CHAPTER 4

CONCLUSION

The results from this study can be concluded as the followings:

Part 1 : Construction of GFP, Tat/GFP, VP/GFP and VP/Tat/GFP fusion protein expression plasmid

1.1. Five constructs of protein and fusion protein; GFP, N-terminal Tat fusion GFP (Tat-GFP), C-terminal Tat fusion GFP (GFP-Tat), N-terminal VP fusion GFP (VP-GFP) and Tat-GFP-VP were prepared.

1.2. Size of PCR product of GFP, Tat-GFP, GFP-Tat, VP-GFP and Tat-GFP-VP was 737, 764, 764, 788 and 815 bp, respectively.

1.3. GFP, Tat-GFP, GFP-Tat, VP-GFP and Tat-GFP-VP PCR fragments were inserted between *Nhe*I and *Bam*HI restriction site of the expression vector, pET28a(+).

Part 2 : Expression and purification of GFP, Tat/GFP, VP/GFP and VP/Tat/GFP fusion protein

GFP, Tat/GFP and VP/Tat/GFP fusion protein were expressed in *E. coli* BL21 (DE3) by IPTG induction method. All protein and fusion proteins were purified by affinity chromatography using a metal-chelating matrix.

2.1. Molecular size of GFP, GFP-Tat, Tat-GFP, VP-GFP and Tat-GFP-VP protein after resolved in SDS-PAGE was about 28, 30, 30, 30 and 32 kDa, respectively.

2.2. Tat residue could reduce the fluorescent intensity of GFP of about 50-60% and in the presence of VP moiety, the fluorescent intensity was only 20% of GFP

Part 3 : Cellular uptake of GFP, Tat/GFP, VP/GFP and VP/Tat/GFP fusion protein

3.1. The higher uptake of Tat-GFP and GFP-Tat fusion proteins than GFP in the HT-29 and KB cells was observed. The fusion proteins were rapidly uptake and reached the maximum within 1 hr. However, cellular uptake of both GFP-Tat and Tat-GFP were concentration dependent.

3.2. N-terminal fusion protein was selected for further study since Tat-GFP, the N-terminal fusion protein of GFP gave the highest uptake efficiency when compared between GFP, GFP-Tat and Tat-GFP.

3.3. N-terminal fusion of VP residue could also enhance the uptake of GFP into HT-29 and KB cells but the uptake efficiency was still lower than Tat-GFP fusion protein.

3.4. No synergistic effect of Tat and VP on the enhancement of GFP uptake since lower cellular uptake than either Tat-GFP or VP-GFP was observed in Tat-GFP-VP fusion protein.

3.5. Since Tat-GFP fusion protein gave superior cellular uptake than other fusion protein, Tat-GFP was selected for further study in **Part 4**.

Part 4 : Cellular uptake efficiency enhancement strategy

4.1. Entrapment in nanovesicles

4.1.1. Blank liposomes and niosomes were prepared by the freeze dried empty liposome (FDEL) method. After lyophilization, all empty nanovesicular formulations (liposomes/niosomes) were white powder with vesicular size from 50.77±0.89 to 195.78±59.47 nm. The Tat-GFP fusion protein was loaded in non-elastic nanovesicles by reconstitution the lyophilized blank nanovesicles with the Tat-GFP (at 1 µM) in phosphate buffer (pH 7.0), while that loaded in the elastic nanovesicles was from the reconstitution of the dried blank nanovesicles with the Tat-GFP in phosphate buffer containing 25% v/v ethanol. Most Tat-GFP loaded nanovesicles showed larger vesicular size than the non-loaded nanovesicles. The zeta potential of both Tat-GFP loaded and non-loaded nanovesicles were in the range of (-) 17.3±3.33 to 36.6±2.95 mV.

4.1.2. Entrapment efficiencies of Tat-GFP loaded in various nanovesicles were in the range of 7.93 to 100%. The elastic nanovesicular formulations of both liposomes and niosomes gave higher entrapment efficiency than the non-elastic nanovesicles. Both elastic and non-elastic niosomes showed higher entrapment efficiency than liposomal formulations.

4.1.3. Most blank elastic nanovesicles indicated higher elasticity with higher DI values than the non-elastic nanovesicles with the maximum DI of 12.69±1.43 in elastic neutral niosomes.

4.1.4. Tat-GFP loaded in elastic anionic niosomes showed the highest uptake of 14.62±0.07% and 15.32±0.96% in HT-29 and KB cells which was 2.81 and 2.84 folds of free Tat-GFP uptake in each cell lines. However, this nanovesicles formulation demonstrated obviously cytotoxicity effect with cell viability of only 37.29±0.67% and 62.48±5.58 in HT-29 and KB cells.

4.1.5. The low toxic elastic anionic niosomes was developed. All developed elastic vesicles showed similar uptake efficiency of Tat-GFP in the range of 13.28±0.48% to 15.95±0.78% in HT-29 cell and 12.74±1.31% to 16.62±1.53 in KB cells. However, the elastic anionic niosomes containing 1 mol% NaC showed the highest cell viability of 92.32±3.82 and 96.62±5.96% in HT-29 and KB cells, respectively.

4.1.6. Transdermal enhancement of Tat-GFP when loaded in elastic vesicles composed of 1 mol% NaC was observed.

4.2. Tat/GFP, VP/GFP and VP/Tat/GFP mixture

4.2.1. By simple mixing GFP with Tat, the mixture gave superior uptake and permeation efficiency than the Tat-GFP fusion protein. Cellular uptake of Tat-GFP and Tat/GFP into HT-29 cells after 1 hr incubation was 5.21±0.24% and 9.31±0.05%. Tat/GFP also demonstrated higher transdermal absorption through rat skin than

Tat-GFP with cumulative amounts of 0.204±0.021 μg/cm^2 and fluxes of 0.034±0.004 μg/cm^{2}h in the receiving chamber which were about 5.57 times of Tat-GFP.

4.2.2. Both VP and Tat dramatically enhanced the cellular uptake efficiency of GFP into HT-29 and KB cells by co-incubation strategy. By mixing with VP, cellular uptake of GFP into HT-29 and KB cells were 3.89-3.98 folds and 3.90-4.05 folds of GFP, respectively. When mixing with Tat, the uptake efficiency of GFP into HT-29 and KB cells were 4.24-4.59 folds and 4.39-5.09 folds of GFP, respectively. No significant difference in cellular uptake efficiency of GFP when VP or Tat was mixed with GFP at different molar ratio of 6:1 to 1/3:1.

4.2.3. The co-incubation of GFP with both VP and Tat peptide gave similar uptake efficiency as with either VP or Tat alone indicated no synergistic effect of VP and Tat on GFP translocation into HT-29 and KB cells.

Part 5 : *In vitro* calcitonin activity of Tat/sCT, VP/sCT and VP/Tat/sCT mixtures

5.1. The data from dynamic particle sizes determination, DSC thermogram and FT-IR spectrum of Tat/sCT, VP/sCT and VP/Tat/sCT mixture indicated the physico-chemical interaction between sCT and VP or Tat.

5.2. When mixed with Tat, the *in vitro* activity of sCT was increased with the maximum relative intracellular calcium of 116.46±0.57 and 172.14±4.12% from Tat/sCT mixture at 3:1 and 1:1 molar ratio in HT-29 and KB cell lines, respectively.

5.3. VP/sCT mixture at 6:1 molar ratio showed significant increasing of intracellular calcium in HT-29 cells of 152.07% of control from the ligand-receptor mediated cellular delivery of sCT by the interaction between VP and PVR on HT-29 cells which demonstrated the expression of extracellular domain of CD155.

Part 6 : *In vivo* calcitonin activity of Tat/sCT, VP/sCT and VP/Tat/sCT mixtures

6.1. Tat/sCT mixture at 1:1 molar ratio demonstrated hypocalcemic effect with the prolonged activity of over 24 hr with the reduction in the serum calcium level of about 18-31% of the control at the dose of 50 µg/kg.

6.2. Hypocalcemic effect could not observed in VP/sCT mixtures due to the lack of PVR in rat.

6.3. Tat/sCT mixture gave the higher cumulative amounts and fluxes in both VED and receiver compartment than sCT solution in transdermal study. Percentage of sCT penetrate through excised rat skin to the receiver compartment at 6 hr of Tat/sCT mixture was 3.50 times of sCT solution. The physical mixing of Tat with targeted peptide might be applicable for the development of transdermal delivery system.

6.4. Tat/sCT mixture demonstrated superior sCT stability than the sCT solution with sCT percentage remaining after 1 month storage at 4°C, RT and 45°C.

In summary, the delivery system of calcitonin, a peptide hormone has been developed. Green fluorescent protein (GFP) was used as a model protein. Tat peptide from HIV-1 and a segment of VP1 from poliovirus were applied for translocation efficiency enhancement and the binding with PVR on GI epithelium, respectively. The fusion proteins of Tat/GFP, VP/GFP and Tat/GFP/VP were produced and the cellular uptake study was performed. Rapidly uptake of all samples within 1 hr was observed. N-terminal Tat fusion GFP (Tat-GFP) demonstrated highest cellular uptake of 5.21±0.24 and 5.40±0.79% in HT-29 and KB cells which was 2.36 and 2.43 folds of GFP, respectively. However, the uptake efficiency was still too low for the application as drug delivery system. Two different methods, entrapment in nanovesicles and co-incubation strategy were evaluated for the efficiency in cellular uptake enhancement. Elastic and non-elastic with various charged (cationic, anionic and neutral) niosomes and liposomes were prepared and 1 μM Tat-GFP was loaded into each nanovesicular formulation. Cellular uptake study of Tat-GFP loaded in nanovesicles was performed and Tat-GFP loaded in elastic anionic niosomes gave the highest cellular uptake. Low toxic elastic anionic niosomes was developed by using NaC as an edge activator and the formulation containing NaC at 1 mol% gave the lowest cytotoxicity in both HT-29 and KB cells. This nanovesicular formulation could also enhance transdermal delivery of Tat-GFP fusion protein. The mixtures of Tat/GFP, VP/GFP and Tat/GFP/VP could enhance the translocation of GFP with cellular uptake efficiency of about 4-5 folds of GFP in both HT-29 and KB cells. The

system of peptide mixtures was selected for further experiments with sCT. *In vitro* calcitonin activity of Tat/sCT, VP/sCT and VP/Tat/sCT mixture was determined by intracellular calcium measurement. Significantly increase of intracellular calcium in HT-29 and KB cells was detected after the cells were incubated with VP/sCT mixture at 6:1 and Tat/sCT at 1:1 molar ratio, respectively. VP/sCT mixture could not increase intracellular calcium in KB cells due to the lack of PVR expression. Tat/sCT mixture at 1:1 molar ratio demonstrated hypocalcemic effect with the prolonged activity of over 24 hr with the reduction in the serum calcium level of about 18-31% of the control at the dose of 50 µg/kg. The results from this study indicated that these mixtures could be considered as a substituted oral dosage form for sCT administration. The slow release of sCT and Tat from the cage forming by hydrophobic residue of VP led to the delayed and long-acting hypocalcemic activity of VP/Tat/sCT mixture. However, the hypocalcemic activity could not observe after the oral administration of VP/sCT mixture from the lack of PVR in rodent animal model. Tat/sCT mixture at 1:1 molar ratio which gave the highest hypocalcemic activity could also provide the better sCT stability than sCT solution. The potential of this mixture for transdermal sCT delivery application was also performed and 3.50 times higher sCT permeation through rat skin than sCT solution was observed.