). The affinity of 1D4 against MA-CA was 0.45 μM while no interaction was observed with 1D4 against A3 demonstrating the high affinity and specificity of this clone (**Figure 3.19**). Moreover, the stoichiometry value represented the molar ratio between 1D4 and MA-CA around 0.6 suggesting the binding of one molecule of 1D4 to two domains of MA-CA. This result confirmed the dimerization phenomenon of CA domain occur in nature (Scarlata and Carter, 2003; Saad et al., 2006).

The functional activity of ankyrin candidates on the interfering of maturation was evaluated by ELISA-based HIV protease assay technique (manuscript is in preparation). All three tested clone exhibited the same scenario with ankyrin control manifested no interfering effect on HIV-1 protease activity. This result could be due to the recognition site of binders is located at CA domain far away from the area for

protease or the affinity was not high enough to compete the activity of this enzyme. However, more clones will be screened for this application.

The interfering effect on viral assembly was appraised by infecting HIV particles onto Sup-T1 stable lines expressing two variants of ankyrin 1D4, membrane-bound 1D4 and cytoplasmic 1D4. Taking the nature of HIV Gag protein-trafficking to inner membrane *via* myristoylation signal sequence (Bouamr et al., 2003; Ganser-Pornillos et al., 2008; Ono, 2010; Simons and Gerl, 2010), the membrane bound 1D4 version was created by fusing N-terminus of 1D4 with the myristoylation signal sequence (**Figure 3.21**) to direct this protein to the inner membrane where the viral assembly occurs. The cytoplasmic 1D4 was also generated to analyze its effect on viral assembly as compared to the membrane-bound form. The distribution of protein expressed by these two forms was determined under the high resolution fluorescence microscopy. Signal sequence inserted in the construct efficiently translocated 1D4 containing myristyl group to the membrane of the cells as showed by the GFP pattern (**Figure 3.22A, upper panel**). In contrast, non-myristoylated 1D4 dispersed throughout the cytoplasm (**Figure 3.22A, lower panel**). The quantity of ankyrin 1D4 expression in these two SupT-1 stable lines was similar as validated by flow cytometry (**Figure 3.22B**) indicating that these stable cells can be evaluated for the function by challenging with HIV_{NL4-3} viral particles. Interestingly, this preliminary data revealed that both of two stable lines significantly decreased the viral assembly. Moreover, stable cell expressing the membrane-bound 1D4 dramatically inhibited the viral replication as compared with the cytoplasmic-1D4 stable line (**Figure 3.22C**).

The results suggest that directing the therapeutic molecules to the functional area of target molecules may be very efficient to block the function.

In conclusion, the ankyrin repeat protein was used to generate novel artificial molecules for hindering the HIV-1 replication. The artificial ankyrin library was constructed using the special design of consensus motif, amplification process and polymerization of repeats onto vector resulted in the effective library. A number of specific binders against H_6MA-CA were retrieved from this constructed library. Some of isolated clones were evaluated for the binding activity, specificity, and their recognition site. The best candidate (1D4) was chosen for further assessment of its quantitative binding constant charisteristic and its functions. Although, this molecule showed no effect on viral maturation but it exploited the disturbance of viral assembly on membrane-bound 1D4 molecule. The preliminary findings are very convincing to apply these molecules as intracellular agents for HIV life cycle interruption