

## CHAPTER 4

### DISCUSSION

The ultimate goal of antiretroviral treatment is to control HIV replication rate at the lowest level as possible in infected individual. Recently, highly active antiretroviral therapy (HAART) has effectively slowed down the progression of AIDS and reduced HIV-related illness and death (Rachel et al., 2002). Although, HAART dramatically decreases HIV replication, but the use of this regimen is associated with the emergence of a variety of side effects (Carpentier et al., 2005), resulting in 25% of patient to stop taking their treatment within the first year (Cicconi et al., 2010). In addition, since therapy is required lifelong, the development of HIV resistance to at least one class or multiclass of drugs has been shown (Cane, 2009; Clavel and Hance, 2004; D'Aquila and Walker, 1999). Therefore new approaches to control HIV replication are needed. Passive immunization of antibody-based therapeutics and gene therapy based on intrabodies therapy has been introduced as alternative approaches. For instance, extracellular antibodies can neutralize HIV-1 at the early phase of cell attachment (Zwick et al., 2001) and intracellular antibodies can block viral replication by negatively interfering with some functions of the HIV matrix (MA) protein (Tewari, Notkins, and Zhou, 2003). Since the MA plays a key role in several steps during virus replication, both in the early and late stages of the virus life cycle (Bukrinsky et al., 1993; Fäcke et al., 1993; Spearman et al., 1994), it represents as a target protein for neutralizing antibodies against HIV-1 (Naylor et al., 1987; Papsidero, Sheu, and Ruscetti, 1989).

Tewari et al. (1998) constructed a single chain variable fragment (scFv) derived from a monoclonal anti-matrix antibody (MH-SVM33C9/ATCC HB-8975) (anti-MA mAb HB-8975) and it could reduce HIV-1 replication. This antibody specifically recognizes a C-terminal epitope (DTGHSSQVSQNY) of HIV-1 matrix domain (MA) located near the HIV protease (HIV-PR) cleavage site (Tewari et al., 1998; Tewari, Notkins, and Zhou, 2003). The aims of this study were : (i) to characterize the key amino acid residues involving in binding interaction of a single chain variable fragment (scFv) against HIV matrix protein (scFv-MA HB-8975) by computational approaches, (ii) to engineer and display scFv-MA HB-8975 on the surface of baculovirus using novel leader peptide, and (iii) to apply the epitope specificity of the monoclonal anti-MA antibody HB-8975 (anti-MA mAb HB-8975) for the development of a simplified enzyme-linked immunosorbent assay (ELISA) for the HIV-1 protease (HIV-PR) activity.

Improvement of the binding affinity of antibody-antigen complex is a challenging problem that has practical applications in the development of therapeutic molecules. Computational protein design can be used to analyze a large number of antibody designs and accurately predict the properties of the antibody designs with their target. Therefore, computational procedure and structural-based protein analysis have been assessed the relevant information that can guide the design of antibody with enhance affinity and specificity (Barderas et al., 2008; Farady et al., 2009). As a consequence, we used the homology modeling strategy to generate the 3D structure of scFv-MA HB-8975 (**Figure 3.1A**) and further analyze it to predict the binding

interaction of scFv-MA HB-8975 with nine HIV matrix peptides by using molecular docking. The predicted data was calculated to PMF scores (**Table 3.1**). The PMF scores could classify the mutant peptides into two groups. The p17.1, p17.2, and p17.8 were identified as the high affinity binding peptides while p17.3, p17.4, p17.6, p17.7, and p17.9 were designated as the low affinity binding peptide. The different binding property was due to the orientations of peptide toward the binding pocket of scFv-MA HB-8975. All high affinity binding peptides directly bound to scFv *via* the N-terminus whereas most of low affinity binding peptides interacted by the C-terminus (**Figure 3.6B**). Importantly, the mutation at particular region at S125N of p17.2 and S125K of p17.8 retained and enhanced the binding affinity, respectively. In order to investigate the binding activity by computational design, peptide ELISA was employed to evaluate the structure-based analysis by computational assisted modeling. As a result, both scFv-MA HB-8975 formats, scFv-gpIII fusion protein on phage particle and soluble protein, retained the binding activity as occurred in parental antibody (**Figure 3.4**) and recognized wild-type and all mutant peptides in the same pattern (**Figure 3.5**) correlated with PMF scores in **Table 3.1**. Moreover, competitive ELISA data were comparable with calculated binding free energy from MDs (**Table 3.2**). The ability of scFv-HB-8975 that binds to entire natural mutant HIV matrix epitopes will be applicable as therapeutic agent in a broad range of HIV-1 variants. Consequently, computational study provided the imperative data, (i) the conformational structure or origination of antibody-antigen, (ii) and binding property of antibody against various antigens which were consistent with the experimental data.

In order to predict the complete structure of scFv-MA HB-8975 in complex with HIV MA peptides, the key amino acid residues in CDR regions of scFv-MA HB-8975 that play a curtail role in interaction was indicated by decomposing the calculated free energies as shown in **Table 3.3** and **Figure 3.6**. The most common interacting residue found in all four peptides is Met100. The strongest binding residues with p17.1 represented in Met100, Lys101, Asn169, His228, and Leu229 whereas p17.8 exhibited particular residues, Trp50, Phe167, and Thr227 led to interaction improvement. In contrast, there was only one amino acid, Gln231, involved in interaction with p17.3 and no strong binding interaction with p17.7 resulted in a low binding affinity. The particular key residues of scFv-MA HB-8975 with each peptide represented the different structural binding interaction. To verify the calculated binding free energy, the common interacting amino acid Met100 of scFv-MA HB-8975 substituted with glycine (Gly100 or scFv-MA M100G) and glutamate (Glu100 or scFv-MA M100E). As a result, scFv-MA M100G remained its activity when displayed on filamentous phage particle but not in the soluble protein. In contrast, the scFv-MA M100E significantly diminished the binding activity in both scFv-gpII fusion protein on phage particle and soluble protein (**Figure 3.7**). Accordingly, the hydrophilic property of Glu100 might affect in the hydrophobic interaction of antigen-antibody whereas nonpolar amino acid substitution, Gly100, did not influence the binding activity of scFv. Even though hydrophobic interaction is a weak force for the antibody-antigen reaction, it is a major driving force which is supposed to make a significant contribution to the interaction (Tanford, 1978). Moreover, it has been reported that the amino acid largest contributions in antibodies

come from tyrosine, glycine, and serine. These three amino acids are overrepresented on the antibody-antigen interface and formed versatility in different types of interaction (Clark et al., 2006; Conte, Chothia, and Janin, 1999). Our experiment clearly demonstrated that the residue Met100 is a key position as shown by the loss of binding upon one single amino acid substitution of scFv-MA M100E.

Regarding the affinity of phage-displayed scFv-MA HB-8975 and scFv-MA M100G, the competitive ELISA revealed the superior affinity of scFv-MA HB-8975 (**Table 3.4**). Nevertheless, scFv-MA M100G still preserved their binding activity, but this mutation decreased the binding affinity with soluble peptide p17.1 compared to scFv-MA HB-8975. Focusing on the reactivity of scFv-MA M100G in different formats, the scFv-gpIII fusion protein exhibited a binding activity whereas the soluble protein had no reactivity (**Figure 3.7**). As a consequence, the rate of protein production and folding of scFv format might cause these effects. Since most scFv expressed as intracellular proteins are found as cytoplasmic inclusion bodies, the insertion of a signal peptide, *OmpA*, has been employed to secrete protein into the periplasm which promoted the proper condition for correct folding of scFv. However, the overexpression of scFv with signal peptide inhibited cell growth (Sandee et al., 2005). In addition, when bacteria are infected with phage particles, the bacterial proteins are engaged in the propagation of phage progeny resulting in the competition of other proteins and scFv-gpIII fusion protein production (Kirsch et al., 2005). Therefore, the level of scFv-gpIII fusion protein expression was slower compared to soluble protein production that lead to protein folding correctly and preserving their

binding activity (Goodrich and Steege, 1999; Kokoska and Steege, 1998). Although residue Gly100 of scFv-MA M100G retained the reactivity as appeared in scFv-MA HB-8975, this amino acid substitution could reduced not only the binding affinity but also disturbed the activity of soluble scFv-MA M100G. Notably, although phage-displayed antibody exhibits the high binding activity, but soluble scFv does not always represent the same activity as shown by scFv-MA M100G.

Taken together, the compatibility of the information obtained by computational study with the experimental data allowed us to better understand the process of scFv-MA HB-8975 binding to HIV matrix peptide variants, and to identify the amino acid residues which are crucial in the antibody-antigen interaction. These structure-guided computational methods could be useful in the future design of antibodies with affinity and specificity optimized by site-specific randomization. The data from computational study will also be useful in the construction and screening of large virtual combinatorial libraries of scFv against HIV proteins, aimed at interfering with HIV replication, prior to investigate the selected scFvs in *vitro* and in *vivo*.

After performing site-specific randomization for improving the binding affinity and specificity of scFv, the effective selection strategy is required. Recently, phage display method has been proposed to isolate improved antibodies specific to cognate antigen from large combinatorial libraries (Hoogenboom et al., 1998; Marks et al., 1991; Sblattero and Bradbury, 2000). Notably, phage display has some limitations imposed by the production in bacteria host e.g. in the case of expression of large or complex eukaryotic proteins that require glycosylation and particular folding.

Therefore an efficient viral display technology using baculovirus (BV) has been promoted as an alternative display strategy for isolating antibodies hence this technology provides post-translation modifications for production of a variety of complex proteins (Grabherr et al., 2001; Mottershead et al., 1997; Mottershead et al., 2000; Ojala et al., 2001). In addition, baculovirus not only represents a powerful expression system of recombinant proteins in insect cells but also can be used for transduction of dividing and nondividing mammalian cells and tissues *in vitro*, *ex vivo*, and *in vivo* as gene delivery vectors for use in human gene therapy (Ghosh et al., 2002; Kenoutis et al., 2006; Kost, Condreay, and Jarvis, 2005).

Baculovirus (BV) display has been used for almost a decade for immunisation purposes, gene delivery, or development of eukaryotic libraries (Grabherr et al., 2001; Mäkelä and Oker - Blom, 2006; Yu - Chen, 2006). Conventional BV-display involves baculoviral envelope glycoprotein GP64 manipulations (Grabherr et al., 2001; Mäkelä and Oker - Blom, 2006; Mottershead et al., 2000), or the use of the VSV-G stem (Ernst et al., 2006; Mäkelä and Oker - Blom, 2006; Ojala et al., 2004). This differs from the incorporation of foreign proteins or glycoproteins into the baculoviral envelope without fusion to GP64, such as the envelope incorporation and display of functional human beta-2 adrenergic receptor ( $\beta$ 2AR) described in an earlier study (Loisel et al., 1997). In a more recent work, the human CAR glycoprotein could be incorporated into the baculoviral envelope. CAR is the high affinity receptor for adenovirus serotype 5 (Ad5) and is a resident glycoprotein of the human cell plasma membrane. The baculoviral envelope-

incorporated CAR was fully functional at the surface of BV-CAR virions, and enabled the formation of BV-CAR-Ad5 complexes, mediated by the interaction between the adenoviral fiber and CAR. We have used this strategy of BV-CAR-Ad5 duo formation to transduce Ad5-refractory cells (Granió et al., 2009).

It was relatively easy to conceive that human  $\beta$ 2AR and CAR molecules, even though expressed in heterologous system, could be displayed on the baculoviral envelope since both are resident membrane glycoproteins. It was rather unexpected for scFvE2/MA, which was an artificial molecule extrinsic to the BV-insect cell system. Moreover, in the case of scFvE2/MA, the scFv molecule was not constructed for membrane targeting, in contrast to scFvG2/MA which carried the specific Met-Gly dipeptide signal for N-myristoylation by N-myristoyl-transferases. Comparison of the amino acid sequences of scFvE2/MA and scFvG2/MA, which both lacked any consensus leader peptide, showed that they only differed by three residues at their N-terminus, M(EAS)L for scFvE2/MA, versus M(G)L for scFvG2/MA. The results of these minor sequence changes were drastic in terms of scFv solubility, cell compartmentalization and extracellular release. Recombinant scFvG2/MA protein expressed in Sf9 cells was inexpressible since it was insoluble and trapped in the membrane pellet.

Recombinant scFvE2/MA however was recovered simultaneously under two different forms: (i) as soluble scFv molecules from lysates of BV-infected Sf9 cells, and (ii) as BV-displayed scFv in the culture medium of the BV-infected Sf9 cell cultures. Both forms could be used as biological tools for different purposes. Soluble



scFvE2/MA could serve in conventional diagnostic assays for HIV-1 Gag detection, through specific recognition of the conserved MA epitope peptide. The MA domain functions as a structural component of HIV-1 virions, but also as a viral cytokine which binds to a cellular receptor, p17R (Belyakov and Berzofsky, 2004; De Francesco et al., 2002), when released by HIV infected cells. In the case of BV-displayed scFvE2/MA using a novel leader peptide, the potential applications would be different. For example, if one considers the virokine properties of soluble MA protein and the importance of inflammatory response at the mucosal sites of HIV-1 entry (Belyakov and Berzofsky, 2004; De Francesco et al., 2002), one could envisage to use pelletable, BV-displayed scFvE2/MA in experimental models of infected mucosae to deplete soluble MA protein from the extracellular medium, or/and to compete with MA protein for binding to p17R. Importantly, the development of BV-scFvE2/MA as gene delivery vectors would be useful for human HIV gene therapy since the advantages include; the ability to replicate in mammalian cells, apparent lack of cytotoxicity, capacity to sustain large insertions of foreign DNA, ability to target many different cell types, and superior safety features relative to mammalian virus-based transduction systems (Intasai et al., 2009; Peipp et al., 2004; Royer et al., 1997).

To assess the role of the N-terminal domain of scFvE2/MA in the process of membrane addressing and scFv display on the baculoviral envelope, we fused the N-terminal octadecapeptide <sup>1</sup>MEASLAAQAAQIQLVQSG<sup>18</sup> (abbreviated N18E2) to another bioactive scFv molecule, scFv-M6-1B9. The ligand of scFv-M61B9 is M6,

also called CD147 (Intasai et al., 2009; Tragoolpua et al., 2008), a transmembrane glycoprotein highly expressed in various types of malignant cells and tumors, e.g. nasopharyngeal carcinoma (Du et al., 2009). CD147 acts as an inducer of extracellular matrix metalloproteinases (EMMPRIN is another acronym for CD147) to promote tumor growth, invasion, metastasis and neoangiogenesis, and is a prognostic marker for invasiveness in prostate cancer (Han et al., 2009) and thyroid carcinoma (Tan et al., 2008). CD147 is also involved in atherosclerosis plaque instability (Yoon et al., 2005) and in the regulatory inhibition of starvation-induced autophagy in human hepatoma cells (Gou et al., 2009).

We expressed the chimeric scFv-N18E2/M6 molecule in recombinant BV-infected Sf9 cells, and found that the BV progeny displayed scFv N18E2/M6 on the baculoviral envelope. This suggested that the N-terminal octadecapeptide N18E2 carried the function required for BV-display of scFv molecules, and could be considered as a BV envelope addressing/anchoring signal peptide. This was further supported by the comparison of the structural domains of scFv downstream of the N18E2 peptide: scFv-N18E2/M6 and scFvE2/MA differed by the successive order of their variable regions, VL-linker-VH from the N- to C-terminus in scFv-M6-1B9, versus VH linker- VL in scFvE2/MA. This implied that the nature of the variable region downstream of N18E2 had little influence, if any, on the membrane addressing of chimeric, N18E2-fused scFv. Although the molecular mechanism of cell trafficking of our chimeric scFv-N18E2/M6 molecule still remained to be elucidated in molecular terms, our present data provided a novel concept and platform for engineering scFv molecules competent for BV-display.

The baculovirus-displayed antibody using novel leader peptide, N-terminal octadecapeptide <sup>1</sup>MEASLAAQAAQIQLVQSG<sup>18</sup> or N18E2, will be valuable in screening the efficient scFv against its target antigen from a large combinatorial library and further applicable as gene deliver vector for isolated scFv which will act as intrabody in human gene therapy.

In our study, the anti-MA mAb HB-8975 was distinguished by its unique property of specific binding to the free C-terminus of the MA domain, after proteolytic cleavage of the H<sub>6</sub>MA-CA precursor by HIV-PR. Therefore, a facilitated ELISA-based assay for determining HIV-PR activity was generated using this particular property of anti-MA HB-8975. Several HIV-PR activity assays have been developed; however, they are not easily used in resource-limited settings. Similarly, while spectrophotometric and fluorometric assays have been introduced, these assays require a trained operator (Fuse et al., 2006). Additionally, cell-based, colorimetric and chromatographic assays generally have a low sensitivity, which can be problematic for high-throughput screens (Billich et al., 1988; Stebbins and Debouck, 1997). As a result, HIV-PR activity assays based on ELISAs were established (Fournout et al., 1997). However, these assays rely on the use of a modified synthetic peptide substrate conjugated with a chemical reagent (2,4,6-trinitrobenzenesulfonic acid), which can be expensive (Bagossi et al., 2004). In this study, we describe a simplified immunological assay designated “ELIB-PA” that can be directly used to evaluate HIV-PR activity using a substrate that is similar to the natural substrate (recombinant H<sub>6</sub>MA-CA protein).

Because the overproduction of recombinant HIV-PR can inhibit cell growth, obtaining a sufficient amount of HIV-PR to perform the activity assay can be difficult (Krausslich, 1992; Rizzo, Korant, and Lawrence C. Kuo, 1994; Stebbins, Debouck, and Lawrence C. Kuo, 1994). Although protocols for the purification and refolding of HIV-PR have been established, the recovery of active HIV-PR is often inadequate (Cheng et al., 1990; Darke et al., 1989; Goobar et al.). Therefore, the development of an efficient *E. coli* expression system for the production of HIV-PRH<sub>6</sub> was a prerequisite for this study. The strict, controlled expression of HIV-PRH<sub>6</sub> was achieved using T7 transcription/translation and *lacUV5* regulatory elements (**Figure 3.18**). In addition, 0.1 mM IPTG and an optimized temperature of 16°C were used during induction in order to retain a high cell density during HIV-PRH<sub>6</sub> production. As a result of these modifications, the expression and solubility of HIV-PRH<sub>6</sub> were remarkably improved (**Fig. 3.19A**). Importantly, the production of HIV-PRH<sub>6</sub> I54V mutant was significantly enhanced compared to wild-type HIV-PR, indicating that one amino acid substitution at position 54, from isoleucine to valine had a great influence on protein production and solubility (**Figure 3.19B**).

The N-terminal His<sub>6</sub> of HIV-PRH<sub>6</sub> substrate was used to orient the substrate such that the CA domain protruded from the surface of the well (**Figure 3.17A**). This particular design was essential for the detection of HIV-PRH<sub>6</sub> proteolytic activity using two individual monoclonal antibodies. The decreased detection of the CA domain, in combination with the detection of the newly generated free C-terminal epitope of the MA domain, was observed following HIV-PRH<sub>6</sub>-mediated proteolysis

of the immobilized substrate (**Figure 3.17D**). A mAb that recognized the CA domain (anti-CA mAb G18) was used to detect the residual, uncleaved H<sub>6</sub>MA-CA substrate. By contrast, the unique interaction of anti-MA mAb HB-8975 with the free C-terminal epitope of MA that is exposed following the removal of the CA domain was crucial in confirming the activity of HIV-PRH<sub>6</sub>. The complete inhibitory effect of LPV against HIV-PRH<sub>6</sub> demonstrated that this assay was reliable (**Figure 3.22**). Additionally, The ELIB-PA was capable of detecting the decreased activity of the HIV-PRH<sub>6</sub> I54V variant and the susceptibility of HIV-PRH<sub>6</sub> I54V to LPV was also observed (**Figure 3.22**). Therefore, the ELIB-PA has the potential to elaborate the degree to which particular mutations affect HIV-PR activity. Even though the amino acid substitution of Ile to Val at position 54 of HIV-PR mutant was able to significantly augment its production and solubility (**Figure 3.19A**). However, the proteolytic activity of HIV-PRH<sub>6</sub> was two-fold higher than that of HIV-PRH<sub>6</sub> I54V (**Figure 3.22**). It has been reported that accumulation of 6 or more amino acid mutations associated with LPV resistance strains (Kempf et al., 2001; Masquelier et al., 2002). The position Val32, Ile47, Lys76, and Val82 are major mutations that caused the resistance to LPV (Friend et al., 2004; Mo et al., 2005). The specific mutations of V32I, I47A, and L76V confer the high-level resistance. However, V82A also appeared in RTV-resistant strains, likely due to the low dose of RTV that is currently combined to LPV. This addition was meant to boost the concentration of LPV in plasma by inhibiting its hepatic metabolism (Hurst and Faulds, 2000; Qazi, Pozniak, and Morlese, 2002). As a consequence, multiple mutations are necessary to select HIV-PR mutants that resist to LPV.

Moreover, the efficacy of the ELIB-PA was validated by determining the  $IC_{50}$  of LPV and other HIV-PR inhibitors, RTV and NFV. As a result, LPV, which belongs to the second generation of PIs, predominated in several properties; (i) the effective diminution of HIV-PRH<sub>6</sub> activity, (ii) the efficient inhibition demonstrated by  $IC_{50}$ , and (iii) the potential binding affinity indicated by accelerated abolishment. By contrast, the first generation of PIs, RTV and NFV, were significantly inferior in all properties (**Figure 3.23**). RTV required the isopropyl side chain of Val82 for specific interaction. However an initial mutation of Val82 to Ala, Thr, and Phe, appeared in patients whose viral RNA load rebounded on RTV therapy (Molla et al., 1996). These mutations reduced the PR affinity *via* hydrophobic interaction of RTV to isopropyl group of Val82, by modifying its side chain to a hydroxyl side chain in the Thr82 and to an aromatic ring in the case of Phe82. Therefore, LPV was developed by replacement of isopropyl-thiazolymethyl urea unit of RTV by shortened cyclic urea unit to eliminate the interaction with Val82. However, LPV still presented the same retention inhibitory potency toward wild-type HIV-PR. Thus LPV can be used for treatment not only in RTV resistant patients but also in common HIV-infected patients (Sham et al., 1998; Stoll et al., 2002). Regarding the inhibition property of the first generation PI, the distinct inhibition mechanism, C2 symmetric inhibitor of RTV and peptidomimic inhibitor of NEV, had no influence on the inhibitory effect (Bühler et al., 2001). Accordingly, the ELIB-PA may aid in screening and selecting novel candidate HIV-PR inhibitor compounds from computerized HIV-drug design by observing the fold change in  $IC_{50}$  and the increment of accelerated inhibition.

Clinically, resistance to HAART is frequently observed in patients receiving long-term treatment (Arrivé et al., 2007; Booth and Geretti, 2007), which often necessitates changes in the drug regimens used to control viral replication. The identification of drug-resistant strains prior to the use of a new therapeutic regimen would therefore provide valuable information. Furthermore, new generations of PIs that inhibit HIV-PR variants must be developed. Currently, a large number of novel PIs have been comprehensively examined using computational molecular modeling (Chang et al.; Ghosh et al., 2007). However, the screening of these compounds must be performed both *in vivo* or *in vitro*, and this approach is complex and time consuming (Dautin et al., 2000; Steindl et al., 2007). Therefore, due to its relative simplicity and ability to be used in high-throughput screens, the ELIB-PA is an attractive, alternative assay that can be used to identify and screen novel PIs resulting in acceleration of the drug screening process in the pharmaceutical-industry.