ELUCIDATING THE DIAGNOSTIC AND THERAPEUTIC POTENTIAL OF AN RNA APTAMER AGAINST HUMAN PITUITARY TUMOUR TRANSFORMING GENE 1

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS AND ABBREVIATIONS

A	Adenine
aa	Amino acid
ALISA	Aptamer-Linked Immunosorbent Assay
APS	Ammonium persulfate
ATP	Adenosine 5’-triphosphate
Bis	N, N’-methylene bisacrylamide
bp	Base pair (s)
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
CTP	Cytidine 5’-triphosphate
CXCR2	C-X-C Motif Chemokine Receptor 2
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
DAB	3,3'-Diaminobenzidine Tetrahydrochloride
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	And others
EtBr	Ethidium bromide
ECL	Enhanced Chemiluminescence
FDA	USA Food and Drug Administration
FFPE	Formalin Fixed Paraffin Embedded
g	Gram
G	Guanine
GFP	Green Florescent Protein

GTP	Guanosine 5’-triphosphate
GST	Glutathione S-transferase
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidise
IDP	Intrinsically Disordered Proteins
IPTG	Isopropyl-β-D-thiogalactopyranoside
KCl	Potassium chloride
K_d	Dissociation constant
kDa	Kilodalton
KOH	Potassium hydroxide
LB	Luria Bertani medium
LiCl	Lithium Chloride
LINA	Lithium Chloride and Sodium Chloride
M	Molar, [(Mole)/(Litre)]
Mg^{2+}	Magnesium ion
Min	Minute (s)
mL	Milliliter
mM	Millimolar
MWCO	Molecular Weight Cut-Off
Na^{+}	Sodium ion
NaCl	Sodium chloride
$NaOAc.3H_2O$	Sodium acetate trihydrate
NaOH	Sodium hydroxide
ng	Nanogram
nM	Nanomolar
nt	Nucleotide (s)
N-terminal	Amino-terminal
-OH	Hydroxyl
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
pmol	Picomole

PTTG1	Human Pituitary Tumour Transforming Gene 1
RLU	Relative Luminometer Units
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcription-PCR
s	Second (s)
SELEX	Systematic Evolution of Ligands via Exponential Enrichment
SECURA	Securin RNA Aptamer
ssDNA	Single-stranded DNA
T	Thymine
TAE	Tris–Acetic Acid–EDTA
TBE	Tris-Boric Acid-EDTA
TEMED	N,N,N’,N’-Tetramethylethylenediamine
TMB	3,3',5,5'-tetramethylbenzidine
Tris	Tris-(Hydroxymethyl)-Aminomethane
tRNA	Transfer RNA
U	Units of enzymatic activity
UTP	Uridine 5’-triphosphate
UV	Ultraviolet
V	Volt (s)
VEGF	Vascular Endothelial Growth Factor
v/v	Volume per volume
w/v	Weight per volume
X–gal	5’-Bromo-4’-Chloro-3’-Indolyl-β-Dgalactoside
x g	Relative Centrifugal Force
µg	Microgram
µL	Microliter
µM	Micromolar
γ32P	Gamma Phosphorus
°C	Degrees Celsius
%	Percentage

ELUSIDASI POTENSI DIAGNOSTIK DAN TERAPEUTIK APTAMER RNA TERHADAP HUMAN PITUITARY TUMOUR TRANSFORMING GENE 1

ABSTRAK

Aptamer asid nukleik merupakan sejenis unsur pengecaman molekul yang mampu berinteraksi dengan sasaran pada spesifisiti dan keafinan yang tinggi. Kajian ini bertujuan untuk menghasilkan aptamer RNA terhadap "Human Pituitary Tumor Transforming Gene 1" (PTTG1) yang mempunyai potensi berfungsi sebagai unsur diagnostik dan terapeutik. Gen *PTTG1* terlebih dulu diklonkan ke vektor pET14b untuk menggabungkan jujukan teg 6X histidin pada gen tersebut. Pengekspresan dan purifikasi protein tersebut dilaksanakan menggunakan strain *E. coli* Rosetta 2(DE3)pLysS. Identiti protein disahkan dalam blot 'Western' menggunakan antibodi monoklon anti-PTTG1. Analisis spektra pendarfluor intrinsik dan analisis interaksi PTTG1 dengan GST-p53 memberi tanda-tanda pelipatan protein yang berjaya. 11 kitaran SELEX dilaksanakan mengunakan dua jenis strategi pemetakan yang dikesan memberi tanda-tanda pemerkayaan. Penjujukan himpunan asid nukleik kitaran 8 menunjukkan kehadiran aptamer (SECURA-3) yang menunjukkan interaksi dengan PTTG1. SECURA-3 mempunyai pemalar pemisahan yang dianggarkan pada 16.41 ± 6.4 nM. SECURA-3 digunakan untuk menghasilkan asai diagnostik berasaskan aptamer iaitu ALISA, blot titik berasaskan aptamer, blot 'Western' berasaskan aptamer, dan "aptahistostaining". SECURA-3 yang ditambah dengan ekor poli-A pada hujung 3'nya untuk dilabel dengan biotin melalui hibridisasi dengan oligo dT yang berlabelkan biotin. Aptamer SECURA-3 menunjukkan pengesanan PTTG1 sehingga 0.0256 nM dalam ujian ALISA langsung dan dengan konfigurasi sandwich pula, SECURA-3 menunjukkan pengesanan PTTG1 sehingga 3.2 nM dengan aptamer

sebagai ejen penangkap dan 16 nM dengan aptamer sebagai ejen pengesanan. Dalam asai blot titik berasaskan aptamer, sehingga 2.5 pmol protein PTTG1 berjaya dikesan. SECURA-3 juga berjaya mengesan PTTG1 yang dimurnikan serta dalam lisat sel HeLa dan MCF-7 di asai blot 'Western' berasaskan aptamer. Selain itu, SECURA-3 berjaya mengesan PTTG1 dengan penembusan yang lebih baik berbanding dengan antibodi monoklon dalam blok paraffin sel difiksatif formalin HeLa dan MCF-7. Seterusnya, dalam meneliti potensi terapeutik SECURA-3, ujian antagonis dengan jujuran CXCR2 yang ditransaktifkan oleh PTTG1 dilaksanakan. SECURA-3 memberi kesan antagonis terhadap urutan CXCR2 dalam asai ikatan bersaing *in vitro* berturaskan nitroselulosa. Sehubungan dengan itu, potensi terapeutik dikaji dalam sel HeLa berlandaskan jujuran CXCR2 yang sama dalam asai pelapor dwi lusiferase dan aptamer SECURA-3 sebagai agen antagonis. Kesan antagonis berkeperluan dos seterusnya ditunjukkan SECURA-3 dengan mengurangkan aktiviti lusiferase relatif berbanding dengan RNA olokan menggunakan asai pelapor tersebut dalam sel HeLa.

ELUCIDATING THE DIAGNOSTIC AND THERAPEUTIC POTENTIAL OF AN RNA APTAMER AGAINST HUMAN PITUITARY TUMOUR TRANSFORMING GENE 1

ABSTRACT

Nucleic acid aptamers are a class of molecular recognition elements capable of target interaction at high specificity and affinity. This study was aimed to generate an RNA aptamer against the Human Pituitary Tumour Transforming Gene 1 (PTTG1) oncoprotein which can serve as a potential diagnostic and therapeutic tool. *PTTG1* gene was first cloned into the pET14b vector to incorporate the small 6X Histidine tag. Protein expression and purification ensued with the *E. coli* Rosetta 2(DE3)pLysS strain under the urea denaturing method. The protein identity was confirmed by western blot using monoclonal anti-PTTG1 antibody. Intrinsic florescence spectra analysis and GST-pulldown interaction analysis with GST-p53 conferred signs of successful protein refolding. 11 SELEX cycles with dual partitioning strategy were performed with positive indication of enrichment. Sequencing of the cycle 8 pool revealed a putative aptamer (SECURA-3) which depicted binding to PTTG1. SECURA-3 had a dissociation constant estimated at 16.41 ± 6.4 nM. SECURA-3 was employed for development of aptamer-based diagnostic assays namely ALISA, aptamer-based dot blot, aptamer based western blot and aptahistostaining. SECURA-3 was extended with a poly A-tail at the 3'-end to functionally labelled with the biotin by hybridization with the biotin labelled oligo dT sequence. SECURA-3 aptamer demonstrated detection of up to 0.0256 nM PTTG1 in the direct ALISA assay and with the sandwich configuration, SECURA-3 demonstrated detection of PTTG1 up to 3.2 nM with the aptamer as the capturing agent and 16 nM with the aptamer as the detection agent. In

the aptamer-based dot blot assay, up to 2.5 pmol PTTG1 was detected. SECURA-3 successful detected purified PTTG1 in aptamer assisted western blot as well as in the HeLa and MCF-7 cell lysates. In the aptahistostaining assay, SECURA-3 successfully detected PTTG1 with better penetration compared to the monoclonal antibody in the HeLa and MCF-7 formalin fixed paraffin embedded cell blocks. SECURA-3 demonstrated antagonistic effects towards PTTG1 and the CXCR2 regulatory sequence in the *in vitro* competitive nitrocellulose filter binding assay. The antagonistic effect was further demonstrated in a dose dependent manner by decreased relative luciferase activity compared to the mock RNA under the luciferase reporter assay in HeLa cells.

CHAPTER 1

INTRODUCTION

1.1 Introduction to Aptamers

One of the hallmarks in the field of applied sciences lies in translating research from bench to bedside. New pursuits in designing better and faster responses to upcoming threats are a core focus (Van Damme et al., 2018). Using combinatorial libraries in screening for potential ligands has been one research focus that lead to the development of diagnostic or therapeutic applications. One such combinatorial library is the nucleic acid combinatorial library which generates target binders called aptamers (Gold, 1995).

Aptamers, termed after the Latin word ‘aptus’ which means ‘to fit’ and ‘meros’, which means ‘particle’ are single stranded nucleic acids that exhibits ligand binding phenotype with high affinity and specificity (Ellington et al., 1990). Aptamers has been generated over an extensive array of targets such as small metal ions and organic molecules, peptides, proteins, microorganisms, and cells. They have demonstrated dissociation constants in the low nanomolar and high picomolar levels (Nimjee et al., 2017; Vance et al., 2014; Kensch et al., 2000). Additionally, they are capable of discriminating the target difference between a single amino acid mutation, a single functional group difference, target enantiomer or even conformational changes in the target (Chen et al., 2015; Zichel et al., 2012; Geiger et al., 1996; Jenison et al., 1994).

The phenotype exhibited by the aptamers are dictated by the structural motifs such as stemloop, G-quadruplex and pseudoknot (Hoinka et al., 2012; Patel, 1997). These

motifs collectively aid in the unique three dimensional structure of the aptamer that interacts with their target through either hydrogen bonding, hydrophobic interactions, electrostatic interactions, Van der Waals forces, base stacking or a combination of them (Rozenblum et al., 2016; Hermann et al., 2000; Patel, 1997).

Molecular recognition elements are typically required to cater to the demands of various applications such as durability across a variety of sample conditions or specific assay dependent functionalization to name a few. In line with the demands, aptamers are subjectable to a myriad of modifications and labelling with functional groups, reporters or signalling compounds, and linkers as commonly applicable with standard oligonucleotide probes (Da Pieve et al., 2009). Several combinations of modifications can simultaneously be applied as well. For example, in the case of Macugen, the FDA approved commercialized RNA aptamer against age-related macular degeneration, the nuclease-resistant analogues, 2'-fluoropyrimidines, and 2'-O-methyl purine nucleosides were utilized (Ng et al., 2006). Additionally, Macugen was also modified with an inverted 3' terminal nucleotide for added nuclease resistance and a 40 kDa polyethylene glycol at the 5'-end for improved aptamer retention in the body through a reduced renal filtration (Ng et al., 2006).

1.2 Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

Aptamers are generated from a process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Tuerk et al., 1990). The key idea in SELEX lies in the directed evolution of sequences from an initial highly diverse pool of sequences which are subsequently deconvoluted into an enriched pool of sequences under selective pressure derived from the binding conditions such as target protein concentration, salt concentration and temperature. The enriched pool of sequences comprises the potential

binders that impart binding onto the target (Dunn et al., 2017). SELEX requires the use of a combinatorial library consisting of single stranded nucleic acids, whereby the library was designed to contain a randomized region in the middle flanked at the ends with a pair of conserved sequences for amplification. The sequence diversity of the combinatorial library is imparted by the length of randomized bases, thus giving birth to as many as 4^n sequences where n is the length of the randomized region (Kopylov et al., 2000).

A three-staged process holds basis to the fundamentals of SELEX (Figure 1.1) ; (i) incubation of the randomized oligonucleotide library with the target (ii) separation of the unbound sequences from the target-bound sequence (iii) amplification of the bound sequences (Dunn et al., 2017; Irvine et al., 1991). This iterative cycle is repeated under a selective pressure typically consisting of stringent binding and washing conditions such as varying ratios of target/nucleic acid competitor such as Salmon Sperm DNA or yeast tRNA to allow the propagation of the high affinity sequences amongst the weak binders (Marshall et al., 2000).

With the foundations of SELEX in mind, various approaches have been made to tailor SELEX in order to fit the criteria and specific assay requirements set by the researcher. Cell-SELEX is a form of selection in which whole cells were used as a target to allow isolation of aptamers against surface biomolecules in their native conformation, with no prior knowledge of the surface biomarkers (Ohuchi, 2012). Capillary electrophoresis-SELEX utilizes differential rate of migration between the target-bound sequences and unbound sequences, thus minimizing the SELEX rounds to merely 2-4 rounds (Mosing et al., 2009). Cell internalization SELEX involves selection of aptamers capable of internalizing into the cells, thus creating a powerful tool in cancer targeted therapy (Gray et al., 2018; Yan et al., 2014)

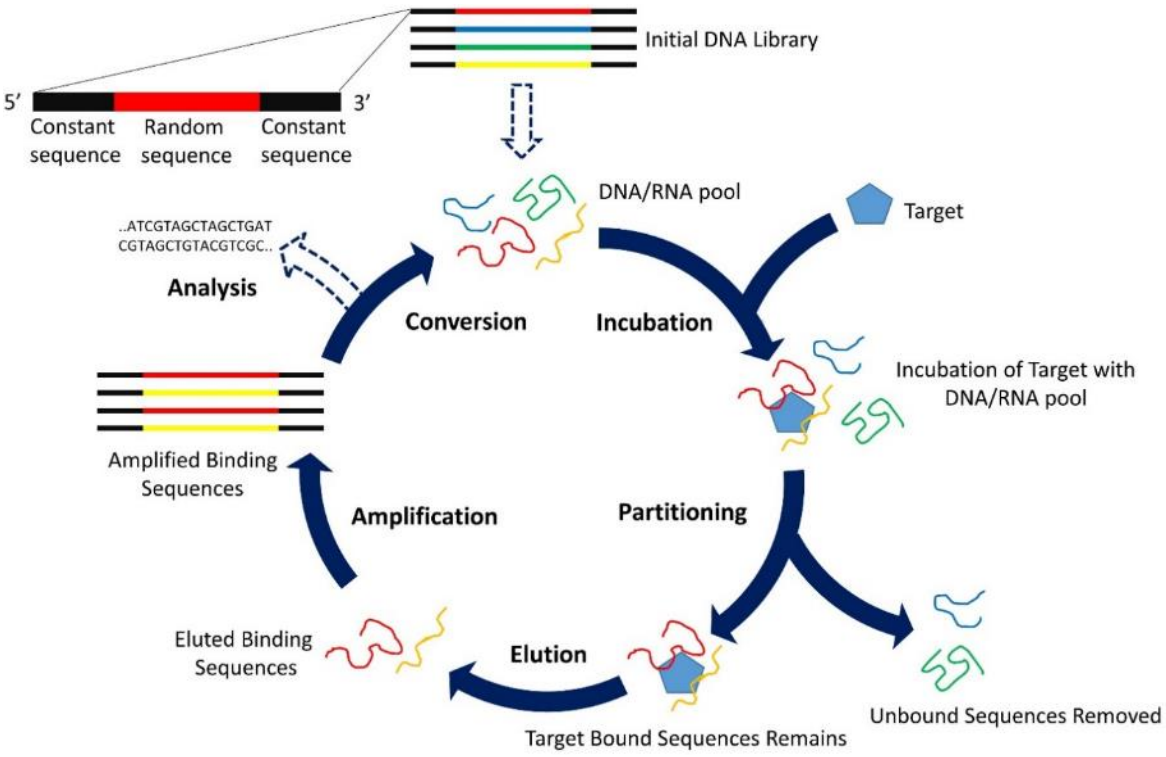

Figure 1.1 The conventional SELEX cycle.

The initial degenerate library (whereby the diversity is accounted for by the randomized sequences flanked by conserved sequences at both ends) is directly used for DNA aptamer selection or *in vitro* transcribed into RNA for RNA aptamer selection. The nucleic acid pool is then incubated with the target protein and the unbound nucleic acids are partitioned out. The bound nucleic acids will then be eluted from their target and subjected to amplification. In the amplification step, the eluted sequences are amplified by PCR for DNA aptamer or RT-PCR for RNA aptamer generation, thus obtaining the nucleic acid pool in the double stranded form for the use in the next SELEX cycle. A conversion process then takes place to convert the double stranded DNA into ssDNA pool in case of DNA aptamer generation. For RNA aptamer generation, *in vitro* transcription was carried out to convert the double stranded DNA into the RNA pool. This process occurs for 8 to 15 rounds after which the nucleic acids sequences will be analysed to unveil the putative aptamer sequences.

1.3 Advantages of Aptamers Over Antibodies

As molecular recognition elements, aptamers are often compared to antibodies and are hence termed as ‘chemical antibodies’ (Chen et al., 2017; Jayasena, 1999). However, there are distinguishing differences between the aptamers and antibodies apart from their molecular construction.

Aptamers are significantly smaller than antibodies in which a typical aptamer of 100 nt has a size at about 30 kDa, while an antibody has a size of around 150 kDa (Zhou et al., 2017; Sun et al., 2014). This is especially helpful in terms of penetrability of aptamers such as across the blood-brain barrier (Monaco et al., 2017) and tissue penetration in immunostaining based applications (Zeng et al., 2010).

The production of the aptamers are in a controlled biochemical setting which proves to be easier and without the potential batch-to-batch variation as compared to the biological requirement for antibody production (Marx, 2013). Furthermore, as a synthetic ligand, aptamers can be generated against targets that are toxic to cells (Rozenblum et al., 2016). Aptamers also reaps benefits in the heat stability aspect such as from reversible heat denaturation as well as a lower shipping cost relative to antibodies as it does not require cold-chain logistics (Dunn et al., 2017).

As therapeutics, aptamers exhibit low to no immunogenicity when applied to humans and rodents (Eyetech Study Group, 2003). Furthermore, reversal of drug activity is possible with aptamers in therapeutic applications by administration of ‘antidotes’ which are antisense oligonucleotides complementary to the aptamer binding domain (Rusconi et al., 2004). The structure of the active aptamer will be distorted from the ‘antidote’ binding and rendered inactive. However in comparison to antibodies,

aptamers remain to date an early technology that require further work and development to ensure growth within the next 25 years (Dunn et al., 2017).

1.4 Aptamers in the Diagnostics Platform

Aptamers holds vast potential in the diagnostics platform where some has achieved commercialisation. New England Biolabs uses a DNA aptamer as an inhibitor to their polymerase in their Hot Start *Taq* DNA polymerase (Cat No. M0496S and M0490S). Their *Taq* DNA polymerase is rendered active when the aptamer is released at higher temperatures thus catering for room temperature PCR component set up.

For the agro-food industry, Neoventures Biotechnology Inc. commercialized their product OTA-Sense which detects for the presence of mycotoxin, Ochratoxin A, at parts per billion quantities usually found in contaminated wheat products (Cruz-Aguado et al., 2008). The kit utilizes Terbium based-florescence which forms a cation bridge with the aptamer-ochratoxin complex and its florescence intensity is measured.

Aptamers have also been developed to cater for human disease diagnostics as well. Sekisui Diagnostics, GmbH released their product, OLIGOBIND (https://www.sekisuidiagnostics.com/products/722-thrombin-activity-assay) which features an aptamer-based enzyme-capture florescent assay to detect true levels of active thrombin *in vivo*. This is especially important to diagnose patient predisposed to bleeding complications. The kit achieved detected picomolar levels of thrombin and showed a 100-fold selectivity to thrombin than prothrombin (Konigsbrugge et al., 2017; Muller et al., 2011).

In an attempt for non-invasive cancer diagnosis, Li et al, (2017) has successfully isolated six aptamer candidates that distinguishes the serum between lung cancer

patients and healthy individuals using an aptamer-based PCR assay. The method utilises selection of aptamers from the magnetic beads-immobilized serum proteins from the lung cancer patients and negative selections with those of healthy individuals, thus giving rise to populations of aptamers specifically targeting the surface biomarkers present in the diseased serum (Li et al., 2017a).

Aptamers were also developed towards the pathological assessment of tissues. Ahirwar et al. (2016) demonstrated successful histochemistry and cytochemistry staining for Estrogen receptor alpha in breast tissues using their DNA aptamer . Zeng et al. (2010) demonstrated the detection of the CD30 biomarker in formalin-fixed paraffin embedded tissue sections using an RNA aptamer in a lower antigen retrieval temperature of 37 °C and a shorter incubation time of 20 minutes compared to the tested antibody which require 95 °C antigen retrieval temperature and a 90 minutes incubation time .

1.5 Aptamers in Therapeutics

The ligand binding property of aptamers provides potential antagonistic abilities that endows them with therapeutic potential. At the moment, one aptamer has successfully been commercialized (Kaur et al., 2018; Morita et al., 2018; Ng et al., 2006). Macugen is the only FDA approved aptamer that was developed for the treatment of age-related macular degeneration (AMD). The aptamer serves as an anti-angiogenic drug by antagonizing Vascular Endothelial Growth Factor (VEGF) 165 isoform (Ng et al., 2006). It is intravitreally injected into the eyes in a 0.3 mg dose once every six weeks and is approved for use in several countries including Australia, Brazil, Canada, Europe and the United States (Kaur et al., 2018).

Studies on Programmed Cell Death Protein 1 (PD-1) and Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) have elucidated their role in the eventual deactivation of T-cell response that limits their action against tumours (Freeman et al., 2000; Krummel et al., 1995). From this emerged a derivative of immunotherapy called T-cell checkpoint therapy which utilised monoclonal antibodies to antagonize these proteins that resulted in enhanced anti-tumour response (Sharma et al., 2015; Keir et al., 2008; Okazaki et al., 2007; Leach et al., 1996). The edge aptamer has over antibodies has sparked an interest to utilise aptamers as potential antagonists against these proteins (Huang et al., 2017; Lai et al., 2016). Lai et al. (2016) showed inhibition of PD-1 protein binding from its ligand PDL-1 using their aptamer which resulted in supressed *in vivo* tumour growth as well as a matching immune profile seen with monoclonal anti PD-1 antibody treatment, such as increased CD4+ and CD8+ infiltration with elevated levels of cytokines . Similarly, Huang et al. (2017) generated a CTLA-4 DNA aptamer antagonist which demonstrated identical potency as anti CTLA-4 monoclonal antibody in a mouse model with a lesser degree of weight loss in the mouse .

As molecular recognition elements, aptamers are also utilized in delivering therapeutic cargo to target cells. Gray et al. (2018) harnessed the intrinsic properties of aptamers by selecting aptamers capable of internalizing into prostate cancer cells without affecting the normal prostate cells . They demonstrated the potency of aptamer-based targeted chemotherapy by chemically conjugating cytotoxic agents to their aptamer and this tool was able to specifically kill malignant prostrate cells *in vitro* and reduced tumour growth *in vivo* (Gray et al., 2018). Additionally, they showed the unique capability to tune the cytotoxicity level using a nucleic acid ‘antidote’ that destabilizes the aptamer structure through complementary base pairing, thus deactivating its binding ability (Gray et al., 2018; Rusconi et al., 2004).

1.6 Pituitary Tumour Transforming Gene 1 (PTTG1)

Human Pituitary Tumour Transforming Gene 1 (PTTG1) is a proto-oncogene with an open reading frame of 609 bp that encodes for a 23 kDa protein (Kakar, 1999; Domínguez et al., 1998). Rat *PTTG1* mRNA from rat pituitary tumour cells was initially found to be highly expressed and the protein was capable of inducing cellular transformation both *in vitro* as well as *in vivo*. In 1998, the human homolog of rat PTTG1 was structurally identified and demonstrated evidence of transcriptional activity (Domínguez et al., 1998; Pei et al., 1997). Southern blot analysis revealed two additional gene homologues namely *PTTG2* (576 bp) with 91 % similarity and *PTTG3* (609 bp) with 89 % similarity to *PTTG1*. However, their expression levels are determined to be nil or very low with unclear significance nor roles determined yet for these homologues (Zhou, 2013; Chen et al., 2000). The expression level of PTTG1 is weak or undetected in normal cells such as pancreas, brain, lung, colon, and small intestine but was found to be high in actively proliferating organs such as the adult testis and thymus (Kakar, 1999; Zhang et al., 1999; Domínguez et al., 1998). PTTG1 was found to be localized in both nucleus and cytoplasm (Mu et al., 2003; Zhang et al., 1999; Domínguez et al., 1998).

PTTG1 is extremely hydrophilic and consists of an acidic transactivation domain at the C-terminus, a DNA binding domain and a basic N-terminus (Zhou, 2013; Kakar et al., 1999; Zhang et al., 1999) (Figure 1.2). The transactivation domain involves SH3-binding sites and together with the DNA binding domain, are responsible for PTTG1 to modulate cellular proliferation, transformation and exhibit transactivation effects (Boelaert et al., 2004; Pei, 2001). A destruction (D) box and KEN box, involved in the degradation of PTTG1, are situated in the N terminus (Zou et al., 1999). PTTG1 falls into the family of proteins categorized as intrinsically disordered proteins (IDP)

(Sánchez-Puig et al., 2005; Kakar et al., 2001). It is natively unfolded and devoid of a tertiary structure with a minimal secondary structure consisting of only a small amount of poly-(L-proline) type II helix (Chu et al., 2014; Csizmok et al., 2008; Sánchez-Puig et al., 2005).

Initially recognized as an oncoprotein, Zou et al. functionally identified PTTG1 to be linked to the cell cycle regulation as the Separase inhibitor or Securin (Zou et al., 1999). An upsurge of *PTTG1* mRNA expression during the cell cycle S phase that peaks at the S-G2 transitions and declines thereafter, suggests that PTTG1 adopts a cell cycle dependent expression (Vlotides et al., 2006). PTTG1 has been shown to lead towards the accumulation of inactive Separase at the nucleus which is in line with its role as the Separase inhibitor predominantly throughout the cell cycle (Han et al., 2013; Hornig et al., 2002; Zur, 2001; Nasmyth et al., 1999). At the onset of anaphase, PTTG1 is targeted for proteasomal degradation which allows Separase to create a loss of chromosome cohesion resulting in sister chromatids separation (Han et al., 2013; Zur, 2001; Nasmyth et al., 1999). Apart from that, PTTG1 was shown to have a role for the transition of cells into G1/S and G2/M phases by interaction with the transcription factor Sp1. In another study, it was found out that PTTG1 affects the stability of cyclin B, compelling the modulation of cell cycle timing into M phase in mouse oocytes (Marangos et al., 2008; Tong et al., 2007).

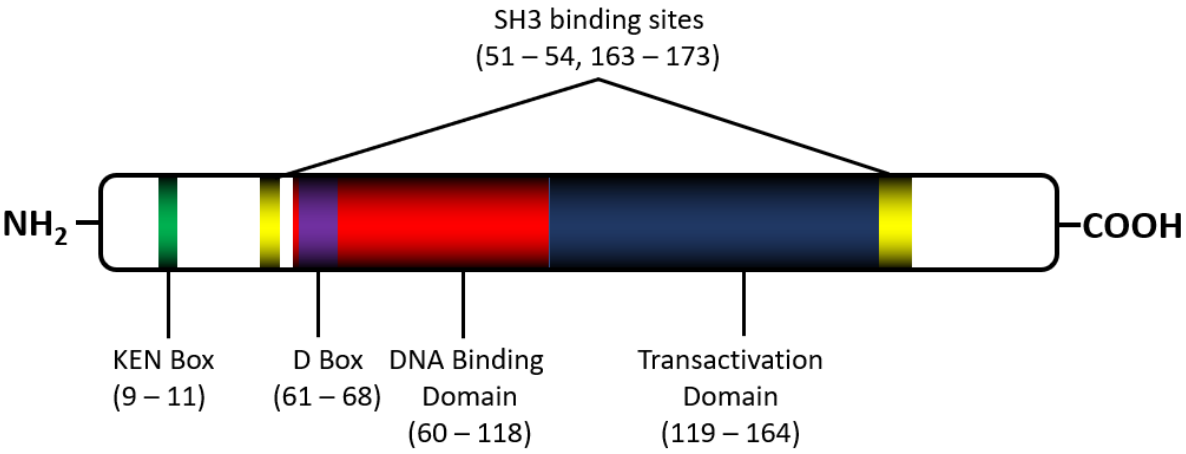

Figure 1.2 Schematic illustration of PTTG1 Protein Structure

PTTG1 consists of 202 amino acids. At the N-terminal, a KEN box is situated at the 9 to 11 aa while a D Box is situated at the aa residues 61 to 68. A DNA binding domain is present from aa residue 60 to 118 while the transactivation domain is located at the aa residue 119 to 164. SH3 binding sites were located at residues 51 to 54 and 164 to 173 (Tong et al., 2009; Tong et al., 2008; Vlotides et al., 2007).

1.7 PTTG1 as a Biomarker for Cancer Diagnostics and Prognostics

With its role in maintaining chromosome stability and its demonstration of cellular transformation effects, PTTG1 overexpression serves as a potential biomarker in the diagnosis of various cancers such as multiple myeloma, clear cell renal cell carcinoma, non-small cell lung cancer, hepatocellular carcinoma, breast cancer, gastric cancer, and adrenocortical carcinoma (Romero Arenas et al., 2018; Wang et al., 2016; Xiea et al., 2016; Xu et al., 2016; Noll et al., 2015; Wei et al., 2015; Fujii et al., 2006; Thompson et al., 2005; Jallepalli et al., 2001). Similarly, PTTG1 was also found to be overexpressed in various cancerous cell lines such as T lymphocyte cell Jurkat, colorectal carcinoma HT-29, HCT-116, SW480 and SW620, cervical carcinoma HeLa, breast carcinoma MCF-7, Burkitt's lymphoma Raji, and promyelocytic leukemia HL-60 (Ren et al., 2017; Kakar, 1999; Zhang et al., 1999).

Several studies have indicated that the level of PTTG1 overexpression resulted in poor prognosis that stems from increased aggressiveness in the tumour behaviour. In a study comprising 210 laryngeal carcinoma patients, 185 showed increased PTTG1 expression in the laryngeal cancer tissues when compared to their carcinoma-adjacent normal laryngeal tissues. The increased expression implied association with lymph node metastasis, tumour grade and level of malignancy (Ma et al., 2018). In colorectal cancer, PTTG1 overexpression serves as an indicator for invasiveness and unfavourable prognosis as the invasive cancer cells had high levels of PTTG1 and patients with these high PTTG1 expressing tumours had a lower overall survival rate (Ren et al., 2017; Heaney et al., 2000).

Ishitsuka et al. (2013) however found PTTG1 to be more significant as a proliferation marker in cutaneous squamous cell carcinoma. They found the levels of

PTTG1 expression does not correlate with the TNM staging, tumour thickness, clinical stage and high-risk clinicopathologic features but positively correlates with the level of expression for Ki67, a known proliferation marker in cutaneous squamous cell carcinomas (Ishitsuka et al., 2013).

Apart from the overexpression, the subcellular localization of PTTG1 may impart additional prognostic value. In a case study involving 470 breast cancer tissue specimens, Gurvits et al. (2016) found PTTG1 staining to be high and predominantly cytoplasmic in aggressive cases particularly triple-negative carcinomas . In addition, patients with sparse PTTG1 cytoplasmic staining survived 11.2 years after diagnosis however, for a majority of the patients with high PTTG1 cytoplasmic staining, the expected survival time was 4.2 years after diagnosis (Gurvits et al., 2016).

1.8 PTTG1 as a Target for Cancer Therapeutics

PTTG1 has been demonstrated to have potent contribution towards tumorigenicity. Genetic instability is one of the tumorigenic factors resulting from PTTG1 overexpression (Hsu et al., 2010; Jallepalli et al., 2001). As a securin, PTTG1 inactivates separase until the onset of anaphase where the latter is degraded. As a result of this degradation, Separase is liberated to promote sister chromatid separation. Cells with abnormal PTTG1 levels therefore demonstrated anomalies in their cell cycle regulation along with the occurrences of deformed chromosome masses, prolonged metaphase to anaphase transition interval and reduced levels of Separase (Hsu et al., 2010; Jallepalli et al., 2001).

The acidic C-terminal hinted the role of PTTG1 as a transcription factor upon experimentation by fusion with a heterologous DNA-binding domain (Domínguez et al., 1998). It was later shown that PTTG1 demonstrated transactivation activity and was

even described to exhibit properties as a “global transcription factor” due to its capability to bind to 746 gene promoters (Tong et al., 2007; Pei, 2001). In 2001, using DNA array and inducible PTTG1 expression on HeLaS3 cells, Pei found that PTTG1 overexpression rapidly induced the transcription of *c-myc* oncogene within 6 hours by directly interacting with its promoter region. The increase in *c-myc* expression resulted in increased cellular proliferation and colony formation (Pei, 2001).

Apart from that, PTTG1 was found to contribute to the metastasis of breast cancer by transactivation of CXCR2 (Ruan et al., 2012). The CXCR2/p21 senescence pathway was found to be activated upon direct transactivation of CXCR2 by PTTG1. Breast cancer cells with ectopically overexpressed PTTG1 that possessed a defective senescence mechanism had demonstrated an increase in metastasis. It was found that, if on the other hand, the senescence pathway is intact, PTTG1 overexpression leads the cells into senescence and restricted the oncogenic potential of PTTG1. Interestingly, these senescent cells were found to create an inflammatory reaction which recruited macrophages and in turn promoted the metastasis of surrounding non-senescent tumour cells (Chen, 2013). In this study, the interaction between PTTG1 and a CXCR2 enhancer sequence will be used as a model to probe the therapeutic potential of the aptamer.

p53 is a tumour suppressor protein which partakes physiological roles towards the suppression of cancer. In an attempt to study the tumorigenesis of PTTG1, Bernal et al. demonstrated that PTTG1 interacts with p53 and hindered its DNA binding ability *in vitro* and further confirmed this using the luciferase reporter assay. Furthermore, decreased transactivating action of p53-induced promoters (BAX, SFN, and CDKN1A) upon PTTG1 overexpression was found, which resulted in the decrease in p53-induced apoptosis of mouse fibroblast NIH3T3 cells (Bernal et al., 2002).

Knockdown of PTTG1 have been demonstrated to lead towards the suppression of cancer. Using PTTG1 targeting siRNA, Kakar et al. found that PTTG1 knockdown had reduced colony forming abilities in lung cancer cells *in vitro* and implantation of these cells *in vivo* demonstrated suppressive effects on the tumour growth in terms of a prolonged time for tumour formation and significantly reduced tumour mass (Kakar et al., 2006). Similarly, miRNAs targeting PTTG1 was shown to have inhibited cell viability and motility as well as induced apoptosis in tumour cells *in vitro* and *in vivo* (Liang et al., 2015; Wang et al., 2013).

1.9 Objectives of the Study

The generation of the aptamer can facilitate the development of aptamer-based diagnostic and therapeutic applications of PTTG1. Therefore, the objectives of this study are as follows:

i) To express and purify recombinant PTTG1

a) Construction of pET14-PTTG1 plasmid
b) Expression and purification of recombinant PTTG1
c) Protein identity confirmation with western blot assay using monoclonal antibody
d) Protein folding analysis to compare dialyzed and denatured PTTG1
e) Functional protein-protein interaction to confirm successful folding of PTTG1

ii) To generate RNA aptamer that has high binding affinity and specificity against recombinant PTTG1

a) Execution of the SELEX cycles to isolate RNA aptamer against recombinant PTTG1
b) Cloning and sequence analysis of aptamer pool
c) Binding analysis of the sequence classes using nitrocellulose filter binding assay to search for the RNA aptamer candidate
d) Dissociation constant and secondary structure determination of the candidate RNA aptamer against PTTG1
e) Interaction analysis between the RNA aptamer, monoclonal antibody and PTTG1 protein

iii) To analyse the diagnostic potentiality of the RNA aptamer

a) Development of Aptamer-linked immunosorbent assay (ALISA)

b) Development of Aptamer-based dot blot assay and aptamer-based western blot assay

c) Development of Aptahistostaining assay with MCF-7 and HeLa cell lines

iv) To study the potential functionality of the RNA aptamer towards therapeutic applications

a) To execute i*n vitro* competitive filter binding assay of PTTG1 and its known binding target, CXCR2 enhancer sequence and the RNA aptamer

b) To analyse the potentiality of the RNA aptamer to antagonize the interaction between PTTG1 and CXCR2 enhancer sequence

CHAPTER 2

EXPRESSION AND PURIFICATION OF RECOMBINANT PTTG1 FOR RNA APTAMER SELECTION

2.1 Introduction

PTTG1 is a 23 kDa intrinsically disordered oncoprotein that lacks a stable tertiary structure and contains minimal secondary structure (Sánchez-Puig et al., 2005; Kakar, 1999; Domínguez et al., 1998). PTTG1 is also alternatively known as human Securin, due to its role in the cell cycle regulation as an inhibitor of Separase (Gurvits et al., 2016; Castilla et al., 2014; Chu et al., 2014; Han et al., 2013; Moreno-Mateos et al., 2011; Zou et al., 1999). This oncoprotein has been implicated from its overexpression, onto the tumorigenicity of many cancers and as such is a highly potential cancer biomarker and therapeutic target (Romero Arenas et al., 2018; Ren et al., 2017; Wang et al., 2016). Development of compounds, peptides or ligands that can potentially antagonize PTTG1 is highly favourable, and this would require a highly purified protein. Thus, to generate an aptamer with high specificity and affinity to PTTG1 as well as for subsequent aptamer-based assays development, it is prudent and cost effective to produce the protein *in-house* which can cater to the relevant purity and yield requirements.

PTTG1 gene was cloned from a GST-tagged plasmid, which bears the gene, into a 6X His-tagged bacterial expression plasmid (pET14b). Subsequent to this, optimized expression and purification recombinant PTTG1 in a bacterial expression system was carried out. Western blot to identify and confirm the protein identity was performed. Protein folding analysis via intrinsic florescence and functional interaction using GST

pulldown assay with a known GST-fused PTTG1 binding partner, p53 was performed to ensure the purified PTTG1 is amenable for SELEX.

2.2 Materials and Methods

2.2.1 Isolation of *PTTG1* Gene via PCR Amplification

PTTG1 gene was isolated via PCR amplification from the pGEX-PTTG1 plasmid (A gift from Prof Ji-Hshiung Chen, Tzu Chi University, Taiwan) (Appendix B) using the forward primer PTTG1NdeI-F (5'-GAG Cca tat gGC TAC TCT GAT CTA TG-3') and the reverse primer PTTG1XhoI-R (5'-GAG Cct cga gTT AAA TAT CTA TGT CAC AG-3'). The forward primer and the reverse primer contain NdeI restriction site and XhoI restriction site, respectively (restriction sites are represented with small caps and underlined). PCR amplification was carried out in a 20 µL reaction mixture containing 1X PCR buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.08 % (v/v) Nonidet P40) (Fermentas, Burlington, Canada), 1.5 mM $MgCl_2$ (Promega Corporation, Madison, USA), 0.2 mM dNTPs (Promega Corporation, Madison, USA), 5 U of Taq DNA polymerase (Fermentas, Burlington, Canada), and 0.1 µM of each primers. The PCR was performed on the T-100 PCR thermocycler (Bio-Rad Laboratories, Hercules, USA) with parameters for PCR cycles as follow: an initial denaturation at 95 °C for 60 s followed by 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, 30 s extension at 72 °C and a final elongation of 120 s at 72 °C.

2.2.2 Agarose Electrophoresis

Following PCR, 5 µL of the PCR product was added with 1 µL of 6X Blue/Orange DNA loading dye (10 mM Tris-HCl pH 7.5, 50 mM EDTA pH 8.0, 0.4 % orange G, 0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 15 % Ficoll® 400) (Promega

Corporation, Madison, USA) which contained 0.5 µg/mL of ethidium bromide (Sigma-Aldrich, St. Louis, USA). The PCR product was run on a 2 % agarose gel (Promega Corporation, Madison, USA) using a mini gel apparatus (Bio-Rad Laboratories, California, USA) with 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 100 V for 30 min. The gel was visualized using the Gel Doc™ XR+ Gel documentation system (Bio-Rad Laboratories, California, USA). The amplicon size was confirmed with a 100 bp DNA ladder (Promega Corporation, Madison, USA).

2.2.3 Cloning of *PTTG1* Gene into TOPO TA cloning vector

The fresh PCR product was cloned into pCR@2.1 cloning vector using TOPO TA cloning kit (Thermo Fisher Scientific, Massachusetts, USA) as per manufacturer's protocol. The reaction mixture contains 2 µL of fresh PCR product, 1 µL of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$), 2 µL ddH_2O, and 1 µL of pCR@2.1 cloning vector. The mixture was incubated for 30 min at RT.

2.2.4 Transformation of TOPO-PTTG1 plasmid into *E.coli* TOP10 cells

Chemically competent *E.coli* TOP10 cells (Catalogue number: C404010) (Invitrogen, Carlsbad, USA) were thawed on ice. The prepared ligation mixture was incubated with the competent cells on ice for 30 min. The bacterial cells were then subjected to heat shock in a water bath (Fisher Scientific, Hampton, USA) at 42 °C for 45 s and immediately incubated on ice. One milliliter of Luria Bertani (LB) medium (Pronadisa, Madrid, Spain) was added and the cells were incubated at 37 °C with shaking at 200 rpm for 60 min (Thermo Scientific, Massachusetts, USA). Meanwhile, 50 µL of 40 mg/mL of X-Gal (Takara Bio, Shiga, Japan) and 50 µL of 100 mM IPTG (Fermentas, Burlington, Canada) were spreaded on LB agar plates (Pronadisa, Madrid, Spain) containing 50 µg/mL Ampicillin (Roche Diagnostics GmbH, Mannheim, Germany).

After incubation, the tubes were centrifuged for 2 min at 13000 x g (Thermo Scientific, Massachusetts, USA) and 1 mL of the LB broth supernatant was removed from the tube. The cell pellets were resuspended in the remaining medium (approximately 200 μL) and was plated onto the selection plates before incubation at 37 °C overnight.

2.2.5 Dual Restriction Enzyme Digestion of Vector and Insert

Reaction mixtures were prepared for the restriction enzyme digestion of pET14b vector (Merck KGaA, Darmstadt, Germany) and TOPO TA cloning vector carrying the *PTTG1* gene. The reaction mixtures contains 15 μL of water, 2 μL 10X FastDigest reaction buffer (Thermo Scientific, Massachusetts, USA), 5 U FastDigest NdeI enzyme (Thermo Scientific, Waltham, USA), 5 U FastDigest XhoI enzyme (Thermo Scientific, Massachusetts, USA) and 1000 ng of vector or 1000 ng of insert PTTG1 DNA fragment. The reaction mixture was incubated at 37 °C for 5 min.

2.2.6 Gel Purification of Digested Fragments

A few microliters of the digested fragments were mixed with 6X loading dye and subjected to agarose electrophoresis (2 % agarose gel for *PTTG1* gene fragment and 0.8 % agarose gel for pET14b plasmid) at 100 V for 30 min. The bands were visualized and excised under a UV transilluminator (Fisher Scientific, Massachusetts, USA). The DNA fragments were then purified using Roche High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. The excised gel was weighed in a 1.5 mL microcentrifuge tube and Binding Buffer was added accordingly. The mixtures were incubated in a heating block (Eppendorf, Hamburg, Germany) at 60 °C for 60 min. The contents were transferred into filter tubes housed within the collection tubes and centrifuged at 13000 x g for 2 min with the flow through subsequently discarded. Next, 700 μL of Wash buffer was

added and centrifuged at 13000 x g for 1 min. The flow through was discarded and the centrifugation was repeated once to remove the residual wash buffer. Hundred microliters of the elution buffer were added, and the tubes were centrifuged at 13000 x g for 1 min. The purified DNA was quantified using a spectrophotometer (Implen Inc, Westlake Village, USA).

2.2.7 Ligation of the Insert and pET14b Vector

The ligation reaction mixture was prepared which contains 1X T4 Ligation Buffer (Thermo Scientific, Massachusetts, USA), 5 U T4 DNA Ligase (Thermo Scientific, Massachusetts, USA), 200 ng vector DNA, 1000 ng insert DNA and topped up to 20 µL with ddH_2O. The reaction mixture was incubated overnight at 4 °C. The cells were transformed into chemically competent *E. coli* TOP10 cells by heat shock and subsequently cultured in a shaking incubator set at 200 rpm, 37 °C for 90 min. The cells were plated on a LB Agar plate containing 50 µg/mL Ampicillin (Roche Diagnostics GmbH, Mannheim, Germany). The plate was incubated overnight at 37 °C.

2.2.8 pET14-PTTG1 Plasmid Extraction

A single colony was picked and inoculated into 4 mL of LB broth containing 50 µg/mL Ampicillin. The culture was incubated overnight with shaking at 200 rpm, 37 °C. The overnight culture was centrifuged at 10000 x g and the supernatant was discarded. The pET14-PTTG1 plasmid was extracted using the Roche High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's protocol. The pellet was resuspended in 250 µL of Resuspension Buffer containing RNAse and incubated for 5 min at RT. Next, 250 µL of Lysis buffer was added and the mixture was gently mixed by inverting. It was then incubated for 5 min on ice before addition of 250 µL chilled Binding buffer. The reaction mixture was mixed gently by

inverting and centrifuged at 13000 x g for 15 min. The supernatant was transferred to the filter tube housed in the collection tube and centrifuged at 13000 x g for 1 min where the flow through was discarded thereafter. Next, 700 μL of Wash buffer was added and centrifuged at 13000 x g for 1 min. The tube was centrifuged again to remove residual wash buffer. 100 μL of elution buffer was added and the plasmid was eluted by centrifugation at 13000 x g for 1 min. The plasmid DNA was quantified using a spectrophotometer (Implen Inc, Westlake Village, USA).

2.2.9 Plasmid Sequencing of pET14-PTTG1

Sequencing of the plasmid (0.5 μg) was carried out using 0.5 μM of PTTG1NdeI-F primer by Apical Scientific Sdn Bhd.

2.2.10 Transformation of pET14-PTTG1 for Protein Expression

Rare codons were found on the *PTTG1* gene sequence which could affect the protein expression level due to codon bias (Refer Section 2.3.2 for detailed explanation). Therefore, Rosetta 2(DE3)pLysS strain was selected due to its possession of the pRARE2 plasmid which encodes for the rare tRNAs. On the other hand, the NiCo21(DE3) strain was used for expression to help in obtaining better purity as previous studies have demonstrated PTTG1 purification was often co-purified with high molecular weight proteins (Refer Section 2.3.3 for detailed explanation). For the transformation process, 1 μL of the purified pET14-PTTG1 plasmid was transformed into chemical competent Rosetta 2(DE3)pLysS cells (Catalogue number: 71403) (Merck KGaA, Darmstadt, Germany) (Genotype: F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE2 (Cam^R)) and NiCo21(DE3) cells (Catalogue number: C2529H) (Genotype: *can::CBD fhuA2 [lon] ompT gal (λ DE3) [dcm] arnA::CBD slyD::CBD glmS6Ala ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21*

Δ*nin5*) (New England Biolabs, Massachusetts, USA) by heat shock. The NiCo21(DE3) strain is a genetically engineered strain with improved IMAC purity to aid in protein purification due to co-purification of host protein issue in PTTG1 purification. The transformed cells were incubated in a shaking incubator set to 200 rpm at 37 °C for 90 min and 100 µL of the cell suspension were plated on a LB Agar plate containing 50 µg/mL Ampicillin (Roche Diagnostics GmbH, Mannheim, Germany) and 25 µg/mL Chloramphenicol (Merck KGaA, Darmstadt, Germany). The plate was incubated overnight at 37 °C.

2.2.11 PTTG1 Protein Expression via IPTG induction

Following transformation, a single colony was picked and inoculated into 4 mL LB broth containing 50 µg/mL Ampicillin and 25 µg/mL Chloramphenicol. The culture was incubated overnight with shaking at 200 rpm, 37 °C. One milliliter of the overnight culture was inoculated into 100 mL of LB broth containing 50 µg/mL Ampicillin and 25 µg/mL Chloramphenicol. Optical density at 600 nm (OD_{600}) of the culture was monitored using the Bio-photometer spectrophotometer (Eppendorf, Hamburg, Germany). Once the OD_{600} reached 0.6, a final concentration of 0.5 mM of IPTG was added to the culture and further incubated. For time course analysis of the protein expression, 1 mL of the culture was taken at one-hour intervals for a total of 20 hours and pelleted by centrifugation at 10000 x g for 1 min. The pellets were boiled in 5X reducing SDS Sample Buffer for 10 min and subjected to SDS PAGE electrophoresis.

2.2.12 SDS PAGE Electrophoresis and Coomassie Blue staining

Protein samples were re-suspended in 5X reducing SDS PAGE Sample Buffer (100 mM Tris-HCl pH 6.8, 10 % SDS, 50 % glycerol, 5 % Beta-mercaptoethanol, 0.05 % bromophenol blue) and heated for 10 min at 95 °C. Samples were resolved on 12 %

(w/v) polyacrylamide gel (Appendix L) at 100 V for 60 min in 1X SDS Running Buffer (25 mM Tris pH 8.3, 0.1 % SDS, 192 mM glycine). Subsequently, the gel was subjected to 0.1 % Coomassie Blue R250 staining solution (Bio-Rad Laboratories, California, USA) which contained 0.1 % Coomassie brilliant blue, 30 % methanol, 10 % acetic acid for 1 hour followed by overnight destaining in destaining solution which contained 30 % methanol and 10 % acetic acid.

2.2.13 PTTG1 Protein Purification

High molecular weight proteins were often co-purified with PTTG1 purification on a bacterial expression system as seen in other published works (Hsu et al., 2010; Chiriva-Internati et al., 2008; Csizmok et al., 2008; Kakar et al., 2001). Therefore, purification with the NiCo21(DE3) strain (genetically engineered strain for higher purity in IMAC purification) and a denaturing purification with urea was employed to aid in obtaining high purity PTTG1.

2.2.13(a) PTTG1 Protein Purification from Rosetta 2(DE3)pLysS cells

After protein expression via IPTG induction, the cells were pelleted by centrifugation at 10000 x g, 4 °C for 15 min (Kubota, Tokyo, Japan). The pellet was re-suspended in cold denaturing Binding Buffer (20 mM Tris pH 8.6, 8 M Urea, 150 mM NaCl, 10 % Glycerol, 1.5X Protease Inhibitor Cocktail EDTA-free) (Roche Diagnostics GmbH, Mannheim, Germany) and lysed by bead-beating (MP Biomedicals, California, USA) using 0.1 mm glass beads (BioSpec, Oklahoma, USA) for 15 s followed by chilling on ice for 30 s intervals for a total of 8 times. The lysate was centrifuged at 10000 x g for 10 min at 4 °C, and the supernatant was collected and re-suspended in chilled Binding Buffer containing pre-equilibrated Talon Resin (Clontech, California, USA) using a rotator for 60 min at 4 °C. The resin was washed five column volumes with cold