IDENTIFICATION OF NOVEL 14-3-3 SIGMA HOMODIMER STABILISER IN NASOPHARYNGEAL CARCINOMA

GHAZI AHMAD AL JABAL

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IDENTIFICATION OF NOVEL 14-3-3 SIGMA HOMODIMER STABILISER IN NASOPHARYNGEAL CARCINOMA

by

GHAZI AHMAD AL JABAL

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LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
Å	Angstrom
h	Hour
mL	Millilitre
nm	Nanometer
ppm	Parts per Million
rpm	Revolution per Minute
v/v	Volume/Volume
µL	Microliter
µM	Micromolar

LIST OF ABBREVIATIONS

1D	One-Dimensional
AMBER	Assisted Model Building with Energy Refinement
CADD	Computer-Aided Drug Design
CHARMM	Chemistry At Harvard Macromolecular Mechanics
CPMG	Carr-Purcell-Meiboom-Gill
DEAE	Diethylaminoethyl
DIPEA	N,N-Diisopropylethylamine
DLS	Dynamic Light Scattering
DMB	1,3-Dimethoxybenzene
DMF	Dimethylformamide
DS	Discovery Studio
EDT	1,2-Ethanedithiol
FP	Fluorescence Polarisation
GCP	Guanidiniocarbonyl Pyrrole
GROMACS	Groningen Machine for Chemical Simulations
GST	Glutathione S-Transferases
HCTU	O-(1h-6-Chlorobenzotriazole-1-Yl)-1,1,3,3-Tetramethyluronium Hexafluorophosphate
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl β-*D*-1-thio-galactopyranoside
ITC	Isothermal Titration Calorimetry
K_D	Binding Affinity
LB	Luria Broth
LC-MS	Liquid Chromatography-Mass Spectroscopy
MD	Molecular Dynamics
MS	Mass Spectrometry
MST	Microscale Thermophoresis
NAMD	Nanoscale Molecular Dynamics
NMR	Nuclear Magnetic Resonance
NPT	Constant Number of Particle (N), Pressure (P) and Temperature (T)

NVT	Constant Number of Particle (N), Volume (V) and Temperature (T)
OPLS	Optimized Potential for Liquid Simulations
PCA	Principal Component Analysis
PDB	Protein Data Bank
PME	Particle Mesh Ewald
PPI	Protein–Protein Interaction
QM/MM	Quantum Mechanics/Molecular Mechanics
RMSD	Root-Mean-Square Deviation
RMSF	Root-Mean-Square Fluctuation
RP-HPLC	Reverse-Phase High-Performance Liquid Chromatography
SDS	Sodium Dodecyl Sulfate
PAGE	Polyacrylamide Gel Electrophoresis
SPPS	Solid Phase Peptide Synthesis
TFA	Trifluoroacetic Acid
TIPS	Triisopropylsilane
TN	Tris-NaCl
UFLC	Ultra-Fast Liquid Chromatography
UHPLC	Ultra-High-Performance Liquid Chromatography
vdW	van der Waals
VS	Virtual Screening
WHO	World Health Organisation

PENGENALPASTIAN PENSTABIL HOMODIMER 14-3-3 SIGMA BAHARU DALAM KARSINOMA NASOFARINKS

ABSTRAK

14-3-3σ ialah protein homodimer berasid dengan lebih daripada seratus pasangan protein yang berbeza yang berkaitan dengan pengisyaratan onkogenik dan pengawalaturan kitaran sel. Kajian terdahulu menunjukkan bahawa 14-3-3σ berfungsi sebagai penindas tumor yang berpotensi dan ekspresinya biasa diturunkan dalam banyak jenis kanser termasuk karsinoma nasofarinks (NPC). Selain itu, oleh sebab 14-3-3σ perlu membentuk homodimer untuk berfungsi secara aktif, penstabil homodimer dijangka juga dapat meningkatkan aktiviti 14-3-3σ. Namun demikian, sehingga kini masih tidak ada ligan yang dilaporkan dapat mengikat pada rongga antara muka dimer 14-3-3σ dan menstabilkan bentuk homodimerinya. Oleh itu, tujuan kajian ini adalah untuk mengenal pasti penstabil homodimer 14-3-3σ baharu yang berpotensi. Tiga pendekatan berbeza telah dilaksanakan untuk mengenal pasti penstabil homodimer 14-3-3σ baharu yang berpotensi, iaitu penyaringan maya *in silico* terhadap pangkalan data komersial sedia ada, mencari dalam literatur untuk sebatian yang dilaporkan menyasarkan antara muka dimer isoform 14-3-3 yang lain, dan reka bentuk rasional penstabil homodimer 14-3-3σ baharu yang berpotensi. Pendokkan molekul *in silico* dan simulasi dinamik molekul (MD) dengan pelbagai alat analisis (telah dilaksanakan untuk mengenal pasti penstabil homodimer 14-3-3σ baharu yang berpotensi. Peptida yang direka dengan rasional dan menunjukkan tenaga bebas ikatan yang lebih rendah pada 14-3-3σ telah disintesis dengan menggunakan pendekatan sintesis peptida fasa pepejal (SPPS) dan dicirikan menggunakan ^{1}H NMR dan LC-MS. Di samping itu, protein 14-3-3σ manusia yang lengkap telah diekspresikan dan dicirikan menggunakan

SDS-PAGE, PAGE asli, ^{1}H NMR, dan analisis penjujukan. Kajian pengikatan peptida yang disintesis pada protein 14-3-3σ yang diekspresikan secara rekombinan dengan menggunakan [^{1}H]-Carr-Purcell-Meiboom-Gill (CPMG) NMR mengesahkan pengikatan peptida pada protein 14-3-3σ. Walau bagaimanapun, analisis kalorimetri pentitratan isoterma (ITC) menunjukkan keafinan ikatan yang agak lemah oleh sebatian terbaik (Peptida 3) daripada kajian pendokkan molekul pada protein 14-3-3σ. Namun demikian, analisis penyerakan cahaya dinamik (DLS) menunjukkan bahawa protein 14-3-3σ hanya mula membentuk agregat pada 48 °C dengan kehadiran Peptida 3, berbanding dengan 43 °C tanpa Peptida 3. Oleh sebab analisis DLS pada protein sahaja pada suhu terpilih (25, 42, 50, dan 60 °C) jelas menunjukkan bahawa bentuk homodimer protein tersebut berpecah kepada monomer sebelum pengagregatan lengkap, keputusan ini menunjukkan bahawa Peptida 3 mampu menstabilkan bentuk homodimer protein 14-3-3σ daripada berpecah menjadi monomer, dan seterusnya menyokong peranan Peptida 3 sebagai penstabil homodimer 14-3-3σ yang berpotensi. Kesimpulannya, kajian ini telah berjaya mengenal pasti penstabil homodimer 14-3-3σ yang baharu. Walaupun keafinan ikatan Peptida 3 tidak sekuat seperti yang dijangkakan *in silico*, kajian ini telah membuktikan keupayaan berpotensi suatu sebatian dengan dua kumpulan berfungsi asas yang dipisahkan oleh jarak tertentu (13 atom karbon) untuk mengikat dan menstabilkan bentuk homodimer 14-3-3σ yang penting untuk fungsi penindasan tumornya.

IDENTIFICATION OF NOVEL 14-3-3 SIGMA HOMODIMER STABILISER IN NASOPHARYNGEAL CARCINOMA

ABSTRACT

14-3-3σ is an acidic homodimer protein with more than one hundred different protein partners associated with oncogenic signalling and cell cycle regulation. Previous studies have shown that 14-3-3σ functions as a potential tumour suppressor and its expression is commonly downregulated in many types of cancer including nasopharyngeal carcinoma (NPC). As the monomers are generally more prone to degradation than dimers, it was hypothesised that a homodimer stabiliser can potentially decrease the degradation of 14-3-3σ. Also, as the 14-3-3σ requires homodimer form to be functionally active, a homodimer stabiliser is expected to also enhance the activity of the 14-3-3σ. However, to date, there is no reported ligand that can bind to the 14-3-3σ dimer interface cavity and stabilise its homodimeric form. Thus, the aim of this study is to identify a potential novel 14-3-3σ homodimer stabiliser. Three different approaches were implemented to identify a potential novel 14-3-3σ homodimer stabiliser, i.e., *in silico* virtual screening of a commercially available database, searching the literature for compounds reported to target the dimer interface of other 14-3-3 isoforms, and rational design of potentially novel 14-3-3σ homodimer stabilisers. *In silico* molecular docking and molecular dynamic (MD) simulation with various analysis tools were implemented for the identification of the potential 14-3-3σ homodimer stabiliser. The rationally designed peptides, which showed lower binding free energy to the 14-3-3σ, were synthesised, using solid phase peptide synthesis approach (SPPS), and characterised using ^{1}H NMR and LC-MS. On the other hand, the full-length human 14-3-3σ protein was expressed and further characterised using

SDS-PAGE, native PAGE, ^{1}H NMR, and sequencing analysis. Subsequent binding study of the synthesised peptides to the recombinantly expressed 14-3-3σ protein using [^{1}H]-Carr-Purcell-Meiboom-Gill (CPMG) NMR confirmed the binding of the peptides to the 14-3-3σ protein. Isothermal titration calorimetry (ITC) analysis, however, indicated a relatively weak binding affinity of the top ranked compound (Peptide 3) from the molecular docking studies on the 14-3-3σ. Nevertheless, dynamic light scattering (DLS) analysis revealed that the 14-3-3σ protein only started to aggregate at 48 ℃ in the presence of Peptide 3, compared to 43 ℃ in the absence of Peptide 3. As the DLS analysis of the protein alone at selected temperature (25, 42, 50, and 60 ℃) clearly showed that the protein's homodimer form dissociated into monomer before complete aggregation, these results seem to indicate that Peptide 3 is able to stabilise the homodimer form of 14-3-3σ protein from dissociating into monomers, thus supporting its role as a potential 14-3-3σ homodimer stabiliser. In conclusion, this study has successfully identified a novel 14-3-3σ homodimer stabiliser. Although the binding affinity of the Peptide 3 was not as good as predicted *in silico*, this study has demonstrated the potential ability of a compound with two basic functional groups separated by a specific distance (13 carbon atom) to bind and stabilise the 14-3-3σ homodimer form which is essential for its tumour suppressing functions.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

1.1.1 Nasopharyngeal carcinoma (NPC)

NPC is a type of head and neck cancer that develops in the epithelial lining of the nasopharynx, commonly originated in the fossa of Rosenmuller, a recess located medial to the medial cruca of the Eustachian tube (Chen et al., 2019). NPC is characterised by Epstein–Barr virus (EBV) infection, hidden anatomical location, remarkable racial and geographical distribution, and high incidence of locoregional recurrence or metastasis (Xiao et al., 2009). According to the International Agency for Research on Cancer, NPC is considered a rare malignancy accounting for only 0.7 % of all cancers diagnosed in 2020 with an incidence rate of around 1 in every 100,000 persons. Nevertheless, NPC is mostly common in eastern and Southeast Asia (Sung et al., 2021; Chen et al., 2019; World Health Organization, 2020). In Malaysia, NPC is the fourth most common malignant tumour. It is common among the Chinese, followed by natives of Borneo (especially the Bidayuh) and the Malay (Loh et al., 2023).

The aetiology of NPC is related to a combination of a genetic, ethnicity, viral infection, and environmental factors (Chang et al., 2021; Cho, 2007). According to the World Health Organization (WHO), NPCs are classified into three pathological subtypes: keratinising squamous, non-keratinising, and basaloid squamous. The non-keratinising NPC can be also divided into differentiated and undifferentiated tumours. The non-keratinising subtype is considered the most common subtype in the endemic areas and is predominantly associated with Epstein-Barr virus (EBV) infection (Chua et al., 2016; Thompson, 2007; Wang et al., 2016a). Current treatment modality of NPC

is radiotherapy and chemotherapy; however, locoregional recurrence and distant metastases are usually considered the causes of treatment failure (Cao et al., 2013; Chua et al., 2003; Li et al., 2014; Wei and Sham, 2005; Xiao et al., 2009). This suggests the need of a new therapeutic agents against NPC.

1.1.2 14-3-3σ (Stratifin, or Sfn)

The 14-3-3 protein family (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein) is a group of acidic polypeptides that are highly conserved in all eukaryotic cells (Leffers et al., 1993; Wang and Shakes, 1996; Prasad et al., 1992). The 14-3-3 protein family was initially described by Moore & Perez in 1967 as an abundant mammalian brain proteins found in vertebrates (Moore and Perez, 1967). 14-3-3 proteins have also been detected in non-vertebrate species such as plants and yeasts (Rosenquist et al., 2001; van Heusden, 2009; van Heusden and Steensma, 2006). The family took its name based on its elution profile, specifically the fraction number of bovine brain homogenate from diethylaminoethyl (DEAE) cellulose column (14^{th} fraction) and subsequent purified fraction 3.3 from gel electrophoresis (Moore and Perez, 1967). The 14-3-3 proteins were initially considered as activators of enzymes in dopamine and neurotransmitter biosynthesis (Ichimura et al., 1988) before its key role in regulating the activity of the protein kinase C and acting as a substrate for c-Bcr serine/threonine kinases were investigated (Reuther et al., 1994). Later, 14-3-3 proteins were shown to interact with many protein kinases such as Raf (Freed et al., 1994).

The 14-3-3 family comprises of seven human isoforms which are encoded by separate genes (YWHAB, YWHAE, YWHAG, YWHAH, YWHAS/SFN, YWHAQ, YWHAZ) located on different chromosomes. The seven isoforms are named after their respective elution positions on high performance liquid chromatography (HPLC) (β-

beta, ε-epsilon, γ-gamma, η-eta, σ-sigma, τ-tau, and ζ-zeta) with more than 1,200 partners forming protein–protein interaction (PPI) in mammalian cells (Sluchanko and Bustos, 2019; Aitken, 2002; Ichimura et al., 1988).

Members of 14-3-3 proteins family share a high degree of sequence similarity (Figure 1.1) with a molecular mass of approximately 28–30 kDa and isoelectric point of 4–5 (Aitken et al., 2002; Fu et al., 2000). In addition, the phylogenetic tree analysis based on the primary sequence of the seven human 14-3-3 isoforms indicates more similarities between certain pairs of 14-3-3 isoforms such as η and γ, β and ζ, as well as σ and τ (Figure 1.2).

The 14-3-3σ protein was first identified in differentiated squamous epithelium by Leffers et al. (1993). 14-3-3σ is unique as it is abundantly expressed in the keratinocytes and epithelial cells (Leffers et al., 1993; Prasad et al., 1992). 14-3-3σ regulates a wide range of proteins which are mostly involved in oncogenic signalling and cell cycle regulation (Huang et al., 2020).

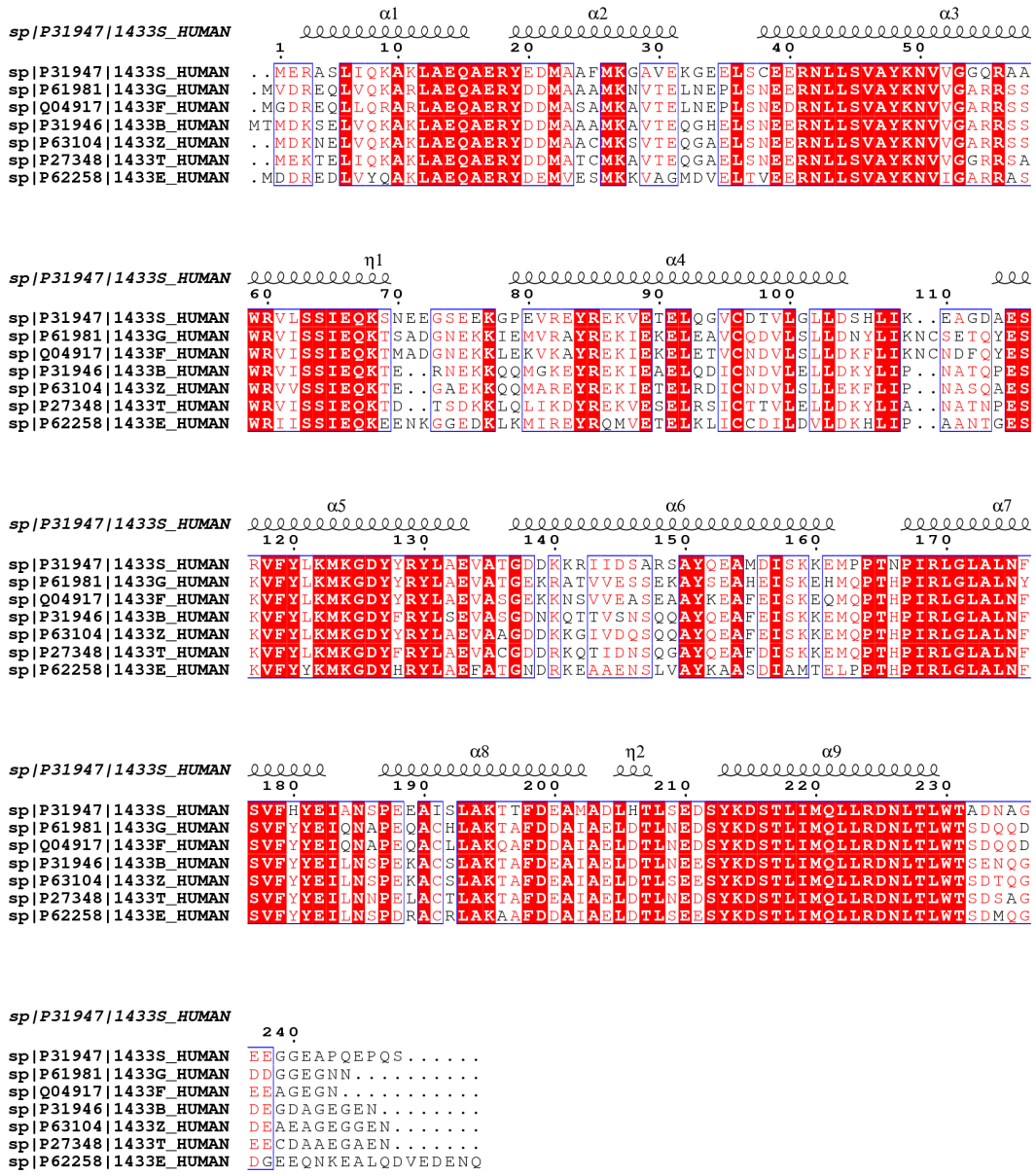

Figure 1.1 Sequence alignment of the seven human 14-3-3 isoforms (UniProtKB codes, h14-3-3ε: P62258; h14-3-3σ: P31947; h14-3-3γ: P61981; h14-3-3η: Q04917; h14-3-3τ: P27348; h14-3-3ζ: P63104; h14-3-3β: P31946), as performed with ClustalW and ESPript 3.0, with red box, white character: strict identity; red character: similarity in group; blue frame: similarity across group. The α-helical regions are indicated above the sequence.

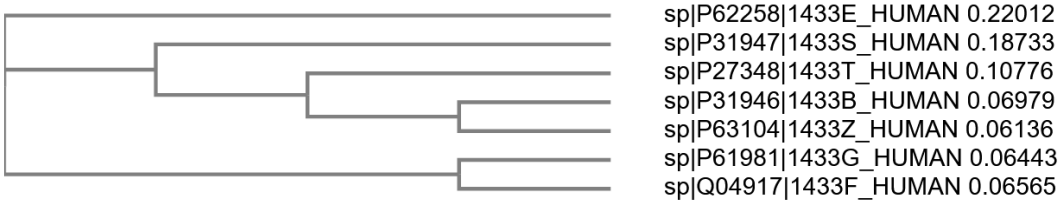

Figure 1.2 Phylogenetic tree of the seven human 14-3-3 isoforms generated using ClustalOmega.

1.1.3 Structure of 14-3-3σ

Crystal structures of 14-3-3 proteins revealed that they are highly helical dimers with a clamp-like or horseshoe conformation (Figure 1.3). 14-3-3 isoforms can form homo- or heterodimers with other isoforms in which the dimer form of 14-3-3 proteins has two amphipathic binding grooves and thus is capable of binding two ligand motifs at the same time, either from the same target or from two different partners (Yang et al., 2006b; Xiao et al., 1995; Gardino et al., 2006). The amino acids residues lining the amphipathic grooves of the 14-3-3 proteins are highly conserved (Figure 1.4, yellow colour) whereas more variations can be seen on the outer surface.

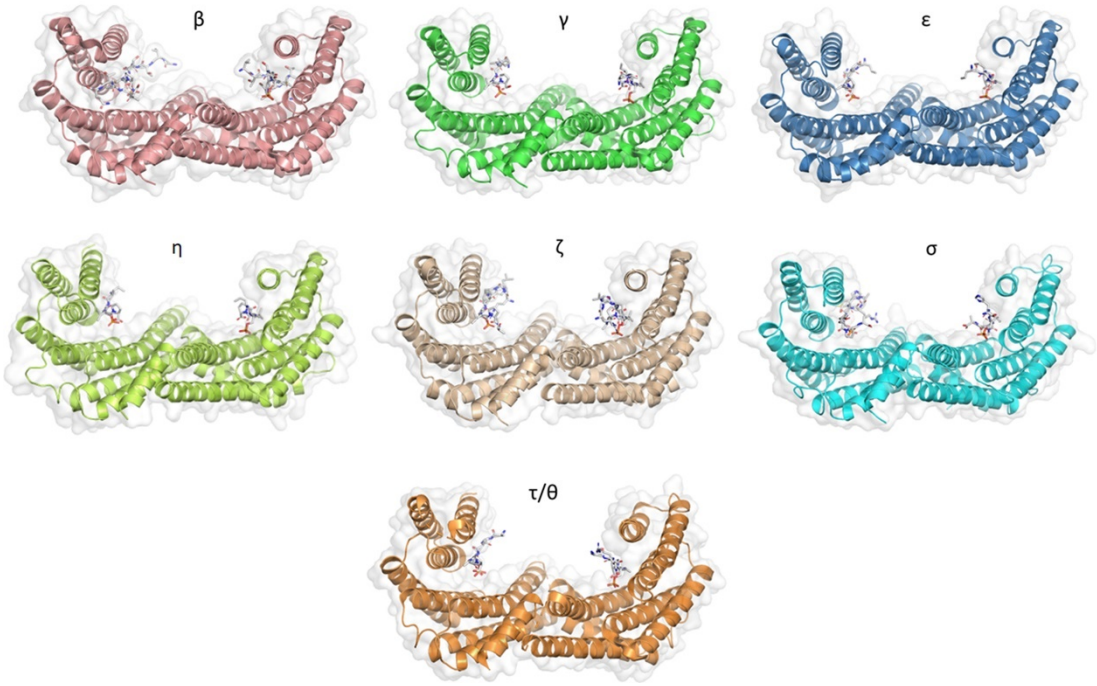

Figure 1.3 Crystal structures of the seven human 14-3-3 isoforms (PDB codes, h14-3-3β: 6A5Q; h14-3-3γ: 2B05; h14-3-3ε: 2BR9; h14-3-3η: 2C63; h14-3-3ζ: 1QJA; h14-3-3σ: 1YWT; h14-3-3τ: 2BTP) (presented as ribbon) with their peptide ligands (presented as stick, backbone colour white) bound to the amphipathic binding groove of both monomers.

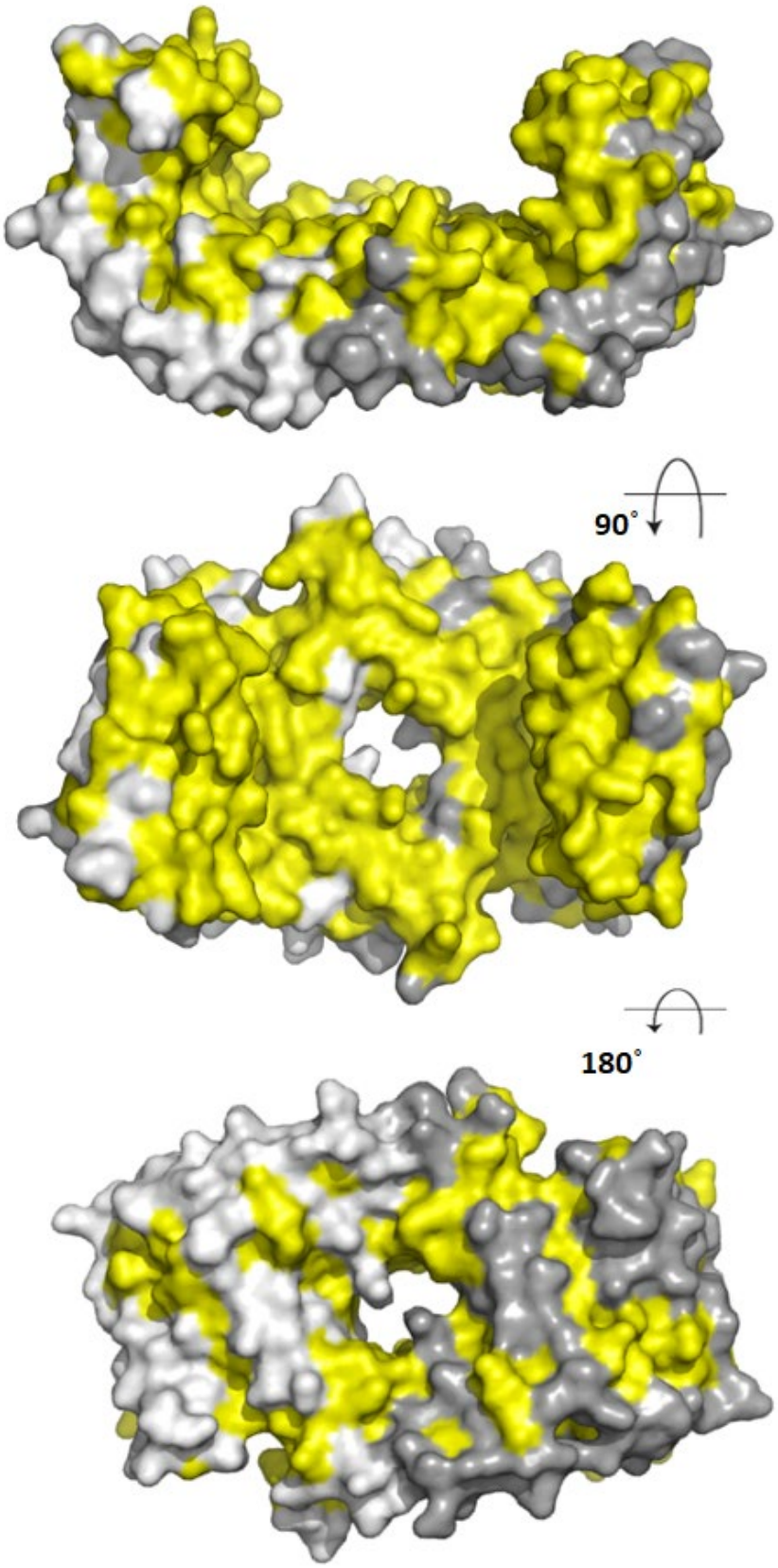

Figure 1.4 Surface representations of the 14-3-3σ homodimer (PDB: 1YWT). The conserved residues are highlighted in yellow colour. The second and third structures are rotated 90° and 180°, respectively, around the x-axis from the previous structure.

Despite the dimeric structural similarity among the mammalian 14-3-3 isoforms, many reports have shown isoform-specific interactions with 14-3-3 binding partners (Aitken et al., 2002; Aitken, 2006; Benzinger et al., 2005). It is believed that the difference in the residues of the region adjacent to the amphipathic groove and the unconserved residues forming the outer surface of 14-3-3 proteins play a key role in 14-3-3 specific binding targets (Gardino et al., 2006; Benzinger et al., 2005). In addition, reports have indicated that the N-terminal as well as the position and angles between the monomers vary among 14-3-3 isoforms, which confer an isoform-specific partner interaction (Benzinger et al., 2005; Yang et al., 2006b).

The 14-3-3 proteins are also classified as phosphoserine/phosphothreonine (pSer/pThr)-recognition proteins, as they generally exert their activity through binding to the phosphoserine/phosphothreonine-containing motifs of a multitude of molecules with various functions such as kinases, phosphatases, transmembrane receptors, and transcription factors (Chen et al., 2018; Lalle and Fiorillo, 2019; Cotelle and Leonhardt, 2016; Fan et al., 2019). In general, there are two high-affinity phosphorylation-dependent binding motifs that are recognised by the amphipathic binding grooves of all 14-3-3 isoforms, i.e., Arg-Ser-Xaa-pSer-Xaa-Pro (R-S-X-pS-X-P, mode I, Figure 1.5a) and Arg-Xaa-Xaa-Xaa-pSer/Thr-Xaa-Pro (R-X-X-X-pS/T-X-P, mode II, Figure 1.5b), where X is any amino acid and pS/T represents phosphorylated serine or threonine (Yaffe et al., 1997). A third binding motif recognised by the C-terminus of 14-3-3 proteins, i.e., pS/pT-X_{1-2}-COOH (mode III, Figure 1.5c) has also been reported (Coblitz et al., 2005). Nevertheless, not all 14-3-3 interactions require a phosphorylated residue as 14-3-3 has also been reported to bind to several non-phosphorylated proteins and peptides, such as exoenzyme S, Cdc25B,

and p190RhoGEF (Petosa et al., 1998; Masters et al., 1999; Henriksson et al., 2000; Mils et al., 2000; Zhai et al., 2001; Xu et al., 2019).

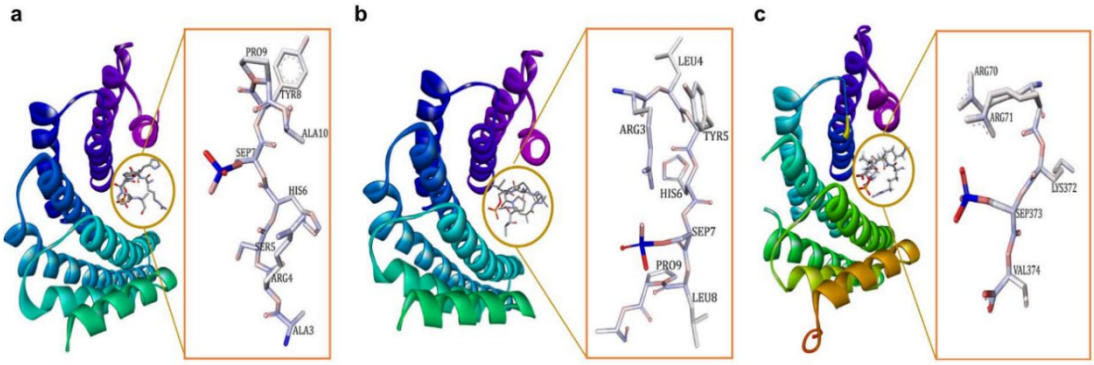

Figure 1.5 (a) 14-3-3ζ/phosphopeptide complex (mode 1, PDB: 1QJB), (b) 14-3-3ζ/phosphopeptide complex (mode II, PDB: 1QJA), (c) 14-3-3σ/TASK3 peptide (mode III, PDB: 6GHP).

The 14-3-3σ isoform only exists as a homodimer because the heterodimer form is destabilised by the force of electrostatic interaction between the residue of Glu in position 80 and the residue of Asp or Glu, that replace Ser in position 5 in the 14-3-3σ structure (Benzinger et al., 2005; Hsu et al., 2018). Like other isoforms, the dimer molecule of 14-3-3σ forms a cup-like shape in which each monomer consists of nine elongated bundles of anti-parallel helices (H1–H9). While four helices (H1–H4) involve in the dimerisation with the other monomer, the remaining five helices (H5–H9) form the amphipathic ligand-binding groove (Figure 1.6) (Benzinger et al., 2005). Earlier reports indicated that the 14-3-3σ protein can be found in either open (apo form) or closed state when it is bound to its target protein or peptides (Hu et al., 2014). While helices H1–H4 were stable in both open and closed state, the intermolecular interactions between the residues in helices H5–H9 were different in the apo-form compared to the bound one. Four hydrophilic residues (Lys49, Arg56, Arg129, and Tyr130) at H3 and H5 have been proposed to contribute to the equilibrium between both states. These residues are believed to form hydrogen bonds with the binding

peptide and drive the transition from the open conformation to the closed conformation (Hu et al., 2014).

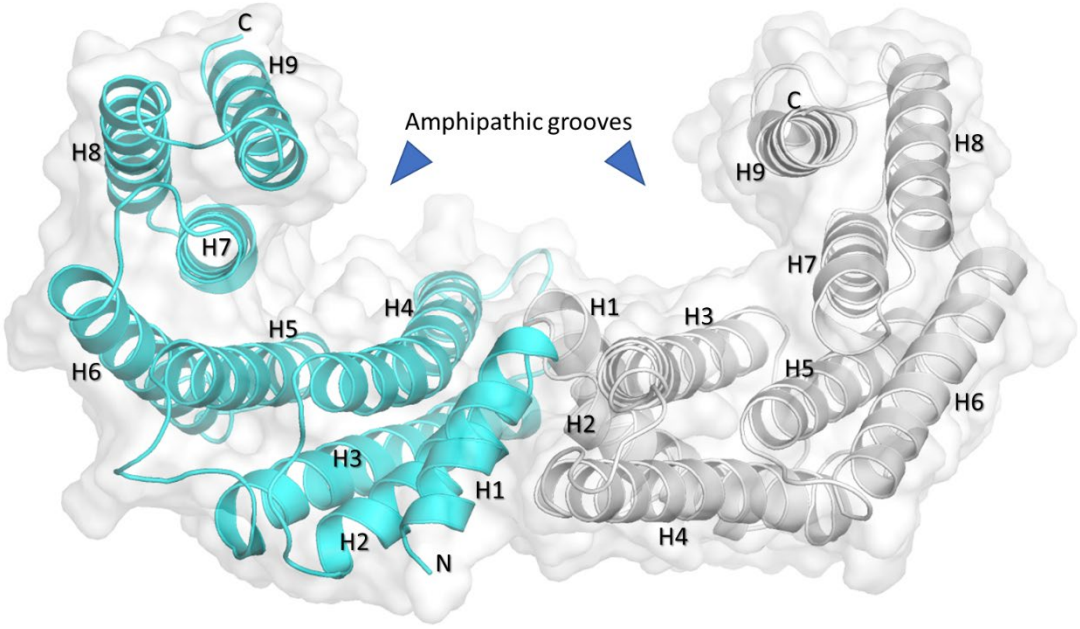

Figure 1.6 Surface representations of the 14-3-3σ homodimer (PDB: 1YZ5). The amphipathic binding grooves are indicated by blue triangles.

Five major non-conserved amino acid residues (Ser5, Glu20, Phe25, Gln55, and Glu80) are located at the interface between both monomers near the N-terminus of the 14-3-3σ isoform. Among them, the residues Ser5, Glu20, Phe25, and Glu80 together with the highly conserved hydrophobic core residues located at the dimer interface, i.e., Leu12 and Tyr84, were found to play a crucial rule in the stabilisation of the homodimer molecule and maintaining the full dimerisation activity of 14-3-3σ (Benzinger et al., 2005; Li et al., 2013). This is evidenced by the dissociation of the dimeric 14-3-3σ into monomers with diminished function upon mutation of these residues (Benzinger et al., 2005; Li et al., 2013; Liu et al., 2011).

1.1.4 Role of 14-3-3σ in cancer

Previous studies have demonstrated the role of 14-3-3σ in suppressing tumour metabolic reprogramming (Phan et al., 2015; Wen et al., 2013; Khorrami et al., 2017;

Ji et al., 2018). In addition, a few reports have also highlighted the crucial role of 14-3-3σ against the cancer cell invasion and metastasis. For instance, a low level of 14-3-3σ has been shown to promote production of lactate which stimulates the migration of epithelial cancer cells to a distant organ through breaking down of extracellular matrices (Gatenby and Gillies, 2004; Phan et al., 2015). Studies have also showed that, among all seven well-known human 14-3-3 isoforms, 14-3-3σ is the only isoform that possesses tumour-suppressing activity (Ferguson et al., 2000; Fu et al., 2000; Lee and Lozano, 2006; Hermeking et al., 1997). It has been shown that 14-3-3σ protein directly controls the G2-M checkpoint of the cell cycle by protecting the tumour suppressor factor P53 against the MDM2-mediated ubiquitination and degradation (Yang et al., 2006a; Hermeking et al., 1997). In addition, 14-3-3σ was also reported to play a crucial role in the cell cycle arrest regulation by acting as a cyclin-dependent kinase (Cdk) inhibitor, i.e., through sequestering the cyclin-dependent kinase 1-cyclin B1 complex from entering the nucleus and initiating mitosis, as well as binding to the cyclin-dependent kinases 2 and 4 (Chan et al., 1999; Laronga et al., 2000). Moreover, 14-3-3σ was also found to negatively regulate the oncogenic activity of the Protein kinase B (also known as Akt) and thus protecting against Akt-mediated tumorigenesis (Yang et al., 2006a). Further, 14-3-3σ has also been reported as a target gene in mammary epithelial cells which regulates the antiproliferative activity of the transforming growth factor-beta 1 (TGF-b1) through the Smad3-dependent mechanism (Hong et al., 2010; Hong et al., 2013). Furthermore, reports have demonstrated 14-3-3σ involvement in controlling cell proliferation and cancer metastasis via the termination of NF-κB signal in mammary cells by regulating the nuclear export of the p65 subunit of NF-κB transcription factor and subsequently inhibits its transcriptional activity (Ingles-Esteve et al., 2012; Wolter et al., 2020). Moreover, 14-3-3σ has also been reported to regulate

the expression of human TASK-3 channel (which is believed to facilitate cancer cell proliferation and survival), by blocking the endoplasmic reticulum retention sequences, and thereby promoting the surface expression of this channel (Aghazadeh and Papadopoulos, 2016; Anders et al., 2013; Zúñiga et al., 2018). 14-3-3σ also regulates the oncogenic activity of transcriptional coactivator TAZ which is an oncogenic protein that promotes cell proliferation and migration. The binding of TAZ to 14-3-3σ leads to cytoplasmic retention of TAZ which subsequently disabling its function (Zhou and Lei, 2016).

Although some studies have also indicated that the 14-3-3σ could be a double-edged sword (Li et al., 2009), whereby its upregulation has been linked with resistance to chemotherapeutic agents (Han et al., 2006; Liu et al., 2006; Zhang and Liu, 2007), overexpression of matrix metalloproteinase 1 (MMP-1), a proteolytic enzyme that degrades native fibrillar collagens, poor prognosis in malignant tumours, including gastric and colon carcinomas (Ghahary et al., 2005; Li et al., 2009; Murray et al., 1996; Inoue et al., 1999), and to binding to the c-Abl protein in chronic myeloid leukaemia, preventing its nuclear translocation and subsequently interfering with its pro-apoptotic effect (Mancini et al., 2009), the vital role of 14-3-3σ in controlling the tumour formations and metastasis in NPC has been fairly consistent (Chen et al., 2015; Huang et al., 2010; Xie et al., 2019).

1.1.5 Expression of 14-3-3σ in NPC

Unlike other isoforms which show elevated expression in many types of cancer, 14-3-3σ protein level is downregulated in chronic myeloid leukaemia, nasopharyngeal carcinoma, as well as lung, breast, oesophageal, uterine, ovarian, and skin cancers (Chan et al., 2010; Fan et al., 2019; Li et al., 2009; Lodygin et al., 2003;

Qi et al., 2014; Zhou et al., 2018). The low expression level of 14-3-3σ protein in many cancer types has been linked to either promoter hypermethylation of Sfn gene (which encodes the 14-3-3σ protein) or direct 14-3-3σ degradation through ubiquitination which eventually aborts the normal physiological role of 14-3-3σ against tumour growth and metastasis (Choi et al., 2011; Ferguson et al., 2000; Umbricht et al., 2001; Urano et al., 2002; Vercoutter-Edouart et al., 2001). Previous research has identified TRIM25 (EFP), RFFL (CARP2), COPS5, and LASP1 as potential partners that could facilitate the degradation of 14-3-3σ protein. (Urano et al., 2002; Yang et al., 2008; Zhou et al., 2018). EFP is a member of the RING-finger B-box coiled-coil (RBCC) motif family. It is an E3 ubiquitin ligase enzyme that has a RING domain located at the N-terminus that is important for its ubiquitination activity while the C-terminus contains the PRY-SPRY domain which has been proposed to be involved in protein–protein interactions (Martín-Vicente et al., 2017; Sanchez et al., 2016). EFP has been shown to be involved in the regulation of 14-3-3σ in breast cancer by Efp-mediated proteolysis (Urano et al., 2002). The direct degradation of 14-3-3σ by EFP caused the dissociation of the protein from cyclin–Cdk complexes, leading to cell cycle progression and tumour growth (Urano et al., 2002). CARP2 is another E3 ligases that has been found to target 14-3-3σ for degradation in HCT116 and HeLa cells, leading to the stabilisation of MDM2, the major negative regulator of tumour suppressor p53 (Yang et al., 2008). LIM and SH3 protein 1 (LASP1) is a specific focal adhesion protein that is known to be involved in numerous biological and pathological processes (Shao et al., 2016). Upregulation of LASP1 has been found in several types of cancer. It has been reported that LASP1 interacted with 14-3-3σ and negatively regulated the expression of 14-3-3σ in colorectal cancer which results in the progression of the tumour (Zhou et al., 2018). Further analysis showed that COPS5 could directly bind

to the SH3 domain of LASP1, and negatively regulate the expression of 14-3-3σ through ubiquitination and protein degradation thus promoting colorectal cancer progression via PI3K/Akt dependent signalling pathway (Zhou et al., 2018; Shao et al., 2016). While the protein partners involved in degradation of 14-3-3σ in various cancer cell types have been reported, it is still unknown whether the same proteins are involved in the degradation of 14-3-3σ in NPC. However, previous iTRAQ-based quantitative proteomic analysis of CNE1 and CNE2 NPC cell lines has identified TRIM25 and LASP1 proteins (Huang et al., 2010), which suggests their possible involvement in 14-3-3σ degradation in NPC.

1.2 Problem Statement

Although many proteins have been reported to be responsible for 14-3-3σ degradation and ubiquitination in various cancer cell types, i.e., TRIM25 (EFP), RFFL (CARP2), COPS5 and LASP1 (Urano et al., 2002; Yang et al., 2008; Zhou et al., 2018), the interaction site of these protein partners on 14-3-3σ and their degradation mechanism, i.e., whether it involves dissociation of 14-3-3σ dimer into monomer, have yet to be determined. Nevertheless, as the monomers are generally more prone to degradation than dimers (Messaritou et al., 2010; Sluchanko et al., 2011), it was hypothesised that a homodimer stabiliser can potentially decrease the degradation of 14-3-3σ. Also, as the 14-3-3σ requires homodimer form to be functionally active, a homodimer stabiliser is expected to also enhance the activity of the 14-3-3σ. Unfortunately, to date, the only modulators of 14-3-3σ are stabilisers and inhibitors of PPI between 14-3-3σ and protein partners at the amphipathic ligand-binding groove, while compounds that were reported to bind at the dimer interface are only for the dimer of 14-3-3ζ and 14-3-3η isoforms which have distinctively different dimer interface than 14-3-3σ.

1.3 Objectives

The main objective of this study is to identify a novel homodimer stabiliser for 14-3-3σ in NPC. The specific objectives of this study are:

a) To design and predict potential 14-3-3σ homodimer stabilisers *in silico*.

b) To synthesise, purify, and characterise selected potential 14-3-3σ homodimer stabilisers designed and predicted from *in silico* study.

c) To recombinantly express, purify, and characterise the 14-3-3σ protein.

d) To analyse the interaction between the synthesised potential 14-3-3σ homodimer stabilisers and the recombinantly expressed 14-3-3σ *in vitro*.

e) To investigate the stabilisation effect of the identified novel 14-3-3σ homodimer stabiliser on 14-3-3σ protein aggregation *in vitro*.

1.4 Scope of the thesis

The 14-3-3σ has gain the attention of many researchers as a potential therapeutic target against cancer growth and metastasis. It regulates a wide array of proteins, many of which are involved in oncogenic signalling and the regulation of the cell cycle. While it is known that the homodimer form of 14-3-3σ is essential for its full activity, and that monomers are more prone to degradation than dimers, there are currently no identified ligands that specifically target the central cavity of the 14-3-3σ dimer interface to stabilise its homodimeric form. Therefore, identifying a homodimer stabiliser for 14-3-3σ could potentially increase its activity and address its downregulation in cancer.

In **Chapter 2**, a literature review on the current status and outlook of drug discovery research against 14-3-3σ, which include the structure–activity relationship and binding interactions of the most recent 14-3-3σ protein-protein interaction (PPI) modulators reported to date is discussed in detail. The strategies and state-of-the-art techniques applied by various group of researchers in recent drug discovery campaign such as CADD and biophysical technologies are also discussed.

In **Chapter 3**, all general materials and methods used in this research are thoroughly described. This includes the methods used for *in silico* design, and prediction of the potential homodimer stabilisers of 14-3-3σ; the synthesis, purification, and characterisation of the selected potential homodimer stabilising peptides; the expression, purification, and characterisation of the recombinant full-length 14-3-3σ protein; the *in vitro* interaction studies (^{1}H-CPMG NMR, ITC) between 14-3-3σ and the peptides, and the *in vitro* stabilisation studies (DLS) of 14-3-3σ by the selected peptide.

In **Chapter 4**, the *in silico* results on the druggability of the central cavity at the dimer interface of the 14-3-3σ, and the identification of the potential 14-3-3σ homodimer stabilising compounds from three independent approaches, i.e., virtual screening (small molecules), repositioning (GCP-Lys-OMe), and rational design (dipeptide) are described. The characterisation of the identity and purity of the synthesised compounds (dipeptides) and the recombinantly expressed full-length human 14-3-3σ protein were also provided in this chapter. The ability of the selected peptides to bind to the 14-3-3σ in *in vitro* ^{1}H-CPMG NMR experiment was also demonstrated. The attempt to estimate the binding affinity of the best dipeptide on 14-3-3σ by ITC as well as the results on the thermal stability and aggregation behaviour

of 14-3-3σ in the presence and absence of the peptide by DLS are also described in this chapter.

Chapter 5 involves discussion on the findings described in Chapter 4. This includes a detailed discussion on the findings from each of the *in silico* design and prediction approach followed by rationalisation of the compound selection for subsequent *in vitro* binding and stabilisation studies. The key observations from the *in vitro* assays are also critically analysed and discussed in this chapter.

In **Chapter 6**, the major findings from this study are summarised. The potential limitations and future directions of this project are also discussed.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The involvement of 14-3-3σ protein in cancer growth and metastasis have been reported in many studies, suggesting the 14-3-3σ as an important target for anticancer drug discovery and development. Consistent with this observation, different chemical classes of 14-3-3σ PPI modulators have been developed as potential therapeutics against cancer. This includes 14-3-3σ PPI stabilisers such as fusicoccanes analogues and fragment-derived small molecule stabilisers (Section 2.2.1(a)), as well as phosphonate and non-phosphonate type 14-3-3σ PPI inhibitors (Section 2.2.1(b)). These modulators were successfully identified using a combination of techniques including *in silico* tools (ligand-based screening, docking, molecular dynamics simulations), biophysical techniques (NMR, X-ray crystallography, isothermal titration calorimetry (ITC), microscale thermophoresis (MST), fluorescence polarization (FP), surface plasmon resonance (SPR)), as well as cell-based assays. However, these modulators generally bind at the amphipathic pockets of the 14-3-3σ which are highly conserved across isoforms, thus limiting their potential use as selective anticancer agents. Thus, recent work has attempted to identify compounds targeting other site such as the dimer interface (Section 2.2.2), though the work in this area is still very limited to date.

2.2 Current status and outlook of drug discovery research against 14-3-3σ

2.2.1 14-3-3 protein-protein interaction (PPI) modulators

14-3-3 PPI modulators are compounds that alter the binding affinity or specificity of 14-3-3 proteins for their target partners. These modulators can be natural

or synthetic compounds, and they can act as stabilisers or inhibitors of 14-3-3 PPIs depending on their mode of action. The modulation of 14-3-3 PPIs has been shown to have a number of biological effects, including the regulation of signal transduction, gene expression, and apoptosis, and it has been implicated in a variety of physiological and pathological processes. Therefore, 14-3-3 PPI modulators have the potential to be used as therapeutic agents for the treatment of a range of diseases, including cancer, neurodegenerative disorders, and metabolic disorders (Stevers et al., 2017; Ballone et al., 2018; Hartman and Hirsch, 2017).

2.2.1(a) 14-3-3σ PPI stabilisers

With the increasing evidence highlighting the role of 14-3-3σ in suppressing cancer cell growth, metabolism, and metastasis, 14-3-3σ PPI stabilisation has begun to gain attention as a promising therapeutic strategy in the discovery of novel bioactive compounds against cancer. In general, PPI stabilisers work as a 'molecular glue' to increase the affinity of the partner protein to 14-3-3σ in order to achieve a positive therapeutic effect. An example of a well-studied 14-3-3σ PPI stabiliser is fusicoccin-A (FC-A (compound 1)) Figure 2.1a), a diterpene glycoside fungal phytotoxin which was initially found to significantly enhance the interaction between the plant analogue of 14-3-3 and the plant plasma membrane H+-ATPase (PMA2) by about 90-fold (Fullone, 1998; Würtele et al., 2003). Recently, compound 1 has been reported to be able to stabilise the interaction between 14-3-3σ and the tumour suppressor gene p53, as confirmed by fluorescence polarisation and isothermal titration calorimetry techniques. Nevertheless, a greater disorder in the ternary complex of 14-3-3σ/p53/compound 1 was observed in the crystallographic data where the C-terminus of the peptide is no longer visible (Figure 2.1b,c), suggesting that either crystal soaking may have forced the p53 peptide to form an unpreferred conformation in the presence

of compound 1 or the fact that compound 1 acts as an allosteric modulator rather than a 'molecular glue' in stabilising the 14-3-3σ/p53 interaction (Doveston et al., 2017).

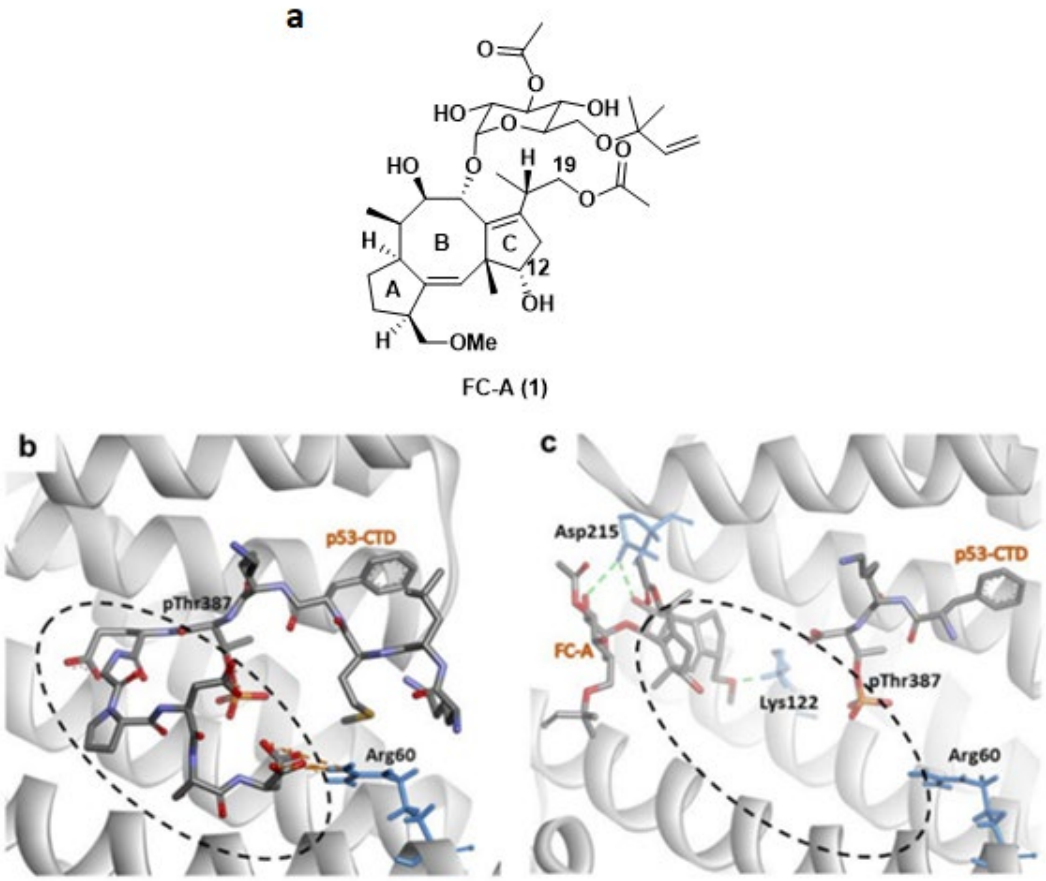

Figure 2.1 (a) Chemical structure of fusicoccin-A (FC-A). Three-dimensional (3D) crystal structure of P53/14-3-3σ complex (b) in the absence of FC-A (PDB: 5MOC) and (c) in the presence of FC-A (PDB: 5MXO), revealed that the C-terminus of the P53 peptide cannot be observed upon binding of FC-A (dashed circles).

Following promising results with FC-A in stabilising the interactions of 14-3-3σ with its protein partner, semi-synthetic analogue to compound 1, FC-THF (compound 2) (Figure 2.2a), in which a tetrahydrofuran ring was added to ring C of compound 1 was generated. However, unlike compound 1, which stabilises the interaction of 14-3-3σ with p53, compound 2 was found to stabilise the interaction between 14-3-3σ and the human potassium channel TASK-3 which is a pro-oncogenic protein that is mainly involved in cancer development. Compound 2 was reported to

be able to increase the binding affinity between TASK-3 binding motif and 14-3-3σ protein by up to 19-fold at 100 μM (Figure 2.2b) (PDB: 3SMN) (Anders et al., 2013).

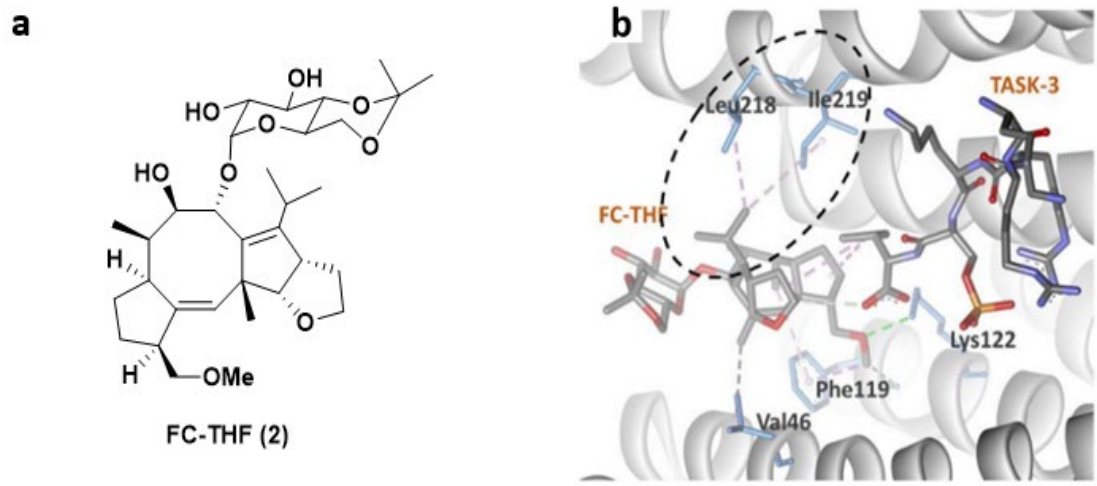

Figure 2.2 (a) Chemical structure of FC-THF. (b) 3D complex of 14-3-3σ/TASK-3 with FC-THF (PDB: 3SMN) revealed hydrophobic (purple dashed lines) and hydrogen bond (green dashed lines) interactions between 14-3-3σ and the isopropyl of FC-THF.

To further improve the affinity of fusicoccanes stabilisers on 14-3-3σ, a series of compound 1 derivatives was designed using molecular dynamic (MD) techniques. As the conserved mode of interaction between compound 1 and 14-3-3σ is mainly in the form of two hydrogen bonds with Asp215 and hydrophobic interactions with Leu218 and Leu222 of 14-3-3σ, it was hypothesised that presenting a third hydrogen bond with Asp215 would increase the potency of the stabiliser. Therefore, the 19-acetoxy moiety of compound 1 was replaced with an isostere, 19-acetamide moiety. In addition, the 3′ acetyl group which does not show a significant potency enhancement was also removed from compound 1 in order to generate a more feasible compound to synthesise. All these modifications led to compound FC-NAc (compound 3), (Figure 2.3a) which showed enhanced potency and biological activity. Further investigation into the structure of the ternary complex: 14-3-3σ, TASK-3 peptide, and compound 3 (Figure 2.3b) (PDB: 6GHP) revealed that Asp215 carboxylate group of 14-3-3σ

adopted a new conformation in order to allow the formation of three hydrogen bonds with compound 3 (Andrei et al., 2018).

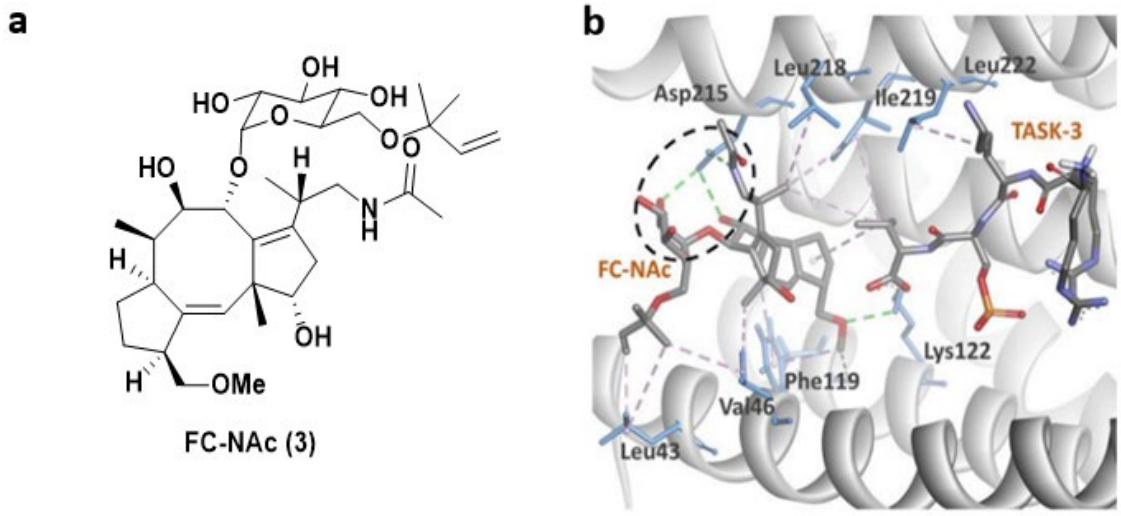

Figure 2.3 (a) Chemical structure of FC-NAc. (b) 3D complex of 14-3-3σ/TASK-3 with FC-NAc (PDB: 6GHP) revealed hydrophobic (purple dashed lines) and hydrogen bond (green dashed lines) interactions between 14-3-3σ and the 19-acetamide moiety of FC-NAc (dashed circles).

Recently, another semisynthetic analogue of compound 1, DP-005 (compound 4) (Figure 2.4a) has been reported. Unlike its predecessors which stabilise TASK-3 or p53 interactions with 14-3-3σ, compound 4 acts as a selective 14-3-3/p65 stabiliser. Structural elucidation of the ternary complex p65_45R/14-3-3σ/ compound 4 (Figure 2.4b) (PDB: 6NV2) revealed that upon binding of compound 4, p65_45R peptide adopted a new orientation allowing compound 4 to form a hydrophobic contact via its isopropyl moiety with Ile46 and Pro47 of the peptide while it binds to Leu218, Ile219, and Leu222 of 14-3-3σ. This additional hydrophobic interaction with p65_45R peptide , however, is not observed in compound 1 as the extra 12-hydroxyl group in compound 1 created a steric and polar clash with the hydrophobic residues of the peptide resulting in unpreferred interactions (Wolter et al., 2020).

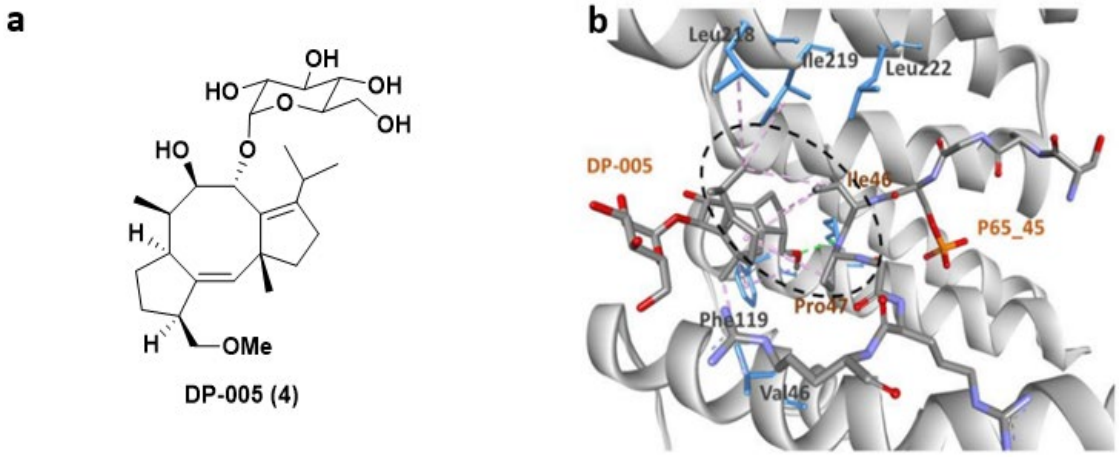

Figure 2.4 (a) Chemical structure of DP-005. (b) 14-3-3σ/DP-005/p65_45R ternary complex (PDB: 6NV2) showed that DP-005 not only forms hydrophobic interactions (purple dashed lines) with 14-3-3σ, but also with P65_45 (dashed circle).

Apart from the fusicoccanes stabilisers, other small-molecule stabilisers of 14-3-3σ have also been reported. This include TCF521-123 (compound 5) and TCF521-129 (compound 6) (Figure 2.5a) which are aldehyde-containing fragment stabilisers of 14-3-3σ/p65 complex. Both compound 5 and compound 6 were identified using the site-directed fragment tethering approach whereby the aldehyde-bearing fragments were found to form an imine covalent anchor (instead of hydrogen bond in fusicoccanes stabilisers) to the side chain of Lys122 residue at the amphipathic binding groove. Additionally, the crystallographic data obtained by crystal soaking experiments also revealed a hydrophobic interaction between the aromatic benzaldehyde ring of compound 5 and compound 6 with Ile46 of p65, while the sulphonamide group makes additional water-mediated contacts with Asn42 and Asp215 of the 14-3-3σ. However, while the morpholine ring of compound 6 is engaged directly with p65 peptides, the piperazine moiety of 5 is pointed away from p65 and only engaged in extra water-mediated contacts with both 14-3-3σ and p65 (Figure 2.5b, c). Nevertheless, both fragments are efficient stabilisers of the 14-3-3σ/p65 complex (Ottmann et al., 2020).

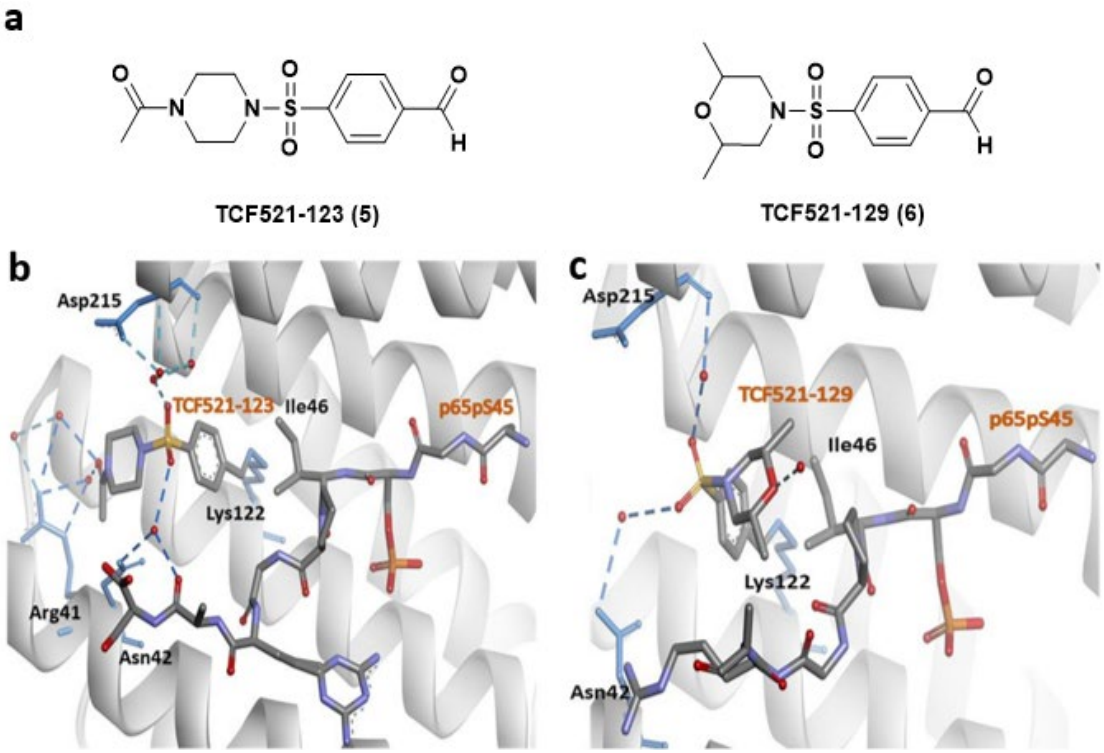

Figure 2.5 (a) Chemical structure of fragment-derived small molecule stabilisers of 14-3-3σ PPI. (b) 14-3-3σ/P65/TCF521-123 ternary complex (PDB: 6YPY). (c) 14-3-3σ/P65/TCF521-129 ternary complex (PDB: 6YQ2).

Using the same covalent imine-based tethering approach and by targeting the lysine residue at the interface of the composite 14-3-3 complex by an aldimine-forming fragments, a reversible covalent molecular glue for the 14-3-3σ/Pin1 interaction was identified from a series of an aldehyde bearing small molecules. By applying a cooperativity analysis of the ternary complex formed between 14-3-3/Pin1 and the tested small molecule, 28 (compound 7) (Figure 2.6a) was developed and found to stabilise the interaction between 14-3-3/Pin1 by more than 250-fold by selective interfacing with a unique tryptophan in Pin1. Crystal structure of 14-3-3σ/Pin1/ compound 7 (Figure 2.6b) (PDB: 7BFW) showed the 2,4-difluorophenyl ring of compound 7 occupied a pocket formed by Cys38, Arg41, and Phe119 on the 14-3-3σ. This arrangement locks the orientation of the 2,4-difluorophenyl ring and cause a conformational change in the Asn42 of 14-3-3, enabling the formation of a direct

hydrogen bond with Gln of Pin1. Additionally, the Trp conformation induced by compound 7 allowed the formation of water-mediated hydrogen bonds between Trp and Gln of Pin1 and Asn42 and Ser45 of 14-3-3σ. Interestingly, compound 7 was found to be a selective 14-3-3σ/Pin1 interaction over other 14-3-3 partners targeting the same binding site such as p65 (Cossar et al., 2021).

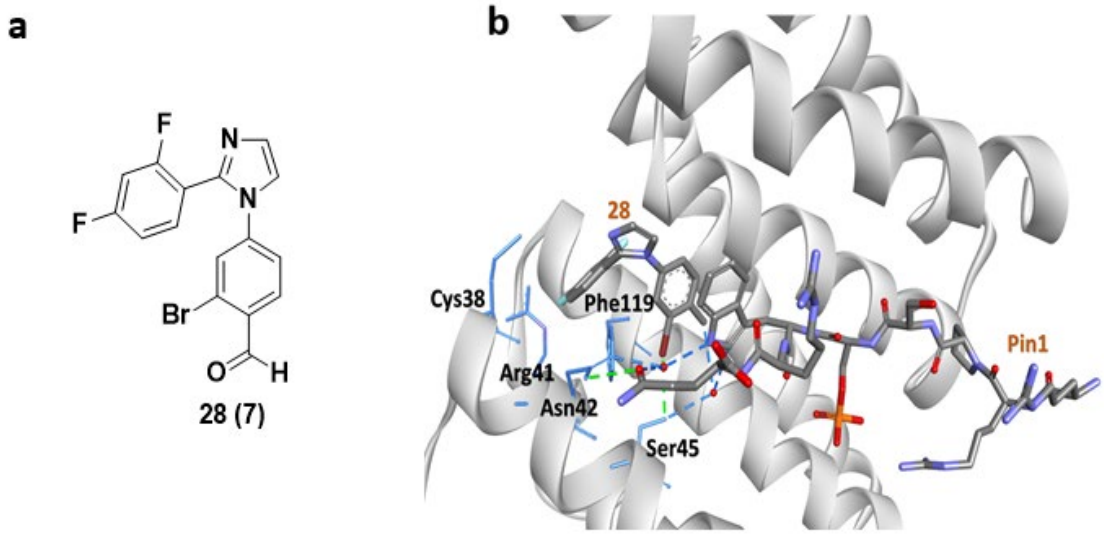

Figure 2.6 (a) Chemical structure of 28. (b) 14-3-3σ/Pin1/28 ternary complex (PDB: 7BFW).

Other examples of covalent small-molecule stabilisers of 14-3-3σ are fragment 1 (compound 8) and fragment 2 (compound 9) (Figure 2.7) which were identified using a site-directed fragment-based screening approach known as disulphide trapping. This approach utilises a cysteine on the target protein as a reactivity handle to capture disulphide-containing fragments that possess a weak intrinsic binding affinity for a target pocket near the cysteine. The bound fragments can then be detected through intact protein mass spectrometry (MS). The disulphide trapping approach was designed to target the hydrophobic pocket at the 14-3-3σ/ERα interface by binding to three 14-3-3σ constructs: a wildtype with a native, surface-exposed cysteine (C38) at the edge of the FC-A pocket, and two other engineered constructs in which the wildtype cysteine was mutated (C38N) and a cysteine was introduced at positions 42