AN ALTERNATIVE *IN SILICO* METHOD TO PREDICT PASSIVE MEMBRANE PERMEABILITY OF POTENTIAL SPSB2-INOS INHIBITOR

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

Å	Angstrom
α	Alpha
β	Beta
nM	Nanomolar
t	Time
F	Force
v	Velocity
N	number of particles
V	volume
E	energy
T	temperature
P	Pressure
ns	Nanosecond

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic Hormone
AI	Artificial Intelligence
AUF1	Au-Rich Element Rna-Binding Protein 1
BAX	Bcl2 Associated X, Apoptosis Regulator
Cav-1	Caveolin-1
CG	Coarse-Grained
CHARMM	Chemistry At Harvard Macromolecular Mechanics
CHIP	C-Terminus of Hsp70–Interacting Protein
COM	Center of Mass
CPPs	Cell-Penetrating Peptides
CV	Collective Variables
DLPC	1,2- Dilaureoyl-Sn-Phosphatidylcholine
DMPC	1,2-Dimyristoyl-Sn-Phosphatidylcholine
DNA	Deoxyribonucleic Acid
DOPC	1,2-Dioleoyl-Sn-Phosphatidylcholine
DPPC	1,2-Dipalmitoyl-Sn-Phosphatidylcholine
ECS(SPSB)	Elongin B/C-Cullin-5-SPRY Domain- And SOCS Box-Containing Protein
EMA	European Medicines Agency
eNOS	Endothelial Nitric Oxide Synthase
FDA	United States Food and Drug Administration
FFT	Fast Fourier Transform
GLP-1	Glucagon-Like Peptide 1
GNU	General Public License
GROMACS	Groningen Machine for Chemical Simulations
HBDs	Hydrogen Bond Donor Groups
HDAC	Histone Deacetylase
HGF	Hepatocyte Growth Factor
HIV	Human Immunodeficiency Virus
hPar-4	Human Prostate Response Apoptosis Protein-4
HuR	Human Antigen R
iNOS	Inducible Nitric Oxide Synthase

K_D	Dissociation Constant
kJ/mol	Kilojoule Per Mole
KSRP	Kh-Type Splicing Regulatory Protein
LINCS	Linear Constraint Solver
MAX	Myc Associated Factor X
MD	Molecular Dynamics
MDM2	Mouse Double Minute 2 Homolog
ML	Machine Learning
MM/PBSA	Molecular Mechanics Poisson-Boltzmann Surface Area
NF-κB	Nuclear Factor-Kb
NLS	Nuclear Localisation Sequence
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NPT	Isobaric-Isothermal
NVE	Microcanonical
NVT	Canonical
PBC	Periodic Boundary Conditions
PME	Particle Mesh Ewald
PMF	Potential Of Mean Force
POPC	1-Palmitoyl-2-Oleoyl-Sn-Phosphatidylcholine
POPE	1-Palmitoyl-2-Oleoyl-Sn-Phosphatidylethanolamine
PPI	Protein-Protein Interactions
PRPs	Proline-Rich Peptides
PTB	Polypyrimidine Tract-Binding Protein
RNA	Ribonucleic Acid
SOCS	Suppressors of Cytokine Signalling
SPRY	SPla and the RYanodine Receptor
SPSB	SPRY Domain-Containing SOCS Box Proteins
SPSB2	SPRY Domain-Containing SOCS Box Protein 2
Src	Proto-Oncogene Tyrosine-Protein Kinase
STAT-1α	Signal Transducer and Activator of Transcription-1α
SV40	Simian Virus 40
TTP	Tristetraprolin

UI	Umbrella Integration
US	Umbrella Sampling
VMD	Visual Molecular Dynamics
WHAM	Weighted Histogram Analysis Method

LIST OF APPENDICES

SUATU KAEDAH *IN SILICO* ALTERNATIF UNTUK MERAMAL KETELAPAN MEMBRAN SECARA PASIF BAGI PERENCAT SPSB2-INOS BERPOTENSI

ABSTRAK

Domain SPRY (Reseptor SPla dan RYanodine) bagi protein kotak SOCS (Penendas Isyarat Sitokin) 2 (SPSB2) didapati bertanggungjawab terhadap degradasi proteasomal nitrik oksida sintase teraruhkan (iNOS). Penyahfungsian SPSB2 pada tikus didapati meningkatkan ekspresi iNOS dan meningkatkan pembunuhan patogen yang berkekalan seperti *Mycobacterium tuberculosis*, mencadangkan bahawa perencat interaksi SPSB2-iNOS adalah agen anti-infektif yang berpotensi. Setakat ini, beberapa perencat peptida SPSB2-iNOS telah dilaporkan. Walau bagaimanapun, peptida-peptida ini (termasuk CP2) didapati mempunyai ketelapan sel yang lemah, menyebabkan aktiviti yang lemah dalam makrofaj hidup. Oleh itu, kajian ini bertujuan untuk mencadangkan perencat telap membran berpotensi SPSB2-iNOS melalui pendekatan *in silico*. Untuk mencapai objektif ini, peptida dwisiklik baru, CPP9CP2, telah direka bentuk, dan simulasi dinamik molekul (MD) telah digunakan untuk meramalkan ketelapan membran peptida tersebut. Teknik analisis dinamik molekul (MD) konvensional untuk meramalkan translokasi peptida, seperti membandingkan profil tenaga bebas (PMF) dan menilai hubungan antara pembentukan liang air dan kecekapan penembusan peptida daripada simulasi MD yang dikendalikan, telah digunakan untuk tiga peptida penetrasi sel (TAT, CPP1, dan CPP9) dan satu peptida bukan telap sel yang diketahui, YDEGE. Walau bagaimanapun, kaedah ini terbukti kurang berkesan dalam menghasilkan semula dengan tepat keputusan eksperimen *in vitro* yang dilaporkan. Secara khusus, walaupun YDEGE tidak telap sel, ia tidak

menunjukkan nilai PMF tertinggi dan membentuk liang air sama dengan TAT dan CPP9, menjadikannya sukar untuk membezakan antara pembentukan liang tulen dan artifak simulasi. Oleh itu, kaedah inovatif menggunakan Molecular Mechanics Poisson-Boltzmann Surface Area (MM/PBSA) telah diterokai untuk mengira halangan tenaga bebas interaksi antara peptida dan dioleoilfosfatidilkolina (DOPC) pada antara muka lipid-air. Terutama, pendekatan ini berjaya membezakan peptida berdasarkan kebolehan penembusan mereka, sejajar dengan keputusan eksperimen *in vitro* yang dilaporkan. Khususnya, CPP9 mempamerkan interaksi paling sedikit dengan DOPC dan penghalang tenaga bebas terendah, diikuti secara berurutan oleh CPP1, TAT, dan YDEGE. Oleh itu, kaedah ini digunakan untuk meramalkan kebolehtelapan membran CPP9CP2 dan dibandingkan dengan CP2. Penghalang tenaga bebas CPP9CP2 didapati lebih rendah pada +143.983 kJ/mol berbanding +192.915 kJ/mol untuk CP2, menunjukkan bahawa CPP9CP2 mempamerkan kebolehtelapan sel yang dipertingkatkan. Penemuan ini menunjukkan bahawa penggabungan peptida penetrasi sel CPP9 kepada CP2 boleh meningkatkan keupayaan CPP9CP2 untuk menembusi lipid dwilapisan, memasuki sel, dan dengan itu meningkatkan keberkesanannya sebagai perencat SPSB2-iNOS dalam makrofaj hidup.

AN ALTERNATIVE *IN SILICO* METHOD TO PREDICT PASSIVE MEMBRANE PERMEABILITY OF POTENTIAL SPSB2-INOS INHIBITOR

ABSTRACT

The SPRY (SPla and the RYanodine Receptor) domain of the SOCS (Suppressors of Cytokine Signalling)-box protein 2 (SPSB2) was found to be responsible for the proteasomal degradation of inducible nitric oxide synthase (iNOS). The knockdown of SPSB2 in mice was found to increase iNOS expression and enhance the killing of persistent pathogens such as *Mycobacterium tuberculosis*, suggesting that inhibitor of SPSB2-iNOS interaction is a potential anti-infective agent. To date, several peptidic SPSB2-iNOS inhibitors have been reported. These peptides (including CP2), however, were found to have poor cell permeability, resulting in their poor activities in live macrophages. Therefore, this study aimed to propose a potential cell-permeable inhibitor of SPSB2-iNOS via *in silico* approach. To achieve the aim, a new bicyclic peptide, CPP9CP2 was designed, and molecular dynamics (MD) simulations were used to predict its membrane permeability. Conventional molecular dynamics (MD) analysis techniques for predicting peptide translocation, such as comparing free energy profiles (PMF) and evaluating the relationship between water pore formation and peptide penetration efficiency from steered MD simulations, were applied to three cell-penetrating peptides (TAT, CPP1, and CPP9) and one known non-cell-permeable peptide, YDEGE. However, these methods proved less effective in accurately reproducing reported *in vitro* experimental results. Specifically, despite YDEGE being non-cell-permeable, it did not show the highest PMF value and formed water pores similarly to TAT and CPP9, making it difficult to distinguish between genuine pore formation and simulation artifacts. Therefore, an innovative method

utilising Molecular Mechanics Poisson-Boltzmann Surface Area (MM/PBSA) was explored to calculate the free energy barrier of interactions between the peptide and dioleoylphosphatidylcholine (DOPC) at the lipid-water interface. Notably, this approach successfully differentiated peptides based on their penetration abilities, aligning with reported *in vitro* experimental results. Specifically, CPP9 exhibited the least interaction with DOPC and the lowest free energy barrier, followed sequentially by CPP1, TAT, and YDEGE. Thus, this method was applied to predict the membrane permeability of CPP9CP2 and compared to CP2. The free energy barrier of CPP9CP2 was found to be lower at +143.983 kJ/mol compared to +192.915 kJ/mol for CP2, indicating that CPP9CP2 exhibited enhanced cell permeability. This finding suggests that conjugating the cell-penetrating peptide CPP9 to CP2 may improve CPP9CP2's ability to traverse the lipid bilayer, enter cells, and thereby enhance its efficacy as an SPSB2-iNOS inhibitor in live macrophages.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Inducible nitric oxide synthase (iNOS) is one of the three isoforms of enzyme nitric oxide synthase (NOS) that were able to generate nitric oxide (NO) by converting the amino acid L-arginine within activated macrophages in response to cytokines or microbial stimuli. The high level of endogenous NO produced played an important role in physiological conditions such as host defense mechanisms against invasive pathogens and other biological processes (Andrabi et al., 2023). The iNOS expression in macrophages is negatively regulated by the interacting partner, the SPRY-domain containing SOCS box protein 2 (SPSB2). SPSB2 protein acts by recruiting an E3 ubiquitin ligase complex to polyubiquitinate iNOS which then leads to the proteasomal degradation of iNOS (Kuang et al., 2010). The study has shown that intracellular killing of persistent pathogens such as *Leishmania major* parasites and *Mycobacterium tuberculosis* was enhanced by the prolonged iNOS expression in SPSB2-deficient macrophages (Kuang et al., 2010). This suggests that inhibitor of SPSB2-iNOS interaction is a potential new class of anti-infectives.

Several cyclic peptide inhibitors of SPSB2-iNOS protein-protein interaction have been developed (Yap et al., 2014, Sadek et al., 2018). These studies showed that cyclic peptides and peptidomimetics containing the DINNN sequence were able to bind to the iNOS binding site of SPSB2 (Yap et al., 2014, Harjani et al., 2016, Sadek et al., 2018). This is in agreement with the previous observation that the SPRY domain of SPSB2 interacts with the N-terminal region of iNOS in a DINNN sequence-dependent manner (Nishiya et al., 2011). These inhibitors have been found to bind to the iNOS binding site on SPSB2 with low nanomolar affinity, and efficiently displace

full-length iNOS from its native state in the macrophage cell lysate (Sadek et al., 2018).

In general, cyclic peptides are more stable and resistant to proteolytic degradation than linear peptides. This is due to their cyclic backbone structure, which can protect them from enzymatic cleavage. However, the permeability of cyclic peptides through biological membranes, such as the plasma membrane of cells, may be limited by their hydrophilic nature and relatively large size compared to small molecules (Czekster and Naismith, 2017). This can complicate the development of cyclic peptides as therapeutics, particularly in diseases where they need to cross the cell membrane and reach intracellular targets.

In the context of SPSB2-iNOS inhibitors, the non-permeability of cyclic peptides may affect their ability to inhibit the protein-protein interaction between iNOS and SPSB2, as this interaction occurs in the cytoplasm of cells. Research showed that these inhibitors were non-toxic when introduced into cells and their lack of effectiveness in live cells was attributed to their poor ability to cross the cell membrane (Babu Reddiar et al., 2021). The inadequate ability of the SPSB2-iNOS inhibitors to pass through cell membranes is due to their highly polar nature. To illustrate, the hydrogen bond donor groups (HBDs) present in the structure of the inhibitors resulted in their strong attraction to water molecules. However, for the inhibitors to move through the cell membranes, they need to traverse a hydrophobic environment, which is not conducive to water interactions. In other words, the energy required to disrupt the hydrogen bonds between the HBDs and water molecules to allow the inhibitor molecules to diffuse through the cell membrane forms a significant obstacle for the inhibitors to cross the cell membrane effectively (Barlow et al., 2020). Thus, the

development and optimisation of cyclic peptide inhibitors of the SPSB2-iNOS interaction could therefore involve strategies to improve their cellular uptake and intracellular distribution.

Several strategies have been employed to improve the permeability of cyclic peptides, such as modifications to the peptide backbone or the addition of lipophilic groups to enhance their ability to cross cell membranes (Wang et al., 2022). These modifications can potentially improve the bioavailability and efficacy of cyclic peptide-based drugs. Apart from these modifications, it is also common to use cell-penetrating peptides (CPPs) to facilitate the delivery of impermeable cyclic peptides across the cell membrane. CPPs are short, cationic peptides that can interact with the negatively charged components of the cell membrane, promoting their internalisation into cells through endocytosis or other mechanisms (Derakhshankhah and Jafari, 2018).

One approach to developing cell-permeable inhibitors of SPSB2-iNOS is therefore to fuse the impermeable inhibitor with a CPP, creating a chimeric molecule that retains the inhibitory activity of the original inhibitor while also possessing cell-penetrating properties. A study has suggested that a fusion of cyclic peptides with a cyclic CPP may produce bicyclic peptides that are cell-permeable, more selective, rigid, and more stable than the linear and monocyclic peptides (Lian et al., 2014). This strategy of combining CPPs with impermeable inhibitors has recently been used in the development of inhibitors of SPSB2-iNOS interaction as described in a study, where the resulting chimeric molecule of a nine residues linear iNOS N-terminal peptide fused with a CPP (R9) was shown to effectively inhibit the SPSB2-iNOS interaction *in vitro* and in cultured cells (Li et al., 2021).

It is, however, worth noting that determining the most suitable and effective CPP to fuse with the inhibitor to deliver it into the cytoplasm of cells is not an easy task. To predict the cell permeability efficiency of different CPPs as well as non-cell permeable peptides, molecular dynamics (MD) simulations have been used (Tran et al., 2021, Ulmschneider and Ulmschneider, 2018). MD simulations involve simulating the motion of molecules in a system, allowing researchers to study the interactions between molecules and their environment (Reid et al., 2019). In such MD simulations, a membrane model is usually prepared with lipids and cholesterol molecules. An initial peptide structure is then placed above the membrane and steered MD simulations are used to compute a mechanistic score of permeability (Tran et al., 2021). MD simulations have been successful in accurately capturing the process of peptide binding, folding, and partitioning into lipid bilayers (Ulmschneider and Ulmschneider, 2018), as well as providing insight into how different types of CPPs affect the membrane's mechanical properties (Grasso et al., 2018).

1.2 Problem Statement

Although the reported SPSB2-iNOS inhibitors can effectively prevent the binding of SPSB2 to iNOS in cell lysates (Yap et al., 2016, Sadek et al., 2018), their application to intact live cells was hindered because they could not penetrate the cell membrane (Rahman et al., 2022, Babu Reddiar et al., 2021). This suggests the need for a new cell-permeable inhibitor of SPSB2-iNOS interaction. Several studies from the past ten years have documented the use of molecular dynamics simulations to predict the mechanism by which CPPs penetrate the lipid bilayer (Ouyang et al., 2022). Few parameters have been frequently used to explain the translocation effectiveness of CPPs including the potential mean force (PMF) for translocation (Sun et al., 2015,

Lyu et al., 2017), the free energy for pore formation (Bennett et al., 2014), and the electrostatic potential of the membrane (Gao et al., 2019). While in principle, using any of these methods would be useful to predict the permeability of a new designed inhibitor of SPSB2-iNOS before embarking into its synthesis and *in vitro* characterisation, these methods and parameters were unfortunately found to be inconsistent in distinguishing the efficiency of different CPPs' translocation across the lipid bilayer.

1.3 Aim of Study

To propose a potential cell-permeable inhibitor of SPSB2-iNOS via *in silico* approach.

1.4 Objectives

a) To identify a suitable method that can differentiate the membrane permeability of different peptides.
b) To design a potentially cell-permeable SPSB2-iNOS peptide inhibitor.
c) To apply the newly identified method to predict the permeability of the designed potentially cell-permeable inhibitor of SPSB2-iNOS.

1.5 Scope of the Thesis

To achieve the aim of this study, this study first attempted to identify an appropriate method for distinguishing the membrane permeability of various peptides. Three different CPPs (CPP1, CPP9, and TAT), along with YDEGE, a known non-cell-permeable peptide (as a negative control), were subjected to three conventional approaches: water pore formation, water density profile, and potential mean force (PMF) profiles (Figure 1.1). As these methods did not yield satisfactory results, an

alternative approach, i.e., the free energy barrier method, was developed. This new method was found to be not only capable of distinguishing the permeability of different peptides investigated but is also less time consuming and less computationally expensive than the conventional approach such as PMF. Therefore, the new method was then used to predict the permeability of the newly designed potentially cell-permeable inhibitor of SPSB2-iNOS (CPP9CP2).

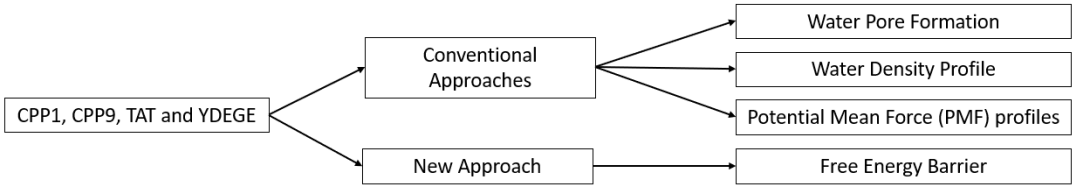

Figure 1.1 Flowchart of the Comparative Evaluation of Membrane Permeability Methods for Different Peptides

1.6 Significance of Study

In recent decades, peptide therapeutics have played a crucial role in today's pharmaceutical and biotech industries, significantly contributing to the development of innovative treatments for a wide range of diseases, including cancer, diabetes, and infectious diseases (Fosgerau and Hoffmann, 2015, Rossino et al., 2023). Since 2000, 33 non-insulin peptide drugs have been approved globally, with over 170 peptides currently in active clinical development. For example, the bestselling peptide drug in the year 2019 is dulaglutide, a glucagon-like peptide 1 (GLP-1) analogue for treating type 2 diabetes, with $4.39 billion in retail sales (Wang et al., 2022, Anand et al., 2023, Muttenthaler et al., 2021). However, the general shortcomings of peptides, such as poor cell permeability and oral bioavailability, have limited the clinical use of certain peptides (Lamers, 2022). Thus, the discovery of new peptide analogues that can penetrate the cell membrane and act as inhibitors of the SPSB2-iNOS interaction may

bridge the gap between theoretical insights and experimental observations, ultimately leading to the discovery of a new class of anti-infectives.

Furthermore, the new method developed in this study to differentiate the permeability of different CPPs as well as non-cell permeable peptides may apply to other inhibitors that are unable to permeate the cell membrane to discover an appropriate carrier. This method could be modified to assess the permeability of other types of carriers to determine which carriers are the most successful at carrying a certain inhibitor. Researchers could potentially save time and resources by employing this method to find an appropriate carrier for a specific inhibitor, which could aid in accelerating the development of new therapies for a variety of diseases and illnesses.

CHAPTER 2

LITERATURE REVIEW

2.1 Protein-protein interaction

Protein-protein interactions (PPI) play crucial roles in life processes. Studies showed that abnormal PPI is related to various diseases such as cancer and infectious diseases. Classic small molecule drug discovery mainly focuses on protein-ligand interactions such as enzymes, ion channels, or receptors. Recently, PPI has gained much attention as a new target for drug discovery. However, targeting PPIs in drug discovery is challenging (Silvian et al., 2013). As PPIs take place on the interface of a specific domain where two proteins associate, the interface area of the protein interaction is usually highly hydrophobic and is larger (1500–3000 $Å^2$) than the usual receptor-ligand contact area (300–1000 $Å^2$) (Ran and Gestwicki, 2018). Besides, the PPI surfaces tend to be flat and are usually less well-defined with shallow and adaptable pockets, making them difficult to bind to small molecules. Thus, classic medicinal chemistry methods may be less effective for discovering and identifying PPI modulators.

In recent years, studies have shown few small molecules that successfully target PPIs and are in the clinical trial phase. However, the discovery of small molecules that target PPIs uses strategies like high-throughput screening, fragment-based drug discovery, and virtual screening to identify potential PPI inhibitors (Lu et al., 2020). Structure-based drug design based on the α-helix secondary structure has also proven successful in discovering inhibitors of c-Myc/Max (Yap et al., 2013), Bcl-2/Bax (Yin et al., 2005), and p53/MDM2 (Chen et al., 2005).

Other than small molecules, peptide-based inhibitors of PPI such as c-helix constrained peptides, α-helix, and β-hairpin-stabilised peptides, cyclic peptides, and bicyclic peptides have also been reported (Nevola and Giralt, 2015). Peptide-based inhibitors are perfect candidates to overcome the difficulties faced as peptides can mimic the main features of the protein. Moreover, peptides can be easily synthesised and modified to make them more resistant to proteases and have higher selectivity and better cellular internalisation.

2.1.1 SPSB-iNOS interaction

Nitric oxide synthases (NOSs) are enzymes that catalyse the production of nitric oxide (NO) by converting the L-arginine to L-citrulline and NO. In general, there are three isoforms of NOS, i.e., neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS), and endothelial nitric oxide synthase (eNOS). Both nNOS and eNOS are calcium-dependent, generating NO in the nervous system's neuron tissues and blood vessels' epithelial cells, respectively. On the other hand, iNOS is calcium-independent and inducible with cytokines or other stimuli in a wide range of cells and tissues, including macrophages (Xue et al., 2018). The production of NO upon iNOS induction is continuous and will only stop upon the degradation of the enzyme. The high level of endogenous NO produced by iNOS plays an essential role in physiological conditions such as host defense mechanisms against invasive pathogens as well as pathophysiological conditions like inflammation and infection (Lechner et al., 2005).

NO production via iNOS expression is regulated at multiple levels in human cells, mainly transcriptional (DNA level) and post-transcriptional (RNA level). At the transcriptional level, transcription factors such as nuclear factor-κB (NF-κB) and signal transducer and activator of transcription-1α (STAT-1α) bind to DNA to activate the iNOS promoter and thereby induce the iNOS expression. At the post-transcriptional

level, the iNOS mRNA stability is modulated via its binding to a complex network of RNA-binding proteins such as AUF1, HuR, KSRP, PTB, and TTP as well as the interaction with their protein partners such as Cav-1, Src, and the proteasomes (Pautz et al., 2010).

Musial and Eissa proved that iNOS undergoes ubiquitination, an essential process for its degradation (Musial and Eissa, 2001). The ubiquitin-proteasome pathway is a crucial regulatory system as NO overproduction could cause harmful effects such as cytotoxicity. Two E3 ubiquitin ligases had been reported to target iNOS for proteasomal degradation, i.e., Elongin B/C-Cullin-5-SPRY domain- and SOCS box-containing protein [ECS(SPSB)] and the C-terminus of Hsp70–interacting protein (CHIP). Unlike CHIP, which was shown to regulate the nNOS, eNOS, and iNOS, the SPRY domain-containing SOCS box proteins (SPSB) were found to only target iNOS for its proteasomal degradation (Matsumoto et al., 2011).

The SPRY domain-containing SOCS box protein 2 (SPSB2), also known as SSB-2, is one of the four mammalian SPSB family proteins (SPSB1 to SPSB4). SOCS family proteins are identified by the SOCS box (approximately 40 amino acid residues) at the C-terminal that recruits the elongin C and B to form an E3 ubiquitin ligase complex and a protein-protein interaction module known as the SPRY/B30.2, which acts as a center domain that targets specific substrates for proteasomal degradation (Perfetto et al., 2013, Kleiber and Singh, 2009).

A study showed that all four SPSB proteins interact with the receptor protein-tyrosine kinase for hepatocyte growth factor (HGF), MET, where the SPSB1 enhances the HGF-MET-induced Erk-Elk-1-serum response element pathway (Wang et al., 2005). Besides, studies also found that the *Drosophila* SPSB1, namely GUSTAVUS,

interacts with the DEAD-box RNA helicase VASA protein (Woo et al., 2006), while human SPSB1, SPSB2, and SPSB4 (except SPSB3) were found to interact with the human prostate response apoptosis protein-4 (hPar-4) (Masters et al., 2006). Intriguingly, a similar sequence was identified in both hPar-4 and VASA, i.e., ELNNNL and DINNNN, respectively, which suggests that the SPRY domain of SPSB1, SPSB2, and SPSB4 recognises a common peptide epitope, [D/E]-[I/L]-N-N-N in these proteins (Nishiya et al., 2011). However, this [D/E]-[I/L]-N-N-N motif is not present in mouse or human VASA or mouse Par-4, thus, suggesting that both VASA and Par-4 proteins are not crucial physiological targets of mammalian SPSB proteins.

Further study by Kuang and co-workers discovered that the DINNN sequence is also found in the N-terminal region of iNOS (residues 23–27) but not in eNOS or nNOS of different species including humans, mice, and goldfish. This suggests that iNOS is potentially a critical physiological target of mammalian SPSB protein. Negative regulation of the iNOS expression in macrophages by SPSB2 confirmed the SPSB-iNOS interaction. In addition, the intracellular killing of persistent pathogens such as *Leishmania major* parasite and *Mycobacterium tuberculosis* were also shown to be enhanced by the prolonged iNOS expression in SPSB2-deficient macrophages. This observation also suggests that inhibitors of SPSB2-iNOS interaction are a potential new class of anti-infective agents (Kuang et al., 2010).

2.1.2 Inhibitors targeting SPSB-iNOS interaction

Researchers have conducted a few studies to design and synthesise inhibitors of the SPSB2-iNOS interaction that could act as possible novel anti-infectives (Norton, 2018). The DINNN motive-containing cyclic peptides CP1 and CP2 were proven to be able to bind to SPSB2 using surface plasmon resonance, and the inhibition of SPSB2-iNOS interaction was demonstrated utilising an *in vitro* macrophage cell lysates assay

(Yap et al., 2014, Yap et al., 2016, Harjani et al., 2016). In a separate study, researchers found that an improved pentapeptide SPSB2-iNOS inhibitor had a higher affinity for SPSB2 with a Kd of 7 nM as evaluated by surface plasmon resonance and is more effective at inhibiting SPSB2-iNOS interaction in macrophage cell lysates (Sadek et al., 2018). This is in agreement with the previous observation that the SPRY domain of the SPSB2 interacts with the N-terminal region of iNOS in a DINNN sequence-dependent manner (Nishiya et al., 2011).

In a recent study by Kuang and co-workers, they discovered the function of several flanking residues in the SPSB2-iNOS interaction, which was previously unsolved structurally. Furthermore, utilising transient transfection and cell-penetrating peptide methods, they were able to investigate the effects of SPSB2-iNOS inhibitors on NO production in RAW264.7 macrophages and discovered that such inhibitors could increase NO production (Li et al., 2021). Apart from that, a study found that by combining the RGD motif with the SPSB2 binding motif (DINNNV), the designed cyclic peptide (CR8) was able to bind to the iNOS binding site on SPSB2 with a Kd of 671 nM, disrupting the SPSB2-iNOS interaction and increasing NO production, and has the potential to be developed into a new antimicrobial and anticancer drug (You et al., 2017).

2.2 Introduction to peptide drugs

Peptides are short chains of approximately 50 or fewer amino acids linked by covalent peptide or amide bonds. Therapeutic peptides commonly act as hormones, growth factors, neurotransmitters, ion channel ligands, or anti-infective agents. The usage of peptides as pharmaceuticals has evolved, and this trend continues as the drug development and treatment paradigms shift. In the early twentieth century, insulin and

adrenocorticotrophic hormone (ACTH) were life-saving drugs developed from natural peptides. In the 1950s, sequence elucidation and chemical synthesis of peptides became feasible, allowing synthetic oxytocin and vasopressin production. As it was discovered that arthropods and squid venoms are rich in bioactive peptides, the isolation of natural products from exotic sources became a favoured method for developing new therapeutic drugs. During the genomic era, numerous essential endogenous peptide hormone receptors were then discovered and molecularly characterised; consequently, corporations and universities began searching for novel peptidic ligands for these receptors (Lau and Dunn, 2018).

The advantages and disadvantages of peptides as therapeutics are listed in Table 2.1 (Craik et al., 2013, Joo, 2012). Generally, peptides have more significant potential as therapeutics than small molecules due to their high selectivity and specificity toward the target protein (Falciani et al., 2011). Peptides, however, suffer from low metabolic stability and oral bioavailability (Falciani et al., 2011). Additionally, peptides generally have low cell permeability (Joo, 2012), which is believed to be caused by the hydrogen bond interactions of the peptide backbone with the water molecules (Qian et al., 2013). Due to these reasons, various modifications are usually made to the therapeutic peptides to make them druggable, which will be discussed in greater detail in the subsequent subsections.

Table 2.1 Advantages and disadvantages of peptide drugs.

Advantages	Disadvantages
High potency	Low metabolic stability
High specificity toward targets	Low membrane permeability
Less toxicity than small molecules	Low oral absorption
Low accumulation in tissues	High production costs
High chemical and biological diversity	Rapidly metabolise in the body

2.2.1 Cyclic peptide

The cyclic peptide is a polypeptide chain in the form of a cyclic ring connected by a linker. Cyclic peptides have been used as therapeutics clinically, such as gramicidin and tyrocidine, which can be found in nature (Craik et al., 2013). To date, 53 cyclic peptides have received approval from various regulatory authorities, with numerous others currently undergoing clinical trials for a broad range of conditions (Costa et al., 2023). Cyclic peptides combine several advantageous properties of a peptide, including high selectivity and affinity for the target, non-toxic, and ease of production, making them promising candidates for development as therapeutics (Abdalla and McGaw, 2018). Because of their structural rigidity, cyclic peptides usually have more biological activity than their linear counterparts. The entropy term of the Gibbs free energy is reduced by the rigidity of cyclic peptides, which then improves their binding towards target molecules and enhances receptor selectivity. Another advantage of the cyclic structure is that it is resistant to exopeptidase hydrolysis due to the absence of amino and carboxyl termini. Additionally, as their structure is less flexible than linear peptides, cyclic peptides can also resist endopeptidases (Joo, 2012).

Peptides, however, are generally not able to cross the cell membrane to target intracellular targets due to their poor cell permeability, and thus, most cyclic peptides used in clinics only act on extracellular targets. For example, vancomycin and daptomycin act by inhibiting and disrupting the cell membrane (Hancock, 2005). Consistent with this observation, there are only two approved cyclic peptides that target intracellular protein in the past two decades with one of them being romidepsin, the first medicine licensed in 2009 for the treatment of lymphoma. Romidepsin is a prodrug in which the disulfide bond is reduced to two thiols within the intracellular matrix and binds to the zinc in the zinc-dependent active site of histone deacetylase (HDAC)

enzymes, thereby suppressing the activity of HDACs and causing cell cycle arrest and apoptosis (Wang et al., 2022).

2.2.2 Cell-penetrating peptides

To efficiently deliver therapeutics into the target site in the cytoplasm or nucleus, the therapeutics must first pass through the cell membrane. However, one of the significant components in the cell membrane is the hydrophobic phospholipid which often acts as a barrier and obstructs the transportation of the therapeutics (Zhang et al., 2019). Thus, developing carriers to improve the cellular uptake of cell-impermeable therapeutics has become a crucial challenge for researchers. Several delivery systems such as liposomes, nanoparticles, and viral vectors have been developed to deliver therapeutics such as peptides, proteins, and small molecules across the cell membrane (Copolovici et al., 2014).

Recently, cell-penetrating peptides (CPPs) have attracted much attention in drug delivery. CPPs are short hydrophilic or amphiphilic peptides, typically with 5-30 amino acids, with plenty of positively charged amino acids, such as lysine or arginine, which can penetrate cell membranes, unlike most peptides (Guo et al., 2016). The first group of CPPs was discovered in the late 1980s and early 1990s when researchers found the Tat peptide (RKKRRQRRR) from the primary domain of HIV-1 Tat protein (Green and Loewenstein, 1988, Frankel and Pabo, 1988) and penetratin from the third helix of the *Antennapedia* homeodomain (Derossi et al., 1994). The ability of these peptides to penetrate the cell membrane has drawn much attention. Over the years, CPPs have been utilised for various applications (Bechara and Sagan, 2013). Some of these applications include the delivery of nanoparticles, double-stranded DNA, liposomes (Gupta et al., 2005), peptides (Gupta et al., 2005, Dietz and Bahr, 2004), proteins (Mae and Langel, 2006, Lehto et al., 2012), antisense oligonucleotides, and small interfering RNA (Meade

and Dowdy, 2008, Mae and Langel, 2006) both *in vitro* and *in vivo*. Conjugation of CPPs onto the cargo can occur either by covalent or noncovalent interactions.

2.2.2(a) Classification of cell-penetrating peptides

CPPs can be classified according to their origin, i.e., peptides derived from proteins, chimeric peptides formed by the fusion of two natural sequences, and synthetic peptides, which are usually synthesised based on structure-activity studies. CPPs can also be classified based on the physicochemical properties of their sequences, i.e., cationic, amphipathic, and hydrophobic. Table 2.2 shows examples of CPPs with their sequences, origin, and physicochemical properties.

Table 2.2 Examples of CPPs.

Peptide	Sequence	Origin	References
Cationic			
Tat peptide	RKKRRQRRR	HIV trans-activator of transcription protein	(Frankel and Pabo, 1988)
Penetratin	RQIKIWFQNRRMKWKK	*Antennapedia* homeodomain	(Derossi et al., 1994)
R9	RRRRRRRRR	Chemically synthesised	(Futaki et al., 2001)
DPV1047	VKRGLKLRHVRPRVTRMDV	Chemically synthesised	(De Coupade et al., 2005)
Amphipathic			
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Chimeric (Galanin and mastoparan)	(Pooga et al., 1998)
P1	MGLGLHLLVLAAALQGAWSQPKKKRKV	Human immunoglobulin and SV40	(Molinaro et al., 2015)
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	HIV gp41 and SV40	(Morris et al., 1997)
Pep-1	KETWWETWWTEWSQPKKKRKV	Tryptophan-rich cluster and SV40 T antigen NLS	(Morris et al., 2001)
MAP	KLALKLALKALKAALKLA	Chimeric	(Oehlke et al., 1998)
SAP	$(VRLPPP)_3$	N-terminal domain of γ-zein	(Martin et al., 2011)
pVEC	LLIILRRRIRKQAHAHSK	Vascular endothelial cadherin	(Elmquist et al., 2001)
ARF(1-22)	MVRRFLVTLRIRRACGPPRVRV	p14ARF protein	(Johansson et al., 2008)
BPrPr(1-28)	MVKSKIGSWILVLFVAMWSDVGLCKKRP	N-terminus of unprocessed bovine prion protein	(Magzoub et al., 2006)
p28	LSTAADMQGVVTDGMASGLDKDYLKPDD	Azurin	(Taylor et al., 2009)
VT5	DPKGDPKGVTVTVTVTVTGKGDPKPD	Chemically synthesised	(Oehlke et al., 1997)
Bac 7 (Bac 1-24)	RRIRPRPPRLPRPRPRPLPFPRPG	Bactenecin family of antimicrobial peptides	(Sadler et al., 2002)
Hydrophobic			
MTS	AAVALLPAVLLALLAP	Kaposi-fibroblast growth factor	(Lin et al., 1995)
C105Y	CSIPPEVKFNKPFVYLI	α1-Antitrypsin	(Rhee and Davis, 2006)
PFVYLI	PFVYLI	Derived from synthetic C105Y	(Rhee and Davis, 2006)
Pep-7	SDLWEMMMVSLACQY	CHL8 peptide phage clone	(Gao et al., 2002)

2.2.2(b) Cationic cell-penetrating peptides

Cationic CPPs are peptides made up of positively charged amino acids such as lysine and arginine. Studies showed that the cell-penetrating activities of CPPs do not depend on the peptide backbone but on the number and position of positively charged residues (Wender et al., 2000). Studies on arginine-based peptides showed that the minimum number of arginine for cell-penetrating activities is eight (R8). The cell-penetrating ability increases with the number of arginines (Tunnemann et al., 2008). For instance, the Tat peptide comprises mainly basic residues, i.e., six arginines and two lysines.

To compare the contribution of basic residues towards the cell-penetrating activities, homo-oligomers of arginine, lysine, and histidine were synthesised and compared. The findings showed that polyarginine peptides with 7-9 residues showed significantly higher cell-penetrating activity than polyhistidine, polylysine, and even the Tat peptide (Mitchell et al., 2000). This is because the guanidinium group of the arginine side chain can form bidentate hydrogen bonds with the cell surface, which increases its penetrating ability. This hypothesis is further supported by other non-peptidic oligomers with guanidinium groups, such as guanidinium-rich oligocarbamates (Wender et al., 2002) and guanidinium-rich oligocarbonates (Cooley et al., 2009) which also showed cell-penetrating abilities. Other examples of cationic peptides are shown in Table 2.2.

2.2.2(c) Amphipathic cell-penetrating peptide

In contrast to the cationic CPPs which mainly consist of arginine and lysine residues, amphipathic CPPs are made up of an alternating sequence of hydrophilic (polar) and hydrophobic (non-polar) residues. Amphipathic CPPs can be divided into four main groups, i.e., primary amphipathic CPPs, secondary amphipathic α-helical CPPs, β-sheet amphipathic CPPs, and proline-rich amphipathic CPPs (Milletti, 2012).

Primary amphipathic CPPs contain a sequence of polar and non-polar domains, while secondary amphipathic CPPs exhibit amphipathic properties under certain conformational states that enable proper positioning of the hydrophilic and hydrophobic residues. In short, the lipophilic and hydrophilic parts in the amphipathic CPPs mediate the translocation of the peptides across the plasma membrane (Bolhassani, 2011).

Primary amphipathic CPPs are chimeric peptides formed by combining a hydrophilic nuclear localisation sequence (NLS) with a hydrophobic domain. NLSs are short peptides formed by lysine, arginine, and proline-rich sequences. NLSs are not good CPPs on their own and therefore are usually combined with a hydrophobic domain to enhance their permeability across the cell membrane. For example, the NLS, PKKKRKV, derived from simian virus 40 (SV40), was fused with hydrophobic domain obtained from signal peptide, P1 (MGLGLHLLVLAAALQGA-WSQ-PKKKRKV), fusion sequence, MPG (GALFLGFLGAAGSTMGA-WSQ-PKKKRKV) and tryptophan-rich sequence, Pep-1 (KETWWETWWTE-WSQ-PKKKRKV) to form amphipathic CPPs via a common linker (WSQP) (Table 2.2). There are also primary amphipathic CPPs that are derived from natural proteins such as pVEC (LLILRRRIRKQAHAHSK), which contains 13 cytosolic and 5 transmembrane residues from murine vascular endothelial-cadherin protein, ARF1-22 (MVRRFLVTLRIRRACGPPRVRV), which is derived from the N-terminal domain of the tumour suppressor p14ARF protein, and BPrPr1-28 (MVKSKIGSWILVFVAMWSDVGLCKKRP), which is taken from the bovine prion protein.

Secondary amphipathic CPPs, on the other hand, can adopt either an α-helical or a β-sheet structure. In the amphipathic α–helix CPPs, hydrophilic and hydrophobic

amino acids are grouped and placed on the opposite side of the helix. For example, the study on the model amphipathic peptide MAP (KLALKLALKALKAALKLA, Table 2.2) showed that the cellular uptake efficiency of MAP peptide correlated with its α-helicity, i.e., the uptake is maintained if the amphipathicity of the peptide is conserved. On the other hand, an amphipathic β-sheet peptide is structured based on the alternating sequence of one hydrophobic and one hydrophilic amino acid that is solvent-exposed. The study on VT5 (DPKGDPKGVTVTVTVTVTGK-GDPKPD) peptide showed that the β-sheet analogue of the aforementioned peptide has a higher uptake efficiency compared to the analogues that are unable to adopt a β-sheet(Oehlke et al., 1997).

Proline-rich peptides (PRPs) are an additional unique group of secondary amphipathic CPPs with effective cellular uptake and noncytotoxic properties. PRPs are a large and variable class of small to medium-sized peptides characterised by the presence of proline residues, which frequently form unusual sequences. This property gives them a typical structure that specifies the numerous biological functions these molecules possess. Specifically, the left-handed polyproline-II helix is required to express antibacterial, immunomodulatory, and antioxidant properties and the fine modulation of protein-protein interactions, thereby playing crucial roles in various cell signal transduction pathways (Vitali, 2015). Among reported proline-rich peptides include bactenecin-7 (Bac7) (Sadler et al., 2002), trimer peptide $(VRLPPP)_3$, which is derived from the N-terminal domain of γ-zein (Martin et al., 2011), as well as several synthetic polyproline-based peptides such as (PPR)n and (PRR)n (where n = 3, 4, 5 and 6) (Daniels and Schepartz, 2007).

2.2.2(d) Hydrophobic cell-penetrating peptides

Hydrophobic CPPs contain mostly non-polar residues. In comparison to cationic and amphipathic CPPs, hydrophobic CPPs are relatively less reported. Hydrophobic

CPPs are usually incorporated with cationic residues to improve their cellular uptake. This may be due to the solubility limit of hydrophobic CPPs in an aqueous solution and the difficulties of assembling a peptide with a long hydrophobic amino acid sequence. There are a few hydrophobic sequences that have been previously reported, such as the natural hydrophobic CPP, C105Y (Rhee and Davis, 2006) and its PFVYLI C-terminal portion, hydrophobic sequence AAVALLPAVLLALLAP derived from Kaposi fibroblast growth factor (Lin et al., 1995), as well as the Pep-7 peptide derived from CHL8 (Gao et al., 2002).

2.2.2(e) Cyclic cell-penetrating peptide

Apart from the aforementioned classes of CPPs, cyclic CPPs have also recently emerged as a new class of CPPs. Unlike conventional CPPs (in linear form), cyclic CPPs do not suffer from the drawbacks of linear CPPs, such as poor stability that makes them difficult to reach their target site, and low cell specificity that limits their direct application to targeted cells (Reissmann, 2014, Ali et al., 2014). Cyclic CPPs are primarily synthesised in the laboratory, while some are purified and characterised from natural products. The first cyclic CPPs used as a drug delivery tool was reported in 2011, where several amphipathic homochiral L-cyclic peptides were shown as potential molecular transporters (Mandal et al., 2011). Over the years, numerous cyclic CPPs have been reported to efficiently transport chemotherapeutic, antiviral, and antibacterial agents across the cell membrane. For instance, cyclic [$W(RW)_4$], which contains arginine and tryptophan residues, was conjugated with doxorubicin to enhance its cellular uptake and cellular retention (Nasrolahi Shirazi et al., 2013). Moreover, Pei's group had developed a series of cyclic peptides containing arginine and L-2-naphthylalanine, which showed good prospects as intracellular drug delivery tools (Qian et al., 2013). A study has suggested that a fusion of cyclic peptides with a cyclic

cell-penetrating peptide (CPP) may produce bicyclic peptides that are cell-permeable, more selective, rigid, and more stable than the linear and monocyclic peptides (Lian et al., 2014).

2.2.3 Applications of cell-penetrating peptides

The cell-penetrating properties of CPPs enable CPPs to deliver various cargo into the cells without damaging the cells. Some therapeutic agents like small molecules, peptides, proteins, and nucleic acids have limited membrane permeability, and hence, they exhibit a less therapeutic effect. Thus, the cellular uptake of these molecules was facilitated by CPPs. The application and examples of CPPs are further discussed below.

2.2.3(a) CPPs for nucleic acids delivery

Gene therapy, including nucleic acid-based molecules, was presented as a promising strategy for treating different diseases. Delivering nucleic acid into the cells was one of the first medical applications of CPP. With their extensive and hydrophilic properties, nucleic acids make passing through the cell membrane difficult. The conventional method used to deliver the molecules faced problems like poor efficiency and cellular toxicity. Therefore, CPPs have been widely developed as a versatile vector for *in vitro* and *in vivo* nucleic acid delivery (Ramsey and Flynn, 2015). Few types of nucleic acid cargo have been reported using CPPs as a vector to deliver into mammalian cells. These include DNA plasmid (Rudolph et al., 2003), antisense oligonucleotides (Astriab-Fisher et al., 2002), decoy DNA (El-Andaloussi et al., 2005), and small interfering RNA (siRNA) (Taylor and Zahid, 2020, Margus et al., 2012).

2.2.3(b) CPPs for peptide and protein delivery

Proteins and peptides are widely discovered as therapeutics. However, their hydrophilic property makes them impermeable to the cell membrane. A strategy like

fusing the CPP with peptide and protein-based therapeutics is exploited to enhance their permeation across the cell membrane. The first CPP-protein fusion was demonstrated in 1994, where large proteins like β-galactosidase, horseradish peroxidase, and RNase A were conjugated with Tat peptide to be translocated into the cells (Fawell et al., 1994). Also, CPPs are widely discovered as delivery agents for anti-tumour agents. For instance, Tat peptide, polyarginine CPP, and penetratin are fused with tumour suppressor p53 peptide or its analogues to improve their uptake and activities in inhibiting the growth of cancer cells (Snyder et al., 2004, Michl et al., 2006, Araki et al., 2010).

2.2.3(c) CPPs for small molecule delivery

Many small molecule drug candidates in drug discovery pose attractive activities *in vitro*. However, like the previously discussed macromolecules, small hydrophilic molecules do not possess sufficient lipophilicity to pass through the membrane. In this case, small molecules had been reported to show improvement in activity when conjugated with CPPs. For instance, a study showed that doxorubicin conjugation with polyarginine inhibits tumour growth *in vivo* with lesser side effects. The interaction of the cationic CPP with glycosaminoglycans was also found to improve the accumulation of the drug in the tumour (Nakase et al., 2012). Besides, cyclosporine A, a systemically active drug that has poor penetration through the skin, when conjugated with polyarginine, was able to transport into human skin cells and inhibit cutaneous inflammation (Rothbard et al., 2000).

2.2.4 Cell-penetrating peptide internalisation mechanism

Although the cellular uptake mechanism of CPPs has been the subject of recent investigations, the pathway involved has yet to be justified clearly (Trabulo et al., 2010). However, it is widely accepted that the uptake mechanism of CPPs varies according to

different CPPs, and some CPPs may have two or more pathways depending on the experimental condition (Guo et al., 2016). Generally, the entry routes of CPPs can be divided into two types, i.e., (1) endocytosis-mediated translocation and (2) direct penetration (Madani et al., 2011) (Figure 2.1)

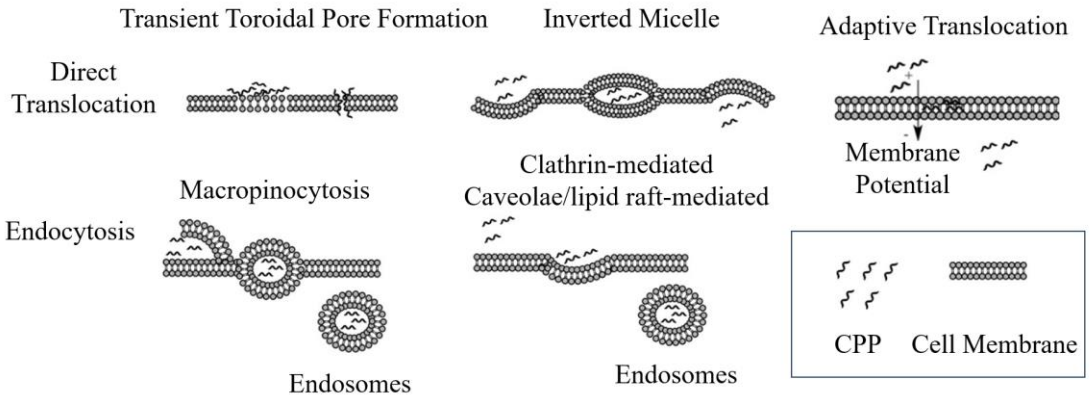

Figure 2.1 The proposed mechanism for cellular entry of CPPs (Ramsey and Flynn, 2015)

2.2.4(a) Endocytosis

Endocytosis includes a few different cellular mechanisms, such as phagocytosis and pinocytosis, that mediate the uptake of large particles and solutes, respectively (Madani et al., 2011). Unlike phagocytosis, which occurs only in specialised cells like macrophages, pinocytosis, which covers a set of various internalisation pathways, exists in most cell types. The pathways include macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated pathway, cholesterol-dependent clathrin-mediated pathway, and caveolin/clathrin-independent pathway (Guo et al., 2016). Several CPPs have been shown to use more than one internalisation pathway mentioned above, and sometimes different pathways may be used simultaneously. For instance, the widely explored Tat peptide has been shown to internalise through micropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis (Ramsey and Flynn, 2015).