

Developing the p19 protein as a tool for studying the RNA silencing pathway

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Abstract

RNA silencing is a cellular mechanism of post-transcriptional gene regulation which is highly conserved among the plant and animal kingdoms of life, and plays a critical part of developmental biology, maintenance of homeostasis, and host-pathogen interactions. The pathway is engaged by small double-stranded (ds)RNA molecules (small RNAs), which effect sequence specific gene silencing by targeting complementary RNA sequences. There are several classes of small RNAs which engage the pathway. MicroRNAs (miRNAs) are expressed in the genome as endogenous regulators of gene expression. Short-interfering RNAs (siRNAs) are usually from exogenous sources such as viral-derived short-interfering RNAs, or synthetic siRNAs which are applied to cells or organisms to inhibit expression of specific genes.

The p19 protein is a viral suppressor of RNA silencing (VSR) endogenous to tombusviruses, which binds small RNA duplexes of any sequence with extremely high affinity. Because of its unique binding properties, recombinant p19 proteins are an excellent platform for tool development surrounding the RNA silencing pathway and are used extensively in novel applications for modulating the activity of small RNAs in living systems and for detecting small RNAs in biological samples. Herein we present work that has increased the breadth of p19's utility as a biotechnology tool in three distinct realms. First, we present a chemical biology approach which combines p19 and small molecules for potent inhibition of the RNA silencing pathway in human cells. Secondly, we present the development of a novel fusion protein between p19 and a cell penetrating peptide (CPP), which functions as an siRNA delivery agent to allow gene knockdown in human cells.

Thirdly, we have improved the utility of p19 for detecting and sequestering human miRNAs through rationally designing the binding surface; we describe mutations which dramatically enhance p19's affinity for human miRNA-122. The work presented here adds to the growing repertoire of engineered RNA binding proteins (RBPs) as tools for studying small RNA molecules and modulating their activity for applications in human therapeutics.

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Finally, with the words of M. Francois Jacob, I acknowledge the source for all that drives us,
in research and in life:

Ce qui guide l'esprit alors, ce n'est pas la logique. C'est l'instinct, l'intuition.

C'est le besoin d'y voir clair. C'est l'acharnement à vivre. C'est le courage.

It's a magical world, Hobbes, ol' buddy... Let's go exploring!

- *Bill Watterson, Calvin & Hobbes*

Table of Contents

Abstract	ii
Acknowledgements	iv
Table of Contents	vii
List of Abbreviations	xi
List of Figures	xiii
List of Tables	xiv
Chapter 1 - General Introduction	1
Preface	2
RNA silencing and antiviral defense.....	3
Viral Suppressors of RNA silencing	5
p19: molecular caliper for small RNA duplexes	8
p19 as a tool to probe small RNA pathways	9
Probing small RNA biology in plants	10
Probing small RNA biology in Drosophila	11
Probing small RNA biology in mammalian cells	11
Inhibiting RNA silencing: applications in biotechnology.....	13
p19 for small RNA detection	14
Modifying p19 for enhanced function.....	19
Rationale	20
Statement of objectives	20
Chapter 2 - Suppressing RNA silencing with small molecules and p19 the viral suppressor of RNA silencing	21
Preface	22
Abstract	23
Introduction	24
Materials and Methods	27
Protein expression & purification	27
siRNAs	27
Small molecules	27
Electrophoretic Mobility Shift Assays (EMSA)	28
Cell based assays	29
Results & Discussion	30
Examining small molecule effect on p19 binding siRNA	30

Reversibility of small molecule inhibition	33
Compatibility of suramin and p19 co-treatment in human cells	36
Conclusion.....	38
Acknowledgements	38
Chapter 3 - A novel p19 fusion protein as a delivery agent for short-interfering RNAs	39
Preface	40
Abstract	41
Introduction	42
Materials and Methods	45
DNA Oligonucleotides and siRNAs	45
Cloning 2x-p19-TAT fusion protein.	45
Protein Expression and Purification	46
Electrophoretic Mobility Shift Assay (EMSA) and Data Analysis	47
Cell Culture	48
Gene knockdown in human cell culture	48
Imaging delivery of fluorescently labelled siRNA	48
Luciferase assays.....	49
Cell viability assays.....	49
Results	50
Designing p19 as a siRNA delivery agent	50
p19 fusion protein binds siRNA with high affinity.....	51
p19 fusion protein delivers siRNA in human hepatoma cells.....	53
p19 fusion protein is a non-cytotoxic delivery agent	57
Enhancing delivery efficiency through endosomolysis	58
Discussion	60
Acknowledgements	64
Chapter 4 - Enhanced specificity of the viral suppressor of RNA silencing protein p19 toward sequestering of human miRNA-122	65
Preface	66
Abstract	67
Introduction	68
Materials and Methods	71
DNA Oligonucleotides and Fluorescently-Labelled siRNAs	71
Cell Culture	72
Plasmid Construction and Site-Directed Mutagenesis	72
Protein Expression and Purification	73

Electrophoretic Mobility Shift Assay (EMSA) and Data Analysis	74
Circular Dichroism (CD) and Thermal Melt Analysis.....	75
Transient Expression of His-Tagged p19-WT and p19-T111S in Huh7.5 Cells	75
Western Blot Analysis of Transient Expression of His-tagged p19-WT and p19-T111S in Huh7.5 cells.....	76
Quantification of miRNA Bound by His-tagged p19	77
Results & Discussion	78
Strategy for Mutational Analysis of p19 Binding with miR-122.....	78
Enhanced Affinity of p19-T111S for miR-122 <i>in vitro</i>	83
Mutational Analyses of the Thr111 p19 Residue.....	86
Structural and Thermal Analysis of p19-T111 Mutants with Circular Dichroism	87
p19-Y73S Mutant Incapable of Binding Small RNA	89
Enhanced Affinity of p19-T111S for miR-122 <i>in situ</i>	91
Conclusion.....	93
Acknowledgements	94
Chapter 5 - General Discussion and Future Directions	95
Modulating the RNA silencing pathway.....	96
Applying p19 in human cells	97
Small molecule modulators of RNA silencing.....	99
Engineering protein based siRNA delivery agents	101
Engineering p19 for enhanced affinity for human microRNAs.....	108
General Conclusion.....	112
References	113
Contribution of Collaborators	128
Appendices.....	129
Supplemental Information for Chapter 2	129
Supplemental Information for Chapter 4	130
Rights and Permissions	132
Curriculum Vitae	137

List of Abbreviations

AGO	Argonaute
ATA	Aurintricarboxylic acid
bp	Base pair
CRISPR	Clustered, regularly interspaced, short palindromic repeat
Cas	CRISPR-associated system
CD	Circular dichroism
CE	Capillary electrophoresis
CLIP	Crosslinking immunoprecipitation
CPP	Cell penetrating peptide
CIRV	Carnation Italian Ringspot virus
CSK	Carboxyl-terminal Src kinase
DCL	Dicer-Like enzymes
dsRNA	Double-stranded RNA
DRBD	Double-stranded RNA binding domain
DTT	Dithiothreitol
EMSA	Electrophoretic mobility shift assay
FRET	Forster Resonance Energy Transfer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HEK293	Human embryonic kidney cells
Huh7	Human hepatoma cells
HCV	Hepatitis C Virus
His8-tag	Octa-histidine tag

miRNA	Micro-RNA
nt	Nucleotide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
qPCR	Quantitative polymerase chain reaction
RBP	RNA binding protein
RISC	RNA-induced silencing complex
RNAi	RNA interference
siRNA	Short-interfering RNA
ssRNA	Single-stranded RNA
TBSV	Tomato Bushy Stunt virus
VSRS	Viral suppressor of RNA silencing
TAT	Transactivator of transcription
TAV	Tomato aspermy virus
TCV	Turnip Crinkle Virus
UV	Ultraviolet

List of Figures

Figure 1.1: The RNA silencing pathway and its suppression by the p19 protein of tombusviruses.	4
Figure 1.2: Diversity in structures and mechanism of dsRNA binding among viral suppressors of RNA silencing (VSRS) as determined by x-ray crystallography.	7
Figure 1.3. Applications of p19 in small RNA detection.	18
Figure 2.1 Inhibiting the RNA silencing pathway	26
Figure 2.2 Examining impact of small molecules on p19:siRNA interactions.....	32
Figure 2.3 Suramin and ATA are reversible inhibitors of p19:siRNA interaction	35
Figure 2.4 p19 and suramin co-treatment prevents siRNA-mediated knockdown of gene knockdown.....	37
Figure 3.1 Developing the 2x-p19-TAT fusion protein for siRNA delivery.	50
Figure 3.2 Assessing siRNA affinity of 2x-p19-TAT and control proteins by EMSA	52
Figure 3.3 Live-cell fluorescence imaging of 2x-p19-TAT delivery of Cy3-siRNA in Huh7 cells.	54
Figure 3.4 2x-p19-TAT allows cell entry and gene knockdown of firefly luciferase reporter gene in Huh7 cells.....	56
Figure 3.5 Cell viability assays	57
Figure 3.6 Promoting endosomal escape for enhanced gene knockdown	59
Figure 4.1 Depiction of the site-directed mutagenesis strategy for identifying p19 mutants with enhanced affinity for miR-122.....	71
Figure 4.2: Mutagenesis strategy for altering the p19 binding site based on structural predictions.....	82
Figure 4.3: Electrophoretic mobility shift assay of p19-small RNA interactions.....	84
Figure 4.4: Circular dichroism of p19-WT and p19-T111S	89
Figure 4.5: Schematic of the <i>in situ</i> miR-122 capture experiment	92
Figure S4.1: 12% SDS-PAGE analysis of purified p19-WT and various mutants.....	131
Figure S4.2: Circular dichroism of p19-WT and p19-Y73S.....	131

List of Tables

Table 2.1 Comparing the half maximal inhibitory concentrations (IC_{50}) of inhibitors of RISC and the VSRS protein p19.....	33
Table 4.1 Dissociation constants (K_d) of p19-WT and T111 mutants with small RNAs	86
Table S4.1 p19 mutant constructs and respective mutagenic primer pair used in site-directed mutagenesis.....	130
Table S4.2 Dissociation constants (K_d) of p19-WT and mutants with small RNAs	130

Chapter 1 - General Introduction

Preface

The following introduction serves as a broad introduction to the RNA silencing pathway, VSRS, and the broad applications of the p19 protein *in vitro* and in living systems. The information herein contextualizes the research progress presented in the following chapters. Portions of this introduction, including text and figures, have been adapted from a review article published in *FEBS Letters* entitled “Studying the RNA silencing pathway with the p19 protein” and authored by D.C. Danielson and J.P. Pezacki (PMID: 23376479). It is reproduced in this thesis with permission from the publishers - refer to **Rights and Permissions**. As first author of this publication, I made significant intellectual contributions to this review, wrote the first draft of the manuscript and designed the figures, which was then edited by myself and J.P. Pezacki.

RNA silencing and antiviral defense

The discovery that double-stranded RNA (dsRNA) in the cell elicits sequence-specific inhibition of gene expression through the RNA silencing pathway has revolutionized our understanding of endogenous gene regulation and host-pathogen interactions [1]. In this system, endogenous and exogenous dsRNA precursors are the substrates of RNase III enzymes in the Dicer family, which generate small RNAs 21-24 nucleotides (nt) in length. miRNAs are endogenous small RNAs with nuclear precursors that are processed in the cytoplasm [1]. miRNAs are critical in eukaryotic post-transcriptional gene regulation of nearly all cellular processes and their aberrant expression is linked to several disease states in humans, including cancer [2]. In plants and invertebrates, this pathway serves as a critical defense against invading viruses, and there is increasing evidence that this pathway has antiviral roles in vertebrates as well [3,4] (**Figure 1.1**).

Plants express several Dicer-Like (DCL) enzymes with specialized functions and DCL4 is the primary enzyme responsible for cleaving dsRNA intermediates or fold-back structures in the viral genome, generating virus-derived siRNAs 21-nt long with 3' 2-nt overhangs and 5' phosphates [5-7]. Small RNAs are the effector molecules of the pathway and are incorporated into a RNA-induced silencing complex (RISC) through interactions with members of the Argonaute (AGO) protein family. Plants express a diversity of AGO proteins, but viral-derived siRNAs are predominantly incorporated into AGO-1 or AGO-2 to form the anti-viral RISC effector complex [8-10]. The small RNA-loaded RISC allows sequence specific gene silencing of the target mRNA, primarily through mRNA degradation or inhibition of translation [11,12].

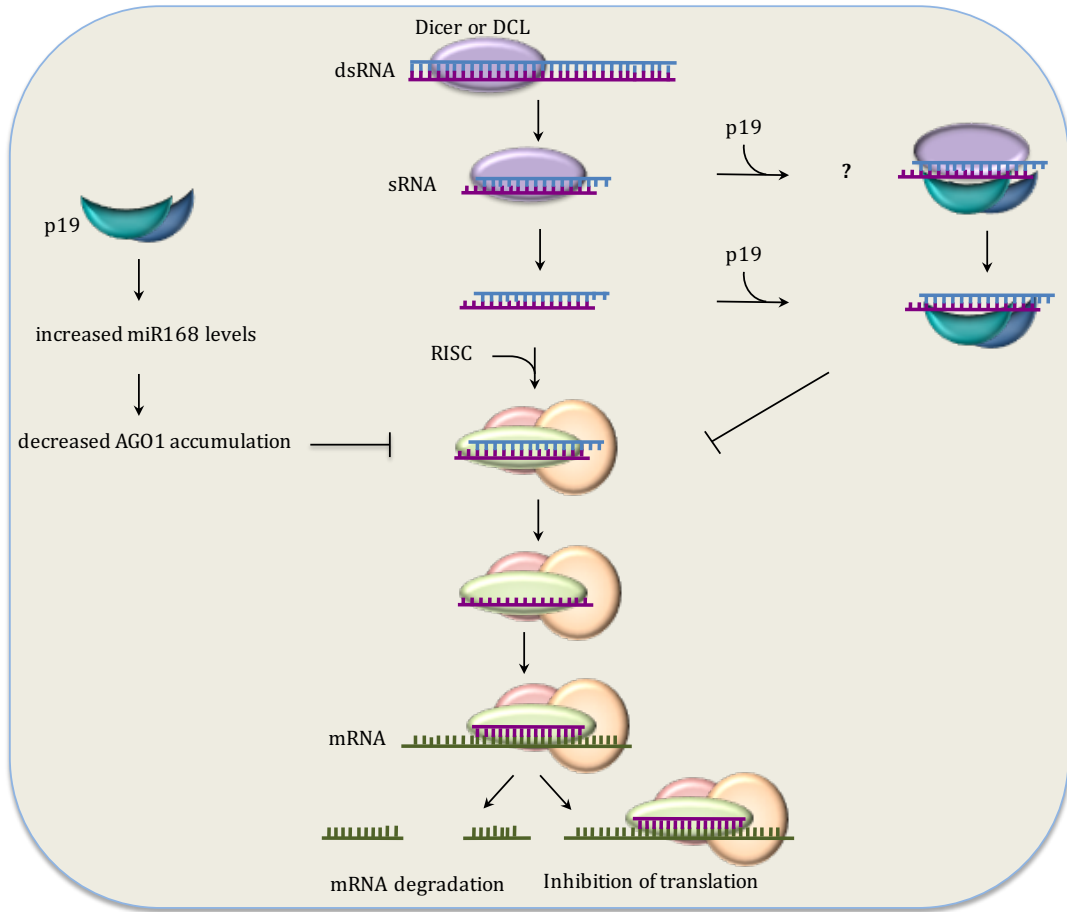


Figure 1.1: The RNA silencing pathway and its suppression by the p19 protein of tombusviruses.

Dicer or Dicer-like (DCL) enzymes cleave double-stranded RNA (dsRNA) into small RNA duplexes 21-24 nucleotides long, which are then incorporated into the RNA-induced silencing complex (RISC), which uses one strand of the duplex to elicit sequence-specific gene knockdown through mRNA degradation or inhibition of translation [12]. p19 is a viral suppressor of RNA silencing, which functions by sequestering viral-derived small RNAs and preventing their incorporation into the RISC complex [13]. Kinetics studies suggest a mechanism of suppression involving a ternary complex between Dicer, small RNA and p19 [14]. p19 also functions to suppress the pathway by preventing the accumulation of AGO-1, a component of anti-viral RISC, by inducing the accumulation of a host miRNA, miR168, which then downregulates AGO-1 expression (left) [15,16].

Viral Suppressors of RNA silencing

To counter the RNA silencing pathway, all plant viruses and several insect and mammalian viruses have evolved VSRS [11,17,18]. VSRS from different viral families have extraordinary diversity in sequence and structure and employ distinct mechanisms to interfere with the host RNA silencing pathway, pointing to their independent evolution [18,19]. Functional annotation of viral genes as VSRS during infection has lent substantial insight into the mechanisms and significance of the RNA silencing pathway in viral defense [11].

Mechanisms of suppression of RNA silencing vary greatly among VSRS. Some VSRS interfere directly with the silencing machinery of the host plant [20], such as the p38 protein of Turnip Crinkle Virus (TCV) and the 2b protein of Cucumber mosaic virus, which both function by interfering with AGO-1-mediated gene silencing, p38 through GW motifs that serve as an AGO 'hook' [21,22] and 2b through interactions with the PAZ domain [10]. Through interactions with AGO-1, they prevent loading of an active RISC complex. Several VSRS interact with dsRNA, either binding long dsRNA to prevent Dicer-mediated cleavage (e.g. Flockhouse Virus B2 protein [23]) or binding siRNAs to prevent active-RISC formation (e.g. p19 protein of tombusviruses [24,25]). Structural analyses have demonstrated diversity in dsRNA binding strategies by these proteins, three of which have been determined by x-ray crystallography in complex with dsRNA, lending insight into the biochemical basis for their ligand specificities and affinities (**Figure 1.2**).

It has been suggested that sequestering small RNAs is a general strategy of VSRS to suppress the pathway [26,27], however it is becoming increasingly evident that *in vitro* small

RNA binding activity does not always contribute to suppressor activity and may be important for other activities of the protein unrelated to VSRS activity. p19's high affinity for small RNA is critical to its function in viral pathogenesis, since point mutations abrogating binding eliminates its silencing activity *in planta* [28-30]. On the other hand, Turnip Crinkle Virus p38 binds dsRNA of variable length [26], but its suppressor activity was completely abrogated by mutating two GW residues responsible for its interaction with AGO-1 [21]. Furthermore, p38 is unlikely to function by small RNA sequestration since its transgenic expression does not prevent the mobility of siRNAs into neighbouring cells, in contrast to what is observed for p19 from tombusviruses and p21 from the Beet Yellow Virus [31].

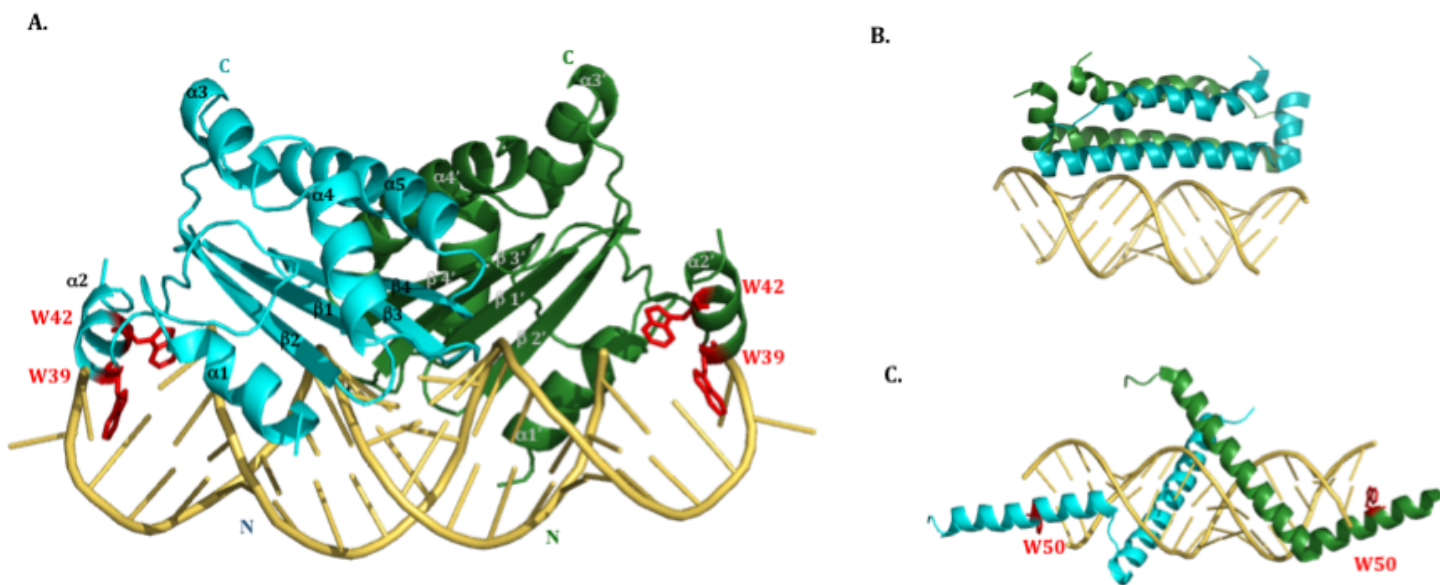


Figure 1.2: Diversity in structures and mechanism of dsRNA binding among viral suppressors of RNA silencing (VSRS) as determined by x-ray crystallography.

(A) Carnation Italian Ringspot Virus (CIRV) p19 in complex with a 21-nt siRNA demonstrates its sequence-independent and size-specific binding of small RNAs, where it functions as a dimer to form a 8-stranded β -sheet binding surface that interacts with one face of the small RNA backbone and N-terminal helices that clasp the ends of the small RNA duplex, with end-capping interactions provided by conserved tryptophan residues (W39 and W42, highlighted in red) (PDB: 1RPU) [28]. Tomato Bushy Stunt Virus (TBSV) p19 in complex with siRNA shows a nearly identical binding strategy (PDB: 19RF) [32]. (B) Flockhouse virus (FHV) B2 protein in complex with an 18-bp dsRNA demonstrates its sequence and length-independent binding of dsRNA, where it functions as a dimer to form a four-helix bundle that interacts with one-face of the sugar-phosphate backbone (PDB: 2AZ70) [23]. (C) Tomato aspermy virus (TAV) 2b protein (1-69) in complex with a 19 bp siRNA demonstrates its sequence-independent binding of dsRNA, where it functions as a dimer to fit its α -helical protein backbones into the major groove of the RNA duplex (PDB: 2ZI0) [33]. TAV 2b preferentially binds dsRNA, with some preference for duplex length conferred by tryptophan stacking interactions with siRNAs (W50, highlighted in red) but also binds long dsRNA with high affinity and dsDNA with weak affinity.

p19: molecular caliper for small RNA duplexes

The p19 protein is expressed by tombusviruses, positive-sense single-stranded RNA viruses, at high levels during infection to allow systemic invasion of the host plant [13]. It is encoded in the 3' proximal open reading frame (ORF) p19, which is embedded in another ORF that encodes a 22-kDa cell-to-cell movement protein. [34,35]. p19 is the most well studied VSRS and its mechanism of suppression is quite unique; it sequesters small RNAs and prevents their incorporation into RISC (**Figure 1**) [24,25]. p19 is in competition with AGO proteins for small RNAs but it can not de-program an already loaded RISC complex [25]. It appears from kinetics studies that p19 efficiently competes for small RNAs through a multiple turnover mechanism, as it exhibits highly reversible binding with a very high dissociation rate constant ($k_{\text{off}} = 0.062 \pm 0.002 \text{ s}^{-1}$) and modeling studies suggest that p19 may intercept RISC formation by forming a ternary complex with Dicer [14].

Delineating p19's biochemical properties *in vitro* have been instrumental to its application as a tool for probing the RNA silencing pathway. The reported crystal structures of p19 from both Tomato Bushy Stunt Virus (TBSV) and Carnation Italian Ringspot Virus (CIRV) in complex with a 21-nt small RNA (**Figure 1.2A**) [28,32] demonstrate how p19 functions as a 'molecular caliper' to bind the small RNA according to the length of the duplex. p19 binds the small RNA as a dimer with N-terminal helices that clasp the edges of the duplex and provide stacking interactions between tryptophan residues and the terminal nucleotides, thereby measuring the length of the duplex. p19 exhibits sequence-independent binding, as the binding surface consists of a continuous 8-stranded β -sheet that forms a concave binding surface, interacting with the small RNA backbone through hydrogen bonding and electrostatic interactions.

p19 evolved to have the highest affinity for the virus-derived siRNA products of the DCL4 enzyme, as this is the main enzyme involved in plant antiviral defense [5,7]. p19 exhibits the highest affinity reported to date of a protein for siRNA, with a dissociation constant of approximately 0.2 nM [14,28,36]. It exhibits drastically reduced affinity for siRNAs longer or shorter than 20-22 nt long, does not bind single-stranded RNA or DNA, and requires 5' phosphates for high affinity binding but does not require 3'-2 nt overhangs [28]. p19 also binds miRNAs (~ 23 nt) with high affinity both *in vivo* and *in vitro* [36-39]. Interestingly, p19 also suppresses RNA silencing by another mechanism unrelated to sequestration of small RNAs; in plants p19 expression induces the expression of a host miRNA, miR168, which reduces levels of AGO-1, thereby inhibiting anti-viral RISC formation [15,16].

p19 as a tool to probe small RNA pathways

p19 has been broadly used as an effective suppresser of RNA silencing through expression in heterologous systems, including non-endogenous host plants [31], *Drosophila* [39], *Drosophila* embryo extracts [25], as well as human cells [30]. Furthermore, its ability to sequester small RNAs in a variety of systems make it an ideal probe for examining the role these molecules play in diverse biological processes, not limited to host-pathogen interactions. Herein we discuss its application as a probe for small RNAs in diverse systems as well as the applications of purified, recombinant p19 proteins in small RNA detection. Furthermore, we discuss how p19 is amenable to modifications including protein fusions and altered binding specificity, which provide novel properties and invite further applications of p19 in biotechnology and therapy.

Probing small RNA biology in plants

Transgenic expression of p19 has allowed interrogation of small RNA biology in the model plant *Arabidopsis thaliana*. Examining small RNA accumulation in plants transgenically expressing cytoplasmic and nuclear variants of p19 gave initial evidence for nuclear processing of small RNA precursors, as nuclear p19 expression repressed the accumulation of 21-nt siRNAs and a 21-nt miRNA [40]. The details of the siRNA and miRNA pathways in *A. thaliana* have been teased apart by applying a ‘molecular toolbox’ approach, where the diverse properties of VSRS allow interrogation of the RNA silencing pathway at different points. Directly comparing the effect of HcPro, p19, p15 and P38 on siRNA- and miRNA-loading of AGO-1 demonstrated that although all VSRS prevented siRNA loading, only p19 prevented miRNA loading into AGO-1, demonstrating its unique ability to sequester both siRNAs and miRNAs *in vivo*. Interestingly, even though TCV p38 interferes directly with AGO-1, it appears to only prevent siRNA loading without affecting miRNA loading, suggesting that there are two distinct cellular pools of AGO-1 that are loaded with siRNA or miRNA and are differentially targeted by VSRS. By employing VSRS, this study uncovers another layer of complexity to these intertwined but functionally distinct pathways [31].

Probing small RNA biology in Drosophila

p19 has been an effective tool in interrogating the RNA silencing pathway in *Drosophila*. It is observed that constitutively expressing cytoplasmic p19 in living flies impairs hairpin-induced RNAi without altering the endogenous miRNA pathway [41]. Furthermore, this study effectively employs the ‘molecular toolbox’ approach to investigate the contribution of RNA silencing via endogenous siRNAs to heterochromatin formation in *Drosophila*; abrogating the pathway through transiently expressing p19 and FHV B2 in S2 cells. It was observed that expressing p19 in the nucleus via a fused nuclear localization signal (NLS-p19) suppressed silencing of heterochromatin gene markers in adult flies, implicating nuclear small RNAs in regulating heterochromatin silencing. Examining the small RNAs bound by p19 through immunoprecipitation determined that there are abundant endogenous siRNAs (endo-siRNAs) in S2 cells derived from transposable elements. Comparing the small RNA immunoprecipitates of cytoplasmic p19 versus NLS-p19 demonstrates the existence of an abundance of cytoplasmic endo-siRNAs in S2 cells as well as moderately abundant nuclear endo-siRNAs, and further suggests that a portion of the cytoplasmic endo-siRNAs are translocated back into the nucleus to elicit chromatin silencing [41].

Probing small RNA biology in mammalian cells

p19 has been used to explore the role of RNA silencing in the defense against the retrovirus Primate Foamy Virus type 1 (PFV). Expressing p19 in human 293T immortalized kidney cells resulted in a marked increase in viral accumulation, suggesting that small RNAs were repressing viral replication. It was subsequently shown that an endogenous miRNA mediates the antiviral defense [42]. p19 was also used to probe the small RNA milieu of mouse

embryonic stem cells through immunoprecipitations of transiently expressed cytoplasmic and nuclear p19 with subsequent small RNA cloning [43]. This study demonstrated that mouse ES cells do not have abundant endogenous siRNAs. Additionally, p19 expression in these cells did not lead to an accumulation of miRNA passenger strands in cell extracts, nor were they detected in the p19 immunoprecipitates. This is in contrast to what has been observed in plants, suggesting that p19 did not have access to mature miRNAs in ES cells [37,44]. Interestingly, it was found that in the absence of siRNAs, p19 is capable of binding non-canonical ligands in which a short RNA species annealed to a longer species (32-35 nt) such that the dsRNA region is 19 base pair (bp) long. The affinity of p19 for these species was reduced 5-10 fold as compared to canonical siRNA ligands, but still exhibits nanomolar affinity. Notably, this study also observes that expressing p19 in HEK293T cells does not prevent exogenous siRNA-mediated gene knockdown.

It appears that p19's ability to suppress RNA silencing in human cells is not universal. The results in HEK293T cells [43] are consistent with another study where transient expression of p19 in HEK293T cells did not show an increase in HIV-1 production, although stable expression of p19 resulted in a modest increase [45]. These findings are in contrast to the increased PFV viral accumulation upon p19 expression in HEK293T cells [42]. We have observed that p19 only has a modest ability to suppress RNA silencing in human hepatoma cells (Huh7), possibly due to high levels of endogenous miR-122 that also acts as a ligand for p19, which is why we explored combining p19 expression with a small molecule approach to inhibiting the pathway in **Chapter 2**. This inconsistency in human cells is likely due to cell-type specific factors that alter p19's function, which have yet to be fully understood and are further discussed in **Chapter 5**.

Inhibiting RNA silencing: applications in biotechnology

Just as the RNA silencing pathway is engaged in response to viral infection, it is also engaged in response to ectopically expressed genes, which are introduced to plants either through viral vectors or agro-infiltration of transfer-DNA. This poses a significant hurdle to expression of foreign genes in plants for either research or biotechnology applications, given that only transient expression (1-3 days) can be maintained [46]. Given that plants are increasingly being applied as vectors for recombinant protein production, especially for therapeutic antibodies, methods allowing long-term gene expression and increased protein yields are being heavily investigated. One commonly applied strategy is to transiently co-express p19 with a foreign gene of interest, which results in a substantial (~25 fold) increase in protein production [46]. This approach has increased the production of therapeutic antibodies [47,48] and for expressing antigens for vaccine development, such as HIV and SARS antigens [49,50].

Insect cells, in particular Sf9 cells, are increasingly important vectors for recombinant protein production both in applications in research and for vaccine development [51]. The most potent vector for gene expression in insect cells is the Baculovirus expression system, but similar to the problem discussed in plants, the engagement of the RNA silencing pathway prevents long-term viral expression and prevents high yields of protein production. It was recently reported that co-expression of p19 increased baculovirus titers ~7 fold and doubled protein expression in Sf9 cells [52]. Thus the widespread use of p19 in plant systems for enhancing foreign gene expression may soon find wider application also in insect cell systems.

In human cells, broadly inhibiting the RNA silencing pathway with p19 is finding applications in gene therapy. Integrating non-viral vectors based on transposable elements is researched heavily as gene therapy approach and has been important in functional genomics studies. Successful integration of DNA transposons has been shown to be inhibited by the RNA silencing pathway, and co-expressing p19 resulted in enhanced integration of the Sleeping Beauty transposase system [53]. Similarly, p19 co-expression was found to dramatically enhance adenoviral titers in human cells (~100-fold) for oncolytic treatments [54].

p19 for small RNA detection

miRNAs are potent endogenous regulators of gene expression, and small changes in their expression patterns have drastic biological outcomes; dysregulated expression of specific miRNAs are linked to several disease states [55]. Thus, highly sensitive detection of the relative amounts of specific miRNAs are required to understand the mechanisms of human disease and for their annotation as diagnostic biomarkers. This is true both for expression patterns in cells and tissues and also in extracellular biological fluids. miRNAs have been detected in stable forms in biological fluids such as human serum and urine, and their relative amounts are being heavily researched as for their diagnostic potential as disease biomarkers [56,57]. Extracellular miRNAs can be circulating as protein:RNA complexes in association with AGO proteins [58] or present in extracellular vesicles such as exosomes, either as protein-RNA complexes or naked RNAs [59].

Typical strategies for miRNA detection and quantification include microarrays, RT-PCR, northern blotting, and next generation sequencing of small RNAs [60]. These techniques,

however, come with several limitations and there is a critical need to develop novel techniques for miRNA detection which combine high sensitivity and amenability to point-of-care diagnostics [61]. Accurate detection and quantification of the relative or absolute amounts of specific miRNAs poses several challenges, both for cellular miRNAs and extracellular miRNAs. The combination of a wide dynamic range of copy numbers, highly similar sequences between miRNAs of the same family, and a low concentration in biofluids (10 fM to 1 pM) pose significant challenges especially for point-of-care diagnostic detection. Applying recombinant p19 proteins to aid in miRNA detection allows for a creative solution to these challenges, which benefit from its unique small RNA binding characteristics; namely its high affinity for small RNAs and its size specificity. Importantly, recombinant p19 proteins are highly amenable to overexpression in bacteria with high soluble yields and the proteins ability to bind small RNAs is not hampered by genetic fusions or chemical conjugations on both the N or C terminus [25,43,62].

p19 has been used as part of an surface-plasmon resonance (SPR) based approach for quantifying miRNA-122 from small RNA isolates of human hepatoma cells, where RNA probes are immobilized on gold surfaces and p19 detects the miRNA-bound probe [63]. This represents a unique strategy that provides a simple alternative to previous SPR-miRNA detection methods that require enzymatic surface modification and gold nanoparticle labels for signal amplification [64]. p19 has also aided miRNA detection as part of a rapid enzyme-linked immunoassay approach which exhibits femtomole sensitivity [63] (**Figure 3A**). This method uses RNA probes conjugated to magnetic microbeads to capture the target miRNA, which is then bound by p19 allowing rapid detection through horseradish-peroxidase linked antibody detection of p19. This method achieves high sensitivity of miRNA detection

without the inherent biases that exist in PCR-based amplification [65].

Magnetic beads with p19 proteins chemically conjugated are commercially available to pull down and concentrate small RNAs from dilute samples (New England Biolabs) (**Figure 3B**) [66]. Detection of specific miRNAs in biological samples such as human serum is possible through applying a sequence specific probe to target a mature miRNA strand and subsequently applying p19 to bind the probe:miRNA duplex. Because low concentration is also the biggest hurdle for accurate detection, this is proving to be a useful strategy for enhancing detection without the need for qPCR based amplification. This approach was applied to enhance miRNA detection in human serum in a technique termed protein-facilitated affinity capillary electrophoresis (proFACE) [67]. The addition of p19 allowed enhanced separation of the of the single-stranded probe and the p19-bound probe:miRNA duplex, allowing very sensitive detection (0.5 fM or 30 000 miRNA molecules per ml) without requiring PCR amplification [67] (**Figure 3B**). p19 has also been applied to fluorescent detection of small RNAs, where a fusion of a linked p19 dimer and a cyan fluorescent protein (CFP) allows reporting on binding of fluorescently labeled siRNA through Förster resonance energy transfer (FRET) [62] (**Figure 3C**). These results suggest the possibility of developing p19 as an intramolecular FRET probe to allow sensing of unlabeled siRNA, which would allow label-free reporting on p19 binding small RNA. This is a strategy that our lab has been exploring. Electrochemical biosensors allowing direct detection of miRNAs are showing great promise towards applicability for diagnostics [61]. A range of approaches have recently been described which rely on p19's ability to bind to probe:miRNA duplexes with high affinity and selectivity over other RNA and DNA

sequences, thereby achieving enhanced sensitivity and dynamic range than previously possible [68-73].

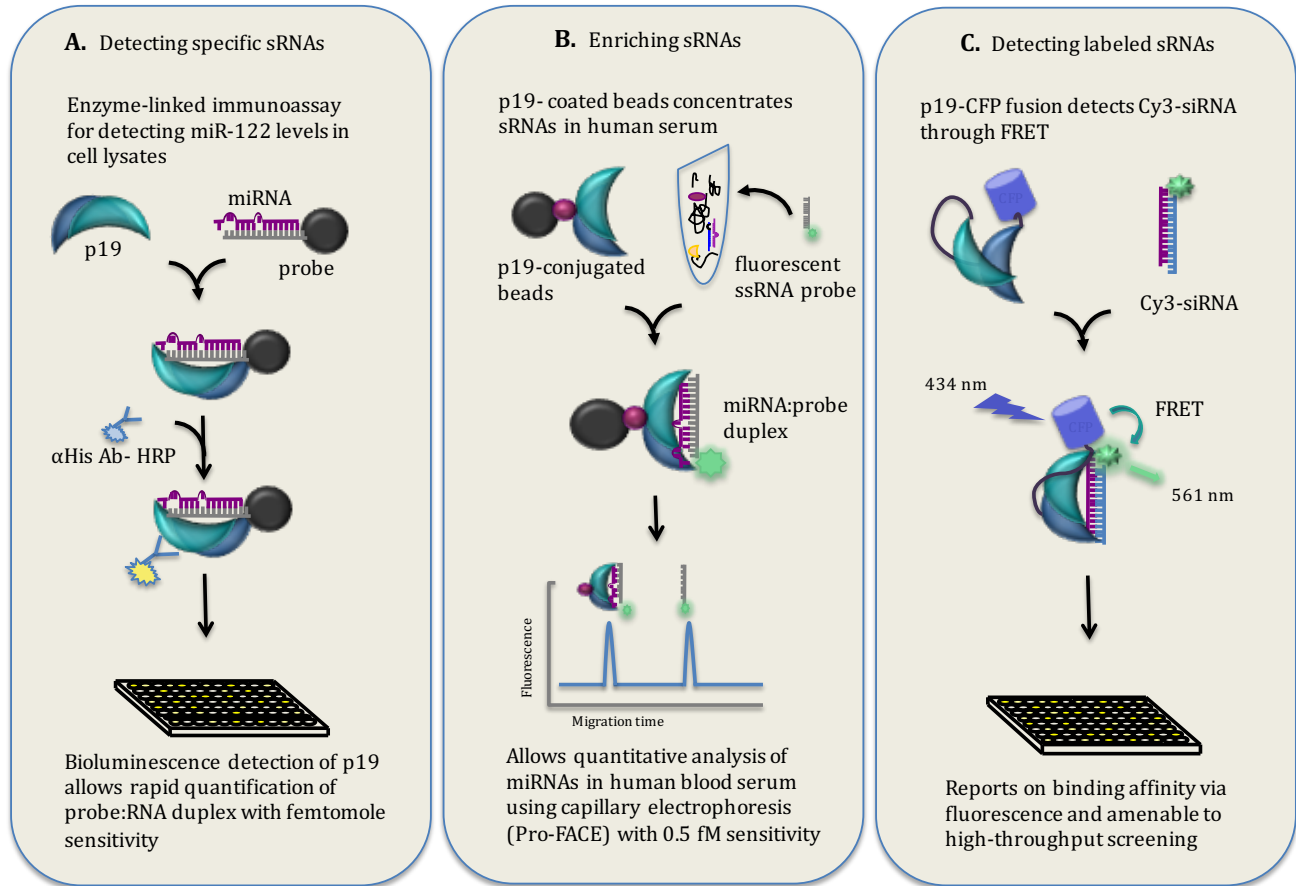


Figure 1.3. Applications of p19 in small RNA detection.

(A) p19 binds probe:miRNA duplexes for quantifying levels of specific miRNAs in biological samples using bioluminescence detection of p19 [63]. (B) p19 fusion protein with N-terminal maltose-binding protein and C-terminal chitin binding domain (MBP-p19-CBD) allows enrichment of small RNAs using chitin magnetic beads (New England Biolabs). Sensitive and quantitative detection of specific miRNAs in human serum by capillary electrophoresis (CE) is attained through pre-concentrating miRNA:probe duplexes using p19-coated beads as well as CE stacking, resulting in 1000-fold enrichment of miRNAs and detection as low as 0.5 fM miRNA without PCR-based amplification [67]. p19 further aids the analysis by enhancing separation of the probe:miRNA duplexes from the excess, unbound probe, a technique termed ProFACE (protein-facilitated CE). (C) A C-terminal cyan-fluorescent protein (CFP)-p19 fusion protein detects fluorophore (DY547) labeled siRNA through Forster resonance energy transfer (FRET), allowing spectrophotometric detection of binding and determination of binding affinity which is amenable to high-throughput screening applications [62].

Modifying p19 for enhanced function

The breadth of scenarios discussed above where recombinant p19 proteins are being applied both for *in vitro* detection of small RNAs and as an inhibitor of RNA silencing in living systems, point to the utility of this molecular caliper. All of these applications benefit from deep understanding of p19s biochemical and biophysical properties, especially in regards to its ability to bind human miRNAs, as well as engineering it for enhanced function.

Recombinant CIRV p19 has been modified for enhanced function through protein engineering for applications in biotechnology and human therapy. Linking the two monomers through a semi-rigid linker (GGGS)₂ between the N-terminus of one monomer and the C-terminus of the other monomer enhances the thermal stability of the construct and its affinity for siRNA 3.5 fold over wild type p19 [74]. Having a linked p19 dimer construct facilitates its use in biotechnology and has been used for quantitative miRNA detection using ProFACE [67]. Mutagenesis studies exploring the importance of cysteine residues in the p19 protein led to the observation that mutating three cysteine residues (C110, C134 and C160) to isoleucine results in improved solubility properties, precluding the need for reducing agents during purification and storage, as is required for purification of wild type p19 proteins to prevent aggregation (unpublished results) [75]. Novel applications of mutant p19 proteins are forthcoming, as there is evidence that mutations in its binding site can alter its binding specificity for certain miRNAs (**Chapter 4**).

Rationale

Understanding the mechanisms of the RNA silencing pathway is essential for research into human disease states, among a multitude of other areas, and is prerequisite to its manipulation for desirable outcomes in therapy and biotechnology. The evolutionary arms race between hosts and pathogens has given us a unique set of proteins that potently abrogate RNA silencing and whose characterization has produced fundamental knowledge of this pathway in diverse organisms. The greatest benefit will come now from expanding this repertoire through protein engineering of these VSRS. The tombusviral p19 protein has been demonstrated to be a unique tool for small RNA sequestration in living systems and *in vitro*, and protein engineering efforts will likely lead to fine-tuning of its ligand specificity and biological effects, thus inviting broader application.

Statement of objectives

The goals of my thesis work have been to develop tools surrounding the p19 protein for applications in sequestering and delivering small RNAs *in vitro* and *in vivo*. **Chapter 2** examines an approach combining p19 and a small molecule inhibitor of the pathway for potent inhibition of the pathway in human cells. **Chapter 3** examines engineering the p19 protein as a delivery agent for siRNAs allowing cell entry of the protein:RNA complex and subsequent gene knockdown. **Chapter 4** examines engineering the binding site of p19 for enhanced affinity for a non-canonical ligand, miR-122.

**Chapter 2 - Suppressing RNA silencing with small molecules and
p19 the viral suppressor of RNA silencing**

Preface

This chapter consists of data previously published in *Biochemical and Biophysical Research Communications* entitled “Suppressing RNA silencing with small molecules and the viral suppressor of RNA silencing protein p19” (PMID: 26079891). It is reproduced in this thesis with permission from the publisher – refer to **Rights and Permissions**. This publication was authored by D. C. Danielson, R. Filip, M. Powdrill, S. O’Hara, and J. P. Pezacki. The individual contributions of each author are detailed below:

As first author of this publication, I made significant experimental and intellectual contributions to this article. J.P. Pezacki and I conceived of the research ideas and experimental plan. I performed the majority of the sample preparation, *in vitro* biochemical assays and cell culture gene knockdown experiments. R. Filip aided in sample preparation and biochemical assays. M. Powdrill and S. O’Hara performed optimization of the cell culture gene knockdown experiments and western blot assay. I wrote the first draft of the manuscript and editing was performed by all authors.

Abstract

RNA silencing is a gene regulatory and host defense mechanism whereby small RNA molecules are engaged by AGO proteins, which facilitate gene knockdown of complementary mRNA targets. Small molecule inhibitors of AGO represent a convenient method for reversing this effect and have applications in human therapy and biotechnology. Viral suppressors of RNA silencing, such as p19, can also be used to suppress the pathway. Here we assess the compatibility of these two approaches, by examining whether synthetic inhibitors of AGO would inhibit p19-siRNA interactions. We observe that aurintricarboxylic acid (ATA) is a potent inhibitor of p19's ability to bind siRNA ($IC_{50} = 0.43 \mu\text{M}$), oxidopamine does not inhibit p19:siRNA interactions, and suramin is a mild inhibitor of p19:siRNA interactions ($IC_{50} = 430 \mu\text{M}$). We observe that p19 and suramin are compatible inhibitors of RNA silencing in human hepatoma cells. Our data suggests that at least some inhibitors of AGO may be used in combination with p19 to inhibit RNA silencing at different points in the pathway.

Introduction

RNA silencing involves post-transcriptional gene silencing pathways fundamental to the regulation of gene expression in eukaryotes. The aberrant function of RNA silencing is widely linked to human diseases, including cancer and viral infections [1,76]. Great strides have been made in determining the mechanisms of RNA silencing pathways [77]. RNA silencing pathways have evolved as a defense mechanism against invading RNA viruses, and remain a critical component of the host-immune response in plants [11]. Plant Dicer enzymes cleave the replicating dsRNA viral genome into viral-derived siRNAs, which are then employed by the RNA-induced silencing complex (RISC) to target the viral RNA for degradation. In response, many viruses evolved distinct mechanisms to suppress RNA silencing in the host, thereby allowing viral propagation. By expressing VSRS, plant viruses are able to block RNA silencing and allow viral propagation [78]. The p19 protein is expressed by tombusviruses as a VSRS. During infection, p19 binds to viral derived siRNAs with very high affinity ($K_d \sim 0.2$ nM) and competes with AGO proteins to prevent their incorporation into RISC. It acts like a molecular calliper, measuring the length of the RNA duplex through end-capping tryptophan residues. The affinity of p19 drops off dramatically for any dsRNA longer or shorter than those generated by the Dicer enzyme [28]. Because of this p19 is becoming an attractive tool in biotechnology [79], in studies of miRNAs [30,36,37,80], in diverse organisms such as human cells, *C. elegans*, and *Drosophila* [27,30,39], and protein overexpression [49,50,81-84].

Chemical biology approaches are increasingly being applied to the RNA silencing pathway, and small molecule screens have identified several modulators of the RNA silencing machinery [85,86]. Compounds have been discovered that modulate specifically the activity

of human miR-21 [87] and miR-122 [88]. Others stimulate RNA silencing with an increase in gene silencing and in miRNA production [89]. Inhibitors of human AGO-2:small RNA interactions have also been identified. Three such small molecules: ATA, suramin, and oxidopamine, prevent RISC loading by this mode of action [90].

Herein we investigate the possibility of targeting the RNA silencing pathway at multiple points for more potent global inhibition (**Figure 2.1**). The compatibility of two different classes of inhibitors of the RNA silencing pathway, p19 and small molecule inhibitors of AGO-2 [90] is assessed. We establish a proof-of-concept chemical biology approach towards potently inhibiting RNA silencing in diverse biological systems.

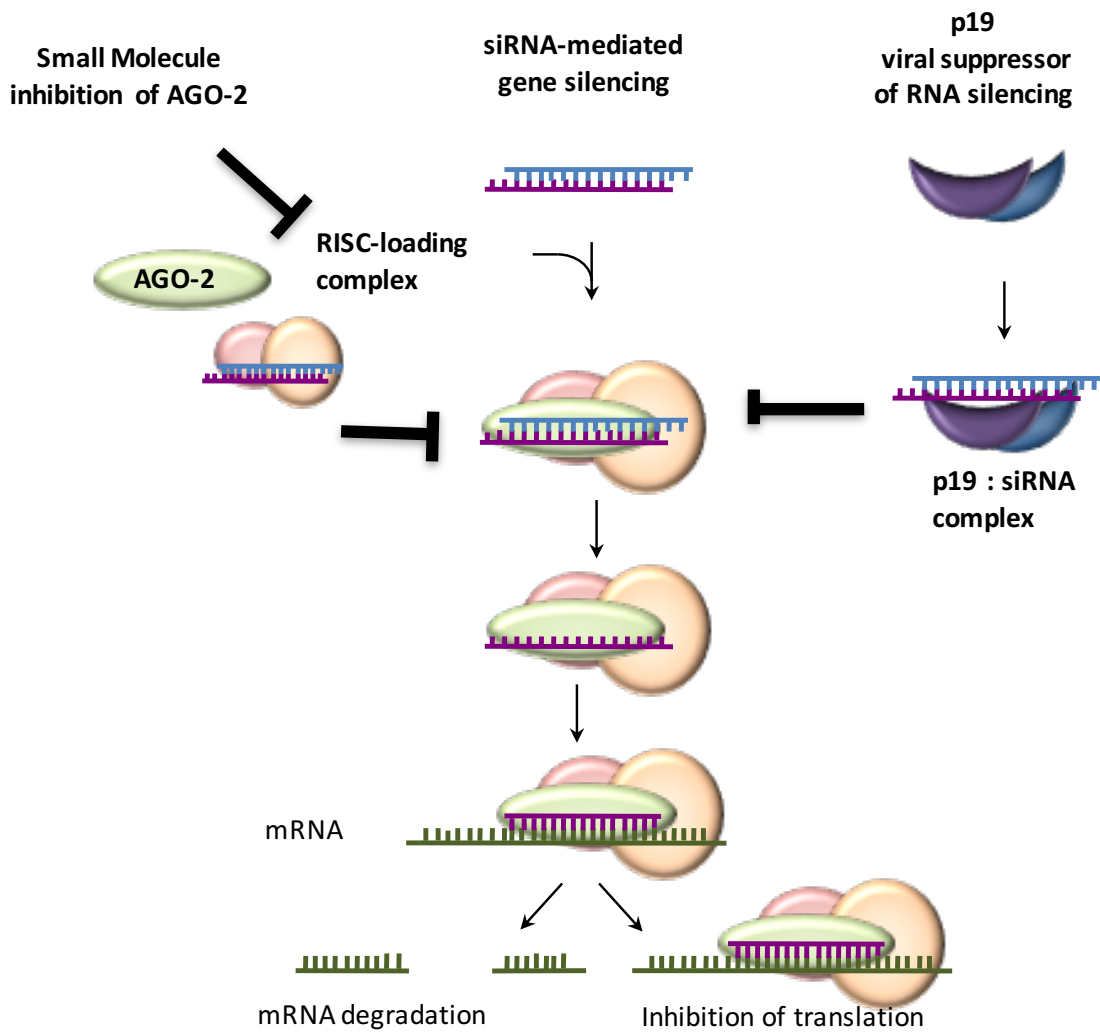


Figure 2.1 Inhibiting the RNA silencing pathway

Scheme depicting the concept of inhibiting the RNA silencing pathway by two distinct classes of inhibitors, to achieve more potent global inhibition of the pathway. The viral suppressor of RNA silencing, the p19 protein, binds siRNAs and prevents their incorporation into RISC. Small molecule inhibitors of the pathway prevent inhibit RISC loading [90].

Materials and Methods

Protein expression & purification

Recombinant histidine-tagged CIRV p19 was expressed from the pTriEX vector in *E.coli* BL21 as previously described [80]. Briefly, transformed cells were grown at 37°C shaking at 220 rpm with 100 ug/ml ampicillin until an optical density of 0.5 was reached, at which point protein expression was induced with 1 mM IPTG and grown at 25°C for three hours post induction. The cells were lysed via sonication and applied to a 1 ml Nickel FF column and washed with 10 ml of was buffer (50 mM Tris, 300 mM NaCl, 60 mM imidazole, pH 8) and eluted with 10 ml in elution buffer (50 mM Tris, 300 mM NaCl, 250 mM imidazole, pH 8) directly into 100 ul of 1M DTT. The eluted p19 proteins were then purified by Size Exclusion Chromatography (SEC) on an S200 via FPLC, in 20 mM Tris, 150 mM NaCl, 1mM TCEP, pH 7.4). The peak fraction was quantified by Bradford protein assay in reference to BSA standards.

siRNAs

siRNAs used for EMSA analysis: Cy3-labeled CSK siRNAs 5'-Cy3-CUA CCG CAU CAU GUA CCA UdTdT-3' and 5'-AUG GUA CAU GAU GCG GUA GdTdT-3' were synthesized, purified by polyacrylamide gel electrophoresis (PAGE), desalted and lyophilized (GE Healthcare) prior to re-suspension in water and storage at -20°C.

Small molecules

Auritracarboxylic acid (ATA), suramin sodium salt and oxidopamine hydrochloride were purchased (Sigma). ATA and oxidopamine stock solutions were prepared in methanol (0.3M), suramin stock solutions were prepared in water (0.1M). All compounds were diluted in the experimental buffer (100-1000 fold) before their application to binding assays.

Electrophoretic Mobility Shift Assays (EMSA)

The compounds of interest were serially diluted in EMSA buffer (20 mM Tris, pH 7, 100 mM NaCl, 1mM EDTA, 0.02% v/v Tween-20, 2 mM DTT). Oxidopamine binding reactions included 2 mM TCEP rather than 1 mM DTT to prevent its oxidation. Purified p19 was added to each concentration of small molecule at a final concentration of 10 nM of p19 dimer. p19 was incubated with the compound for 20 minutes, at which point 10 μ l of 10 nM CSK Cy3-siRNA was added to the binding reaction for a final siRNA concentration of 2 nM and incubated for 1 hour. Competition assays were performed in the same manner, except that a constant concentration of p19 (10 nM) diluted in EMSA binding buffer containing either 0.8 μ M ATA or 900 μ M suramin, to which a range of Bovine Serum Albumin (BSA) concentrations were added by adding a constant volume of serial dilutions.

After the incubation, 5 μ l of 5x TBE loading buffer was added (20 mM Tris, 90 mM boric acid, 2 mM EDTA, 18% Ficoll, 0.02% Xylene) and 10 μ l of each sample was applied to a 6% non-denaturing acrylamide gel (Novex, Invitrogen) and electrophoresed in 0.5X TBE buffer at room temperature at 100V for 50 minutes. The gel was scanned by a Fluorescent Method Bio Image Analyzer III (Hitachi, Japan) for detection of the fluorescently (Cy3) labeled siRNA. The fraction of bound RNA in each lane was determined by quantifying the integrated density of the bands (ImageJ software). Concentration-response titration data was then fitted to the Hill equation (4-parameter), yielding the half-maximal inhibition (IC_{50}) values (Graphpad prism).

Cell based assays

Human hepatoma cells (25×10^4 cells/well in 6-well plate) grown in DMEM + 10% FBS were transfected with either a low (1 ug) or a high amount (2.7 ug) of pTriEX CIRV p19 DNA or pTriEX empty vector (2.7 ug) (no treatment, suramin only) using Lipofectamine (Invitrogen) according to manufacturer's instructions in Optimem medium (Invitrogen).

After 4 hours the media was replaced with fresh media or media with 25 μ M Suramin. After 24 hours the cells were treated with 75 nM siRNA targeting GAPDH (siGENOME GAPD Control siRNA-Human, ThermoScientific) or a non-targeting negative control siRNA (Life Technologies, AM4635) using lipofectamine RNAiMAX (Invitrogen) and Optimem medium with or without 25 μ M Suramin. After 72 hours the cells were harvested in SDS loading buffer and denatured at 95°C. The protein content of the cell lysates were quantified by Bradford assay in reference to BSA standards, and 20 μ g of each sample was analyzed by SDS-PAGE and immunoblot, where the blot probed for GAPDH (anti-GAPDH, Ambion) and PTP-1D (anti-SHP2 Ab, BD Transduction) and visualized using chemiluminescence reagent (ECL prime, Amersham biosciences).

Results & Discussion

Examining small molecule effect on p19 binding siRNA

Three previously characterized inhibitors of AGO-2 were examined to determine their compatibility with the VSRS p19. ATA is a triphenylmethane molecule which inhibits a wide range of nucleic acid binding proteins [91,92]. Oxidopamine is an adrenergic compound applied in research to induce symptoms of Parkinson's disease in animal models [93]. Suramin is a polysulfonated polyaromatic symmetrical urea that is currently clinically applied as an anthelmintic agent [94], and has documented anticancer activity through inhibition of topoisomerases [95] and also inhibits reverse transcriptases of several retroviruses [96]. To examine the compatibility of small molecule inhibitors of AGO-2 with the VSRS p19 protein, as co-treatments for inhibition of RNA silencing, we performed *in vitro* titrations of the small molecules with purified recombinant CIRV p19 proteins, and assessed their impact on p19's siRNA binding activity by EMSA.

We observe that incubating increasing concentrations of ATA (**Figure 2.2A**) with p19 prior to siRNA addition is accompanied with a decrease in the amount of siRNA able to form a complex with p19 (top band) and an increase in the amount of siRNA in an unbound state (bottom band) (**Figure 2.2B**). By quantifying the fraction of siRNA bound by p19 via densitometry, and plotting the effect on siRNA binding as a concentration-response curve, we observe an IC_{50} value of 0.43 μ M (**Figure 2.2C**). The concentration-response curve was a more accurate fit of the data when fit with a variable slope, resulting in a calculated Hill coefficient of -3.8. This suggests that ATA is interacting with several different sites on the p19 binding surface. ATA readily polymerizes in solution and its poly-anionic nature could likely interact with several sites on p19's highly cationic siRNA-binding site, as has been

demonstrated for a variety of nucleic acid binding proteins [97-101]. Our results indicate that ATA is a potent inhibitor of p19:siRNA interactions, inhibiting p19 with the same potency as observed for AGO-2 (**Table 2.1**) [90]. This suggests that p19 and ATA are not compatible approaches for inhibiting the RNA silencing pathway in biological settings [91].

In contrast, we observe that incubating increasing concentrations of suramin (**Figure 2.2D**) with p19 prior to siRNA addition has a much less pronounced effect on p19's ability to bind siRNA (**Figure 2.2E**). Notably, approximately 1000-fold higher concentrations of suramin than ATA are required to inhibit p19 from binding its siRNA ligands as we observe an IC_{50} value of 427 μ M. (**Figure 2.2F**). Our results indicate suramin is a relatively mild inhibitor of p19:siRNA interactions, as it displays approximately 600-fold more potent inhibition of AGO-2 [90] (**Table 2.1**). The dramatically increased potency of suramin for AGO-2 versus p19 suggests that p19 and suramin may be compatible inhibitors of the RNA silencing pathway, if suramin were applied at low concentrations which would impact AGO function but not p19's function. This is hypothesized to be a key determinant for these approaches to be used together for simultaneous suppression of RNA silencing.

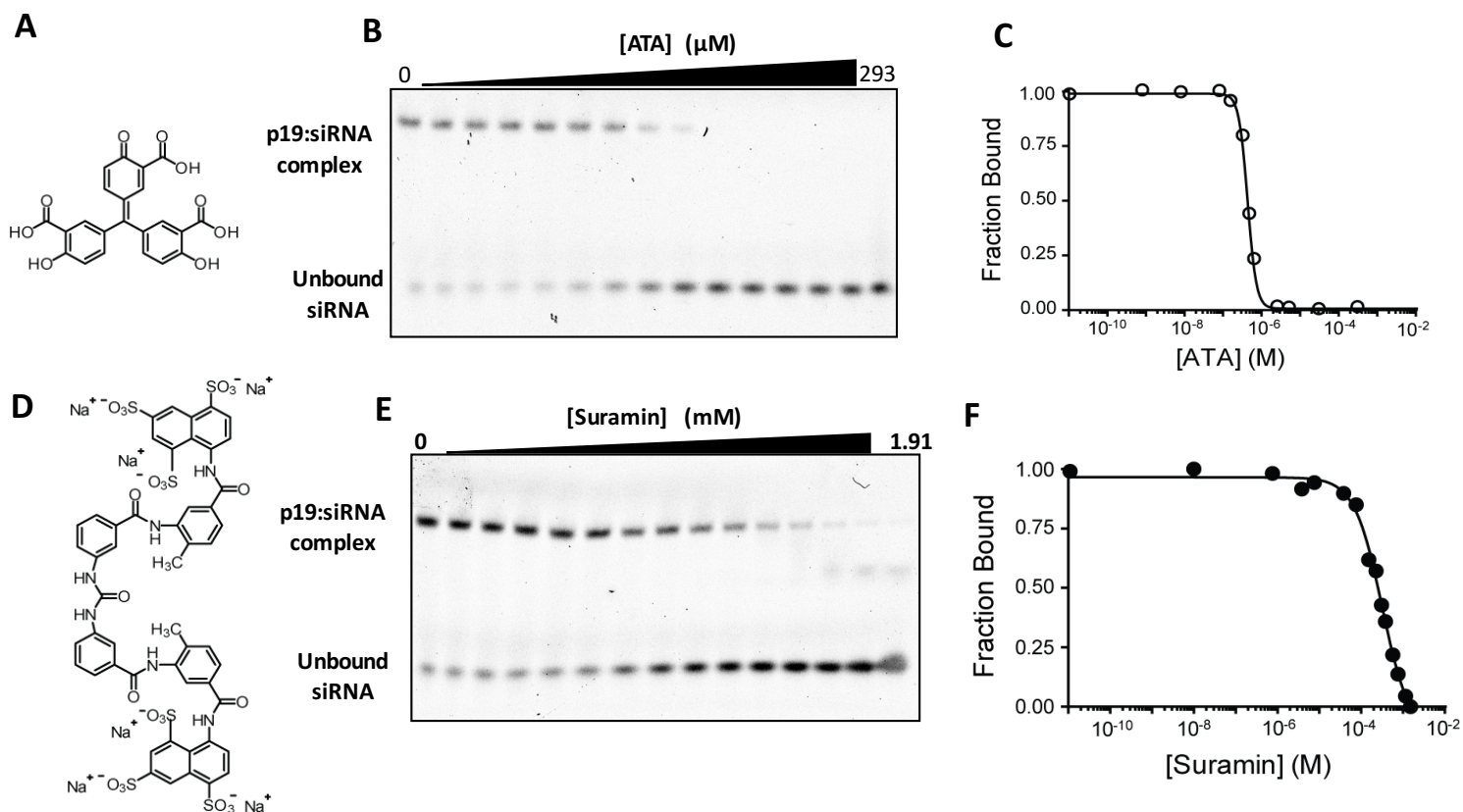


Figure 2.2 Examining impact of small molecules on p19:siRNA interactions

(A) Molecular structure of aurintricarboxylic acid (ATA) (B) Titration of ATA with p19, examining impact of on p19:siRNA interactions by EMSA. Samples of 10 nM p19 dimer incubated with a range of ATA concentrations (0 to 293 μM) and 2 nM Cy3-labeled siRNA. (C) Densitometry of bands in (B) allow quantification of the fraction of RNA bound by p19 at each concentration of ATA. Data is fit with a variable-slope concentration response curve indicating an IC_{50} value of 0.427 μM (95% confidence interval of 0.415 to 0.440 μM) with a Hill coefficient of 3.8. (D) Molecular structure of Suramin. (E) Titration of suramin with p19, examining the impact on p19:siRNA interactions by EMSA. Samples of 10 nM p19 dimer incubated with a range of suramin concentrations (0 to 1.9 mM) and 2 nM Cy3-labeled siRNA. (F) Densitometry of bands in (E) allow quantification of the fraction of RNA bound by p19 for each concentration of Suramin. Data is fit with a fixed-slope concentration response curve indicating an IC_{50} value of 427 μM (95% confidence interval of 326 to 559 μM) with a Hill coefficient of 1.

Upon incubation of p19 with increasing concentrations of oxidopamine (up to 4.5 mM) prior to siRNA addition, we do not observe any impact on the ability of p19 to bind siRNA

(Figure S2.1) Given that oxidopamine inhibits AGO-2 *in vitro* with an IC_{50} value of 1.6 μ M [90], our results suggest that oxidopamine is another candidate for application with p19 as a co-treatment for inhibition of RNA silencing (**Table 2.1**). However, since previous results had indicated that oxidopamine did not display marked inhibition of the RNA silencing pathway in cell culture, we did not pursue any further experiments with oxidopamine.

Table 2.1 Comparing the half maximal inhibitory concentrations (IC_{50}) of inhibitors of RISC and the VSR protein p19.

Values shown at 95% confidence level, showing significant figures only, compared with those for human AGO-2 (*data from ref. [90])

	IC_{50} for p19 (μ M)	IC_{50} for Ago2 (μ M) *	Inhibition of RISC <i>in situ</i> *
ATA	0.43	0.47	~ 100%
Suramin	430	0.69	~ 50%
Oxidopamine	>4584	1.61	N/A

Reversibility of small molecule inhibition

To better understand by what mechanism ATA and suramin prevent p19 from binding its siRNA ligands, we examined whether the effect of the small molecules is reversible. Both ATA and suramin have been described to interact with BSA through electrostatic and hydrophobic interactions [92,102-104], thus we performed a competition experiment and titrated BSA into the binding reactions, to examine its impact on the binding reaction. In this experimental design, we expect that if the inhibitory activity of the small molecule on

p19:siRNA interactions is reversible then adding more BSA will allow an increase in p19:siRNA complex formation, as more of the small molecule interacts with BSA.

We observe that adding increasing concentrations of BSA to the binding reaction of ATA, p19 and siRNA (**Figure 2.3A**) as well as to the reaction of p19, suramin and siRNA (**Figure 2.3B**) is accompanied by an increase in the fraction of siRNA bound by p19, indicating that the small molecules are interacting with BSA and relieving the inhibition of p19:siRNA.

Our results suggest that both ATA and suramin display readily reversible inhibition of p19:siRNA interactions. This is consistent with the general observations of ATA being a general, non-covalent inhibitor of nucleic acid binding proteins, and has been reported to function by blocking the nucleic acid binding site through electrostatic interactions [91,92,99-101]. Thus the binding mode and cooperativity observed in our study is consistent with previous findings. Suramin also binds to nucleic acid binding proteins through non-covalent interactions involving its negatively charged functional groups [94].

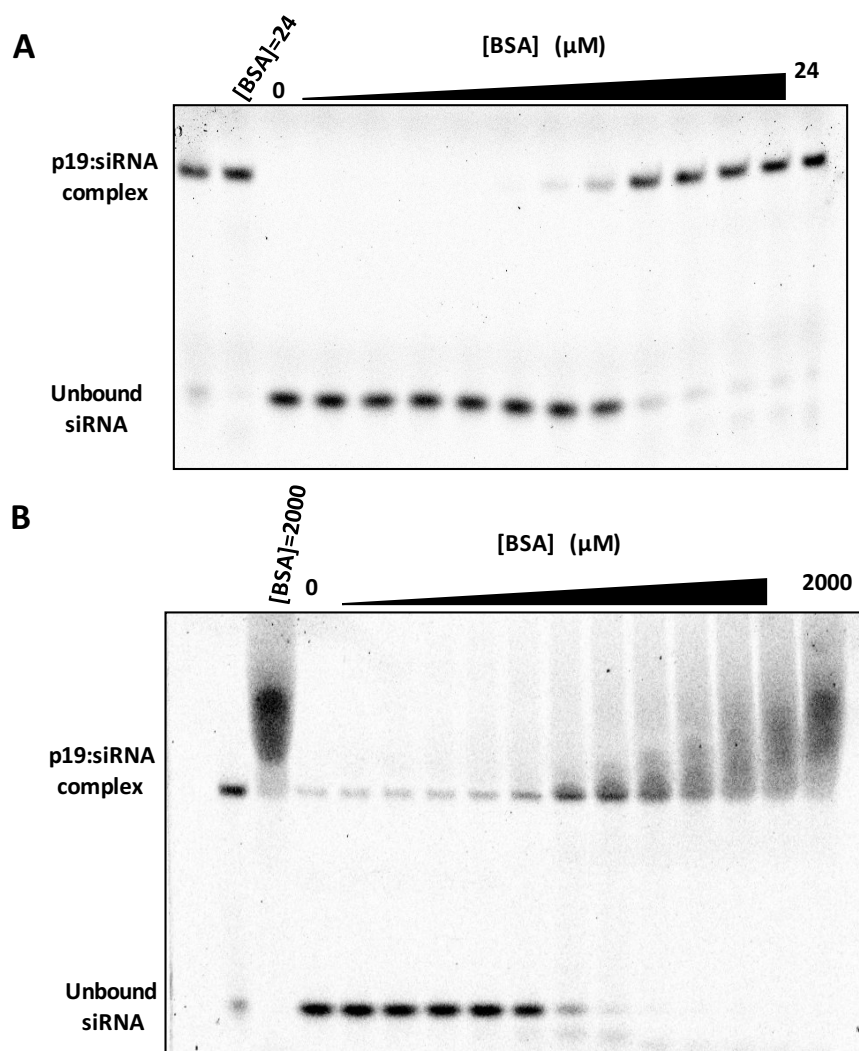


Figure 2.3 Suramin and ATA are reversible inhibitors of p19:siRNA interaction

(A) A representative gel examining the reversibility of inhibition of p19:siRNA interactions with ATA. Increasing concentrations of BSA (0 to 24 μM) (A) are added to binding reactions with a constant concentration of p19 (10 nM) and 0.8 μM ATA (B) Increasing concentrations of BSA (0 to 2 mM) are added to binding reactions with a constant concentration of p19 (10 nM) and 900 μM suramin. The first lanes of each gel are without any BSA or inhibitor. The second lanes of each gel include the highest concentration of BSA, but no inhibitor.

Compatibility of suramin and p19 co-treatment in human cells

Suramin treatment of human hepatoma cells at 25 μM inhibits the RNA silencing pathway ~ 50%, as determined by blocking the function of an exogenously transfected siRNA [90]. At 25 μM treatment *in vitro*, we do not observe any inhibition of p19:siRNA interactions, suggesting that suramin and p19 may be compatible as a co-treatment in human cells at this concentration to achieve more potent inhibition of the pathway.

We examined the compatibility of suramin and p19 as a co-treatment for the inhibition of the RNA silencing in human hepatoma cells. To measure the inhibition of the pathway, we examined the effect of both suramin and p19 on the activity of an exogenous siRNA targeting the endogenous and abundant gene GAPDH, examining the outcome of these treatments on GAPDH protein expression. We observe that suramin treatment and p19 co-expression is accompanied with an increase in GAPDH protein abundance, reflecting that the co-treatment is preventing the action of the added siRNA (**Figure 2.4**). Treating the cells with suramin (and no p19) is accompanied with a marked increase in GAPDH abundance, while expressing p19 but not treating with suramin is accompanied with a modest increase in GAPDH expression. We observe in our experiments that the inhibitory activity of p19 expression in human cell culture can be variable for multiple reasons likely stemming from the fact that the protein originates from a plant virus, however it does suppress RNA silencing in the experiment presented in **Figure 2.4**. Importantly, we consistently observe that the two treatments are compatible and their combination results in potent inhibition of RNA silencing.

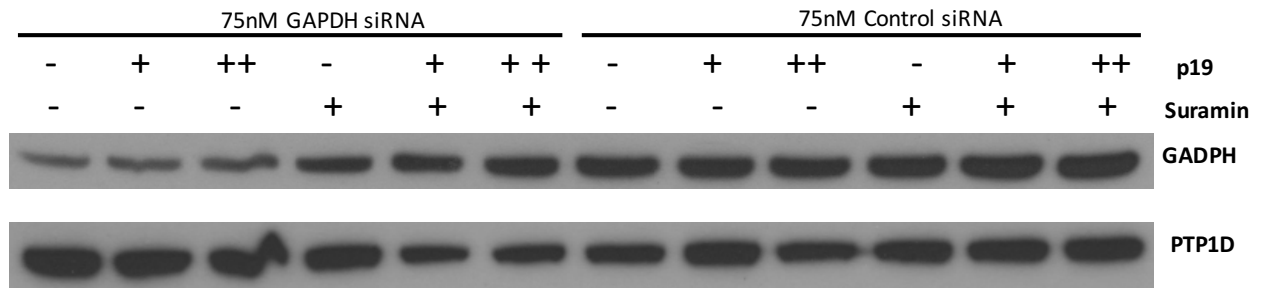


Figure 2.4 p19 and suramin co-treatment prevents siRNA-mediated knockdown of gene knockdown.

A representative western blot examining the compatibility of suramin and p19 as a co-treatment for inhibiting the RNA silencing in human cells. Huh7 cells are transfected with a low (+) or high (++) concentration of plasmid DNA expressing CIRV p19 or the empty vector control (-), and either treated with 25 uM suramin (+) or water control (-). The cells are transfected with siRNA targeting GAPDH (75 nM) or a non-targeting control (75 nM). GAPDH abundance is compared to that of PTP-1D as a reference.

Conclusion

In this study we examine the compatibility of heterologous inhibitors of RNA silencing pathways, the tombusviral p19 protein and small molecule inhibitors of RISC-loading. We identify that two inhibitors, oxidopamine and suramin, are candidates for compatible co-treatments with p19, given that oxidopamine does not inhibit p19:siRNA interactions and suramin only impacts p19:siRNA interactions at high concentrations. Further development of more specific inhibitors of RISC loading then should also work in concert with p19, without unwanted off-target effects. Recently reported high-resolution structural information of human AGO-2 will likely also aid the development of effective small molecule inhibitors [105,106]. Targeting multi-step pathways from multiple points is a strategy often applied for drug treatments, and can result in synergistic inhibition of the pathway where the inhibitory effect of combining two approaches is more potent than the additive effect of each treatment separately. Here we show a proof of concept for targeting the RNA silencing pathway at multiple points using VSRS and small molecule inhibitors.

Acknowledgements

J.P.P would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for funding of a discovery grant supporting this work. D.C.D would like to acknowledge funding from the NSERC and the Ontario Government in the form of graduate scholarships. R.F. would like to thank NSERC for an undergraduate research scholarship.

**Chapter 3 - A novel p19 fusion protein as a delivery agent for
short-interfering RNAs**

Preface

This chapter consists of data previously published in *Molecular Therapy: Nucleic Acids* entitled “A novel p19 fusion protein as a delivery agent for short-interfering RNAs.” PMID: (27045207). It is reproduced in this thesis with permission from the publisher – refer to

Rights and Permissions. This publication was authored by D. C. Danielson, N. Sachrajda, W. Wang, R. Filip and J. P. Pezacki. The individual contributions of each author are detailed below.

As first author of this publication, I made significant experimental and intellectual contributions to this article. J.P. Pezacki and I conceived of the research ideas and experimental plan. I performed the majority of the sample preparation, *in vitro* biochemical assays and cell culture gene knockdown experiments. W. Wang aided in the sample preparation and optimization of the gene knockdown assays, specifically the endosomolytic peptide assays. R. Filip aided in sample preparation of the fusion protein. I wrote the first draft of the manuscript and editing was performed by all authors.

Abstract

RNA interference (RNAi) is the biological mechanism that allows targeted gene knockdown through the addition of exogenous short-interfering RNAs (siRNAs) to cells and organisms. RNAi has revolutionized cell biology and holds enormous potential for human therapy. One of the major challenges facing RNAi as a therapy is achieving efficient and non-toxic delivery of siRNAs into the cell cytoplasm, since their highly anionic character precludes their passage across the cell membrane unaided. Herein we report a novel fusion protein between the tombusviral p19 protein, which binds siRNAs with picomolar affinity, and the 'TAT' peptide (RKKRRQRRRR), which is derived from the transactivator of transcription (TAT) protein of the human immunodeficiency virus and acts as a cell-penetrating peptide. We demonstrate that this fusion protein, 2x-p19-TAT, delivers siRNAs into the cytoplasm of human hepatoma cells where they elicit potent and sustained gene knockdown activity without toxic effects.

Introduction

RNAi is a gene silencing mechanism critical for endogenous gene regulation and host-pathogen interactions in a variety of organisms [12]. In this process, small dsRNA molecules inhibit gene expression post-transcriptionally in a sequence-specific manner. siRNAs are 19-bp duplexes with 3' 2-nt overhangs and 5' phosphates which associate with cellular AGO proteins to form RISC, which then targets complementary RNA sequences for inhibition of gene expression [12].

RNAi is currently an indispensable tool in biotechnology as siRNAs can be made synthetically, designed to target any gene of interest, and added exogenously to cells or organisms to knockdown target gene expression. siRNAs are also attractive as therapeutics, as they are potentially able to knockdown expression of any disease-causing or viral genes. siRNA-based therapeutics have been hindered because they are highly anionic molecules that do not readily cross the cell membrane unaided [107-109]. Effective delivery agents for siRNAs are being pursued for applications in therapy and biotechnology. The standard method of transfecting siRNA into cells *in vitro* involves lipofection (ie- Lipofectamine, RNAiMAX) however these reagents are associated with substantial cytotoxicity. For therapeutic applications, viral vectors and a variety of non-viral approaches are being investigated; including lipid-siRNA conjugation, stable nucleic acid lipid particles (SNALPs), polymers, aptamers, nanoparticles and antibodies [108].

There is an emerging field of cell penetrating peptide (CPP)-mediated siRNA delivery for both *in vitro* and *in vivo* applications [110-112]. CPPs are highly cationic short peptides that facilitate cell entry of attached cargo through association with cell surface glycans and

uptake via macropinocytosis, a type of fluid-phase endocytosis used by all cell types [113,114]. CPPs can have a range of sequences, peptides consisting of a eight or nine arginine residues ('R8, R9') have been reported and widely used for cell entry technologies [115]. CPPs can also be derived from natural sequences such as the 'TAT' CPP, derived from amino acids 49-57 of the HIV-1 TAT protein (RKKRRQRRR) [116,117]. The TAT peptide has been widely used for mediating cell-entry of fusion proteins and other cargos with high efficiency and without cytotoxicity [118,119]. One caveat of CPP-mediated cell entry is that the material can be trapped in the endosome, precluding full delivery to the cytoplasm unless an endosomolytic agent is applied simultaneously [120]. With this in mind, these peptides sequences have still been widely applied to allow cell entry of a variety of cargo and hold therapeutic potential [115,121].

In this work we report a novel fusion protein between a CPP and a siRNA binding protein, p19, to function as a siRNA delivery agent. The 19 kDa protein, p19, is expressed by tombusviruses to bind viral-derived siRNAs and prevent their incorporation into the RISC complex, thereby suppressing the RNA silencing pathway in the host plant [13]. The p19 protein exhibits the highest reported affinity for siRNA duplexes ($K_d \sim 0.2$ nM) and is able to bind siRNAs of any sequence, as it's binding specificity is based on the length of the duplex [28]. The reported crystal structures of p19 in complex with siRNA demonstrate how p19 is able to act as a 'molecular calliper' for siRNA [28,32]. As a dimer, p19 interacts with the sugar phosphate backbone of the siRNA and two N-terminal tryptophan residues on each monomer provide end-capping interactions with the terminal nucleotide of a 19-bp duplex, allowing for size-specific binding. Importantly, p19 does not bind DNA or single stranded RNA [28].

Recombinant p19 proteins with novel properties have been described, such as a p19 linked dimer fusion protein where two monomers are fused by a semi-rigid linker sequence, which displays improved affinity for siRNA and higher thermostability than wild type p19 [74]. Herein, we have fused a CPP sequence 'TAT' to this linked dimer construct to allow cell entry of the p19:siRNA complex. In this work we observe that the fusion protein 2x-p19-TAT is an effective delivery agent for siRNA, allowing for potent gene knockdown in human cells without cytotoxic effects. We investigate the role of endosome-entrapment in the delivery process, and conclude that the 2x-p19-TAT approach would be more potent with the addition of an endosomolytic agent.

Materials and Methods

DNA Oligonucleotides and siRNAs

All DNA oligonucleotides used in the cloning experiments were purchased from Sigma GenoSys (Oakville, ON) and purified by reverse-phase cartridge purification. All RNAs were synthesized by Dharmacon (Lafayette, CO), purified by polyacrylamide gel electrophoresis (PAGE), and desalted using reverse-phase HPLC. The purity was demonstrated to be >95% according to the manufacturer's specifications. The duplexed siRNAs used in the electrophoretic mobility shift assay have the following sense and antisense sequences respectively: Cy3-CSK siRNA (21-mer) 5'-Cy3-CUACCGCAUCAUGUACCAUdTdT-3' and 5'-AUGGUACAUGAUGCG GUAGdTdT-3'. The siRNA used for monitoring gene knockdown via luciferase assays 'luc2' siRNA, targeting the synthetic firefly luciferase gene in the psiCHECK-2 vector (Promega, Madison, WI): 5'-GGACGAGGACGAGCACUU-3' and 5'-GAAGUGCUCGUCCUCGUCCUU-3'. Controls for gene knockdown studies employed negative control siRNA (*Silencer* Negative Control No.1 siRNA (AM4635, ThermoFisher Scientific).

Cloning 2x-p19-TAT fusion protein.

Cloning of the CIRV p19 protein as a linked dimer fusion in the pTriEx plasmid was described previously [74]. The 2x-p19-TAT fusion protein was created by PCR amplification of the p19 monomer with an N-terminal linker and C-terminal TAT sequence with by generating PCR primers to contain the TAT sequence at the C-terminus of the linker-p19 monomer, with a terminal 8-histidine tag flanked by Xho1 restriction sites. The forward primer used was: TTAG CTC GAG GGC GGC GGC GGC TCC GGC GGC GGC GGC

TCT ATG GAA CGC GCT ATC CAA G and the reverse primer used was: TTAG CTC
GAG GCG GCG GCG CTG GCG GCG TTT CTT GCG CTC GCT TTC TTT CTT GAA G.

The PCR product was then digested with the Xho1 restriction enzyme and inserted into pTriEx vector containing the p19 monomer. The resulting construct '2x-p19-TAT' was confirmed by sequencing. The non-binding p19-Y73S control construct was previously described [36] and the 2x-Y373S-TAT construct was cloned using the same primers and restriction enzymes as stated above for the wild type 2x-p19-TAT construct.

Protein Expression and Purification

Bacterial expression of 2x-p19-TAT was performed using *E. coli* strain BL21 (DE3), which were grown at 37°C in LB + 100 ug/ml ampicillin until an OD₆₀₀ of 0.5-0.6, induced with 1mM IPTG, grown for an additional 3h at 25°C. After harvesting, bacterial pellets were resuspended in 50 mM Tris, 300 mM NaCl, 10 mM imidazole, 1 mM dithiothreitol (DTT) and lysed by sonication on ice-bath. Cell lysate was then centrifuged at 20000xg for 20 min at 4°C. The proteins were purified by gravity affinity chromatography using Ni-NTA Fast Flow (GE Healthcare, Piscataway, NJ), the resin washed with 10 CV of 50 mM Tris, 300 mM NaCl, 80 mM imidazole, pH 7.0, and then eluted with 5 CV of 50 mM Tris, 300 mM NaCl, 250 mM imidazole, pH 7.0, with 1 mM of DTT added immediately to the eluate. The eluate was then purified via a Superdex200 size exclusion column (GE Healthcare, Piscataway, NJ). Fractions containing the desired p19 proteins, as determined by SDS-PAGE analyses, were pooled and stored at 4°C for subsequent assays.

Electrophoretic Mobility Shift Assay (EMSA) and Data Analysis

For EMSA binding experiments samples were prepared by incubating 2 nM Cy3-labelled siRNAs with 0 - 2 μ M of purified 2x-p19-TAT in 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.02% v/v TritonX-100, 2 mM DTT, pH 7 for 1 h at room temperature. The samples were then analysed by electrophoresis, where 5X TBE sample buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, 15% Ficoll type 400, 0.02% xylene cyanol) was diluted to 1x in the binding reaction and then 10 μ l applied to a 6% TBE gel and electrophoresed at a constant voltage of 100 V for 50 minutes in 0.5x TBE buffer (Novex, Invitrogen). The gels were imaged with Fluorescent Method Bio Image Analyzer III (Hitachi, Japan) and densitometry performed with ImageJ software (NIH, USA). The fraction of RNA bound by p19 was determined by dividing the band intensity of p19-bound RNA by the sum of the band intensities from the complex and unbound RNA. The data was analyzed by plotting the fraction bound values against p19 concentration and fitted using GraphPad Prism 4 according to the following equation (eq. 1):

$$\Delta P = \Delta P_{\text{Max}} \left(\frac{K_d + np + x}{2np} - \sqrt{\left(\frac{K_d + np + x}{2np} \right)^2 - \frac{x}{np}} \right) \quad (1)$$

where ΔP denotes the change in fluorescence intensity, ΔP_{Max} is the maximal change in fluorescence intensity, K_d is the dissociation constant, n is the number of equivalent sites on the p19 dimer, p is the concentration of labelled small RNA, and x is the concentration of the p19 dimer.

Cell Culture

Huh7 cells were grown at 37 °C with 5% CO₂, in DMEM (Gibco-Invitrogen) with 10% fetal bovine serum (FBS), 100 nM non-essential amino acids, 50 U/mL penicillin, and 50 µg/mL streptomycin.

Gene knockdown in human cell culture

Purified 2x-p19-TAT was concentrated using Amicon Ultra 10-kDa MWCO centrifugal filter device (Millipore, Concord, MA) to 100 µM as determined by the DC protein assay (Bio-Rad, Hercules, CA) and then complexed with luc2 siRNA (Thermo Scientific Dharmacon) with the sequence 5'-gga cga gga cga gca cuu cuu-3', in a 10:1 molar ratio for 45 minutes at room temperature and applied to cells in reduced-serum transfection medium (Optimem, Invitrogen) at a final siRNA concentration of 500nM. After 4h the cells were recovered by adding DMEM with 20% FBS. For the treatments co-incubated with the E5-TAT peptide (GLFEAIAEFIENGWEGLIEGWYGGRRKRRR) (GenScript), the 45-minute incubated treatment mixtures were supplemented with 10 µM E5-TAT, and the treatments were maintained for 24 hours.

Imaging delivery of fluorescently labelled siRNA

Huh7 cells were seeded at 60% confluency in glass-bottom chamber slides and after 24h, the treatments were applied as described above and incubated for 5h or 48 hours. The cells were then washed twice with phosphate buffered saline (PBS) and imaged live-cell in phenol-red free media. The fluorescence imaging was performed using a 100x oil objective on an Olympus 1X81 fluorescence microscope equipped with appropriate filter for Cy3 excitation and emission and a Photometrics (Coolsnap ES) camera, taken with 1-second exposure

times. The images were false coloured with ImageJ and the same brightness and contrast applied to all images of samples within the same time point.

Luciferase assays

To monitor the gene knockdown activity of 2x-p19-TAT, Huh7 cells were seeded into a 100 mM dish and after 24h and at 70% confluency were transfected with a dual luciferase reporter vector, psiCHECK-2 (Promega, Madison, WI) using Lipofectamine2000 (Invitrogen, Burlington, ON) as per manufacturers instructions. After 24 hours, the psiCHECK-2 expressing Huh7 cells were reseeded into a 96-well plate. After another 24 hours, the cells were treated with 2x-p19-TAT:luc2 siRNA complexes as discussed above and after 24h or 72h, the level of knockdown was determined via dual luciferase assays (Promega, Madison, WI) using a Lmax luminometer microplate reader (Molecular Devices, Sunnyvale, CA). Percent gene knockdown was determined by calculating firefly/renilla signal and normalizing to the untreated control.

Cell viability assays

Huh7 cells were seeded in 96-well plates and after 24h and at 70% confluency were treated with protein: siRNA complexes as outlined above or treated with siRNA: RNAiMAX (Invitrogen, Burlington, ON) complexes. Cells were treated for 24h and 72h, the media removed, and 50 μ L of a solution of 2.5 mg/mL MTT in PBS was added to each well. The cells were incubated with the reagent for 3h, aspirated and the formazan crystals dissolved in 150 μ L of DMSO. Absorbance at 570 nM was measured using a Spectra Max M2 (Molecular Devices, Sunnyvale, CA) and recorded using Softmax Pro 4.7 software. The data

was recorded in triplicates and normalized to the untreated control samples to reflect percent cell viability.

Results

Designing p19 as a siRNA delivery agent

In designing the p19 protein as a siRNA delivery agent (**Figure 3.1**), we built upon a p19 construct previously described where two p19 monomers are connected by a semi-rigid linker, resulting in improved thermostability and higher affinity for siRNA than wild-type p19 [74]. To allow cell entry of the linked p19 dimer, we fused the cell penetrating peptide 'TAT' to the C-terminus of the construct. This '2x-p19-TAT' construct also contains a C-terminal 8-histidine tag for affinity purification. The fusion protein 2x-p19-TAT expressed well in *E. coli* and was purified by nickel affinity and size exclusion chromatography.

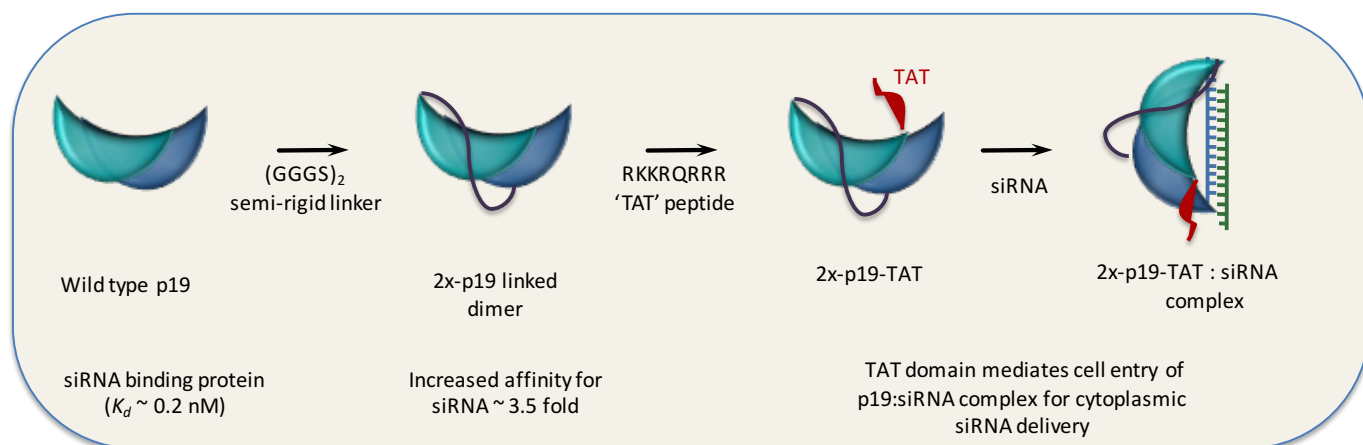


Figure 3.1 Developing the 2x-p19-TAT fusion protein for siRNA delivery.

Linking p19 monomers with a semi-rigid linker to give a linked p19 dimer construct provides improved thermostability and higher affinity for siRNA [74]. For siRNA delivery, a fusion with the cationic TAT peptide on the C-terminus of the linked dimer allows cell entry of the p19:siRNA complex.

p19 fusion protein binds siRNA with high affinity

We hypothesized that p19's high affinity for siRNA would be an asset for its development as a siRNA delivery agent. Therefore, following expression and purification of the 2x-p19-TAT fusion protein we analyzed its ability to bind siRNAs by fluorescence-based EMSA (**Figure 3.2A**). We observed that the addition of the TAT domain to the 2x-p19 construct did not interfere with its ability to bind siRNA with high affinity. We determined the dissociation constant (K_d) of 2x-p19-TAT for fluorescently labelled siRNA as 0.44 ± 0.16 nM (**Figure 3.2D**). We also designed a fusion protein to serve as a negative control, which would distinguish between delivery of p19-bound siRNAs and siRNAs that are electrostatically associated with the cationic TAT peptide. We have previously described the p19-Y73S mutation which completely abrogates siRNA binding [36]. We linked Y73S monomers into a linked dimer construct (2x-Y73S) in the same manner as the 2x-p19 construct and confirmed that the linked Y73S dimer remains incapable of binding siRNA (**Figure 3.2D**). When the TAT peptide is added to the C-terminus (**Figure 3.2D**), the fusion construct is able to bind siRNA with low affinity ($K_d = 1.1$ μ M) (**Figure 3.2E**).

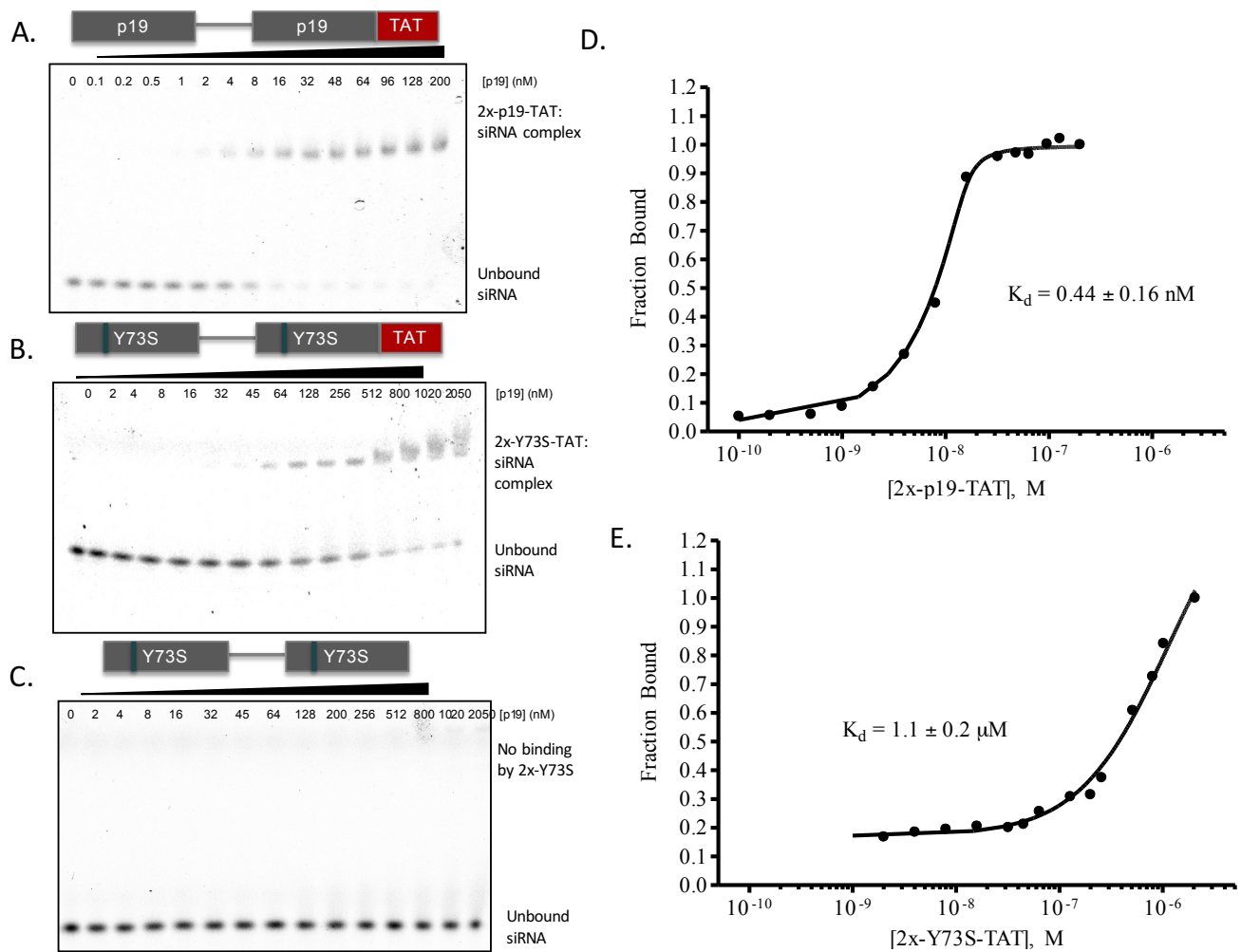


Figure 3.2 Assessing siRNA affinity of 2x-p19-TAT and control proteins by EMSA

(A) Diagram of the p19-based siRNA delivery construct, consisting of a linked p19 dimer with C-terminal TAT domain. EMSA between 2x-p19-TAT and fluorescently labelled (Cy3) siRNA. Cy3-siRNA (2 nM) is incubated with increasing concentrations of 2x-p19-TAT (0-0.2 μM). As the concentration of p19 fusion protein increases, the siRNA mobility is altered due to p19 binding. (B) Construct diagram of 2x-Y73S. The Y73S mutation has been shown to prevent p19 from binding siRNA [36] EMSA assay between the linked dimer construct 2x-Y73S (0-0.2 μM) and Cy3-siRNA (2 nM) indicates that the linked Y73S dimer remains unable to bind siRNA. (C) Construct diagram of 2x-Y73S-TAT, where a C-terminal TAT domain has been added to the linked Y73S dimer from (B). EMSA between 2x-Y73S-TAT (0-0.2 μM) and Cy3-siRNA (2 nM) indicates low affinity binding between the construct and RNA, attributable to electrostatic interactions between the TAT domain and the siRNA (D) Binding affinity determination through plotting fraction of RNA bound versus molar concentration gives a dissociation constant (K_d) of 0.46 nM of 2x-p19-TAT for siRNA (E) Binding affinity determination through plotting fraction of RNA bound versus molar concentration gives a dissociation constant (K_d) of 0.46 nM of 2x-p19-TAT for siRNA. Binding affinity determination of the low affinity interaction between 2x-Y73S-TAT and siRNA gives a $K_d = 1.1 \mu\text{M}$.

p19 fusion protein delivers siRNA in human hepatoma cells

To characterize 2x-p19-TAT's ability to deliver siRNA, we first used live-cell fluorescence microscopy to monitor the entry of fluorescently labeled siRNA complexed with 2x-p19-TAT into human hepatoma (Huh7) cells (**Figure 3.3**). The cells treated with 2x-p19-TAT: Cy3-siRNA complexes for five hours display cytoplasmic Cy3-fluorescence (**Figure 3.3A**) that is not present in control samples where Cy3-siRNA alone was added to the cells (**Figure 3.3B**). We confirm this treatment maintains cytoplasmic fluorescence after 48 hours (**Figure 3.3C**). This indicates that 2x-p19-TAT is allowing cell entry of the Cy3-siRNA. The cell entry is mediated specifically by the TAT domain, as when cells are treated with 2x-p19: Cy3-siRNA complex, we do not see any internalized fluorescence signal (**Figure 3.3D**). Importantly, we observed this cytoplasmic fluorescence in all of the cells treated, thus 2x-p19-TAT delivers siRNA with high transfection efficiency.

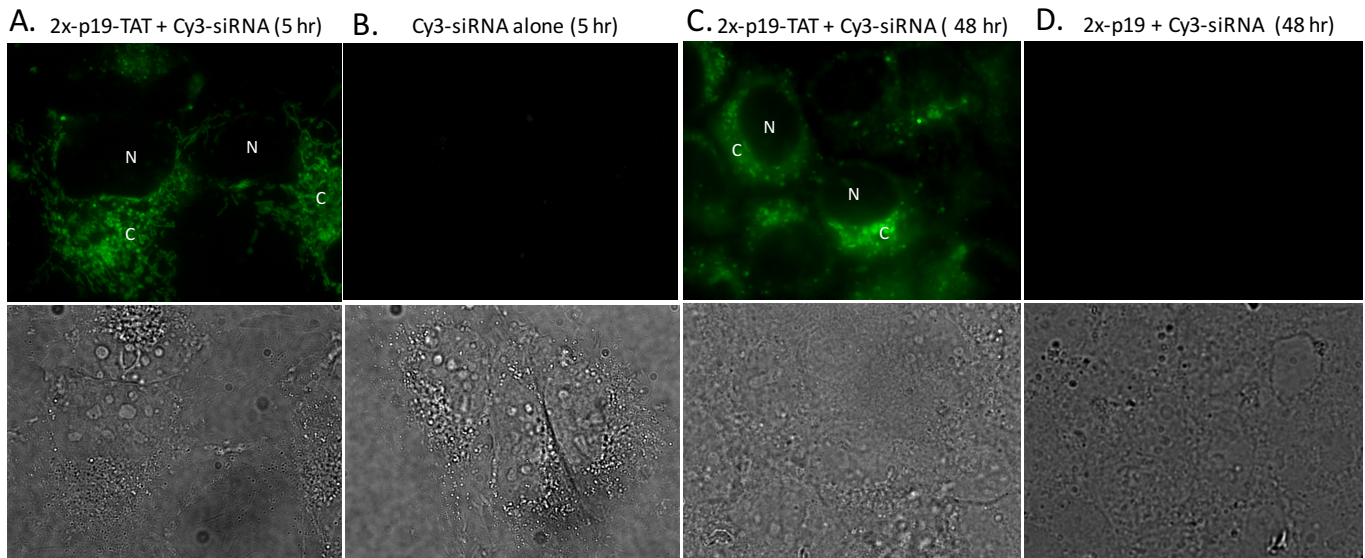


Figure 3.3 Live-cell fluorescence imaging of 2x-p19-TAT delivery of Cy3-siRNA in Huh7 cells.

(A) Cells treated 2x-p19-TAT: Cy3-siRNA for 5 hours show cytoplasmic fluorescence indicating cell entry of Cy3-siRNA (B) Cells treated with the control treatment of Cy3-siRNA alone do not show any fluorescence signal, indicating no cell entry of the naked siRNA. (C) 2x-p19-TAT + Cy3-siRNA for 48 hours display cytoplasmic fluorescence (D) Cells treated with the control treatment of 2x-p19 + Cy3-siRNA for 48 hours do not show any fluorescence, indicating that the TAT domain fusion protein is required for cell entry of the complex.

To demonstrate that 2x-p19-TAT-mediated siRNA delivery results in gene knockdown we employ a luciferase reporter assay. We delivered siRNA targeting the firefly luciferase mRNA to Huh7 cells expressing a dual-luciferase reporter vector (psiCHECK-2) and monitored the response via dual luciferase assays (**Figure 3.4**). The results show that 2x-p19-TAT complexed with luc2 siRNA allows for knockdown of firefly luciferase expression, where after 24 hours the normalized firefly/renilla luciferase signal is reduced to

approximately 40% of the mock samples and after 72 hours show a further reduction to approximately 4% of the mock signal. Adding the fusion protein alone, without siRNA treatment, does not result in a substantial effect on the normalized luciferase signal. We also examine the specificity of the knockdown response by treating with the fusion protein and a negative control, non-targeting siRNA. Importantly, we observe no knockdown response when we perform the same assay with the 2x-p19-Y73S construct complexed with luc2 siRNA and applied to the cells for 72 hours (**Figure 3.4B**). Given that the Y73S mutation to p19's binding site abrogates its ability to bind siRNA, this result suggests that low affinity interactions between the TAT domain and siRNA are not sufficient to allow gene knockdown activity, and the high affinity interactions between p19 and siRNA are required for this activity. These results demonstrate that 2x-p19-TAT functions as a siRNA delivery agent as it allows for potent and sustained gene knockdown in human cells.

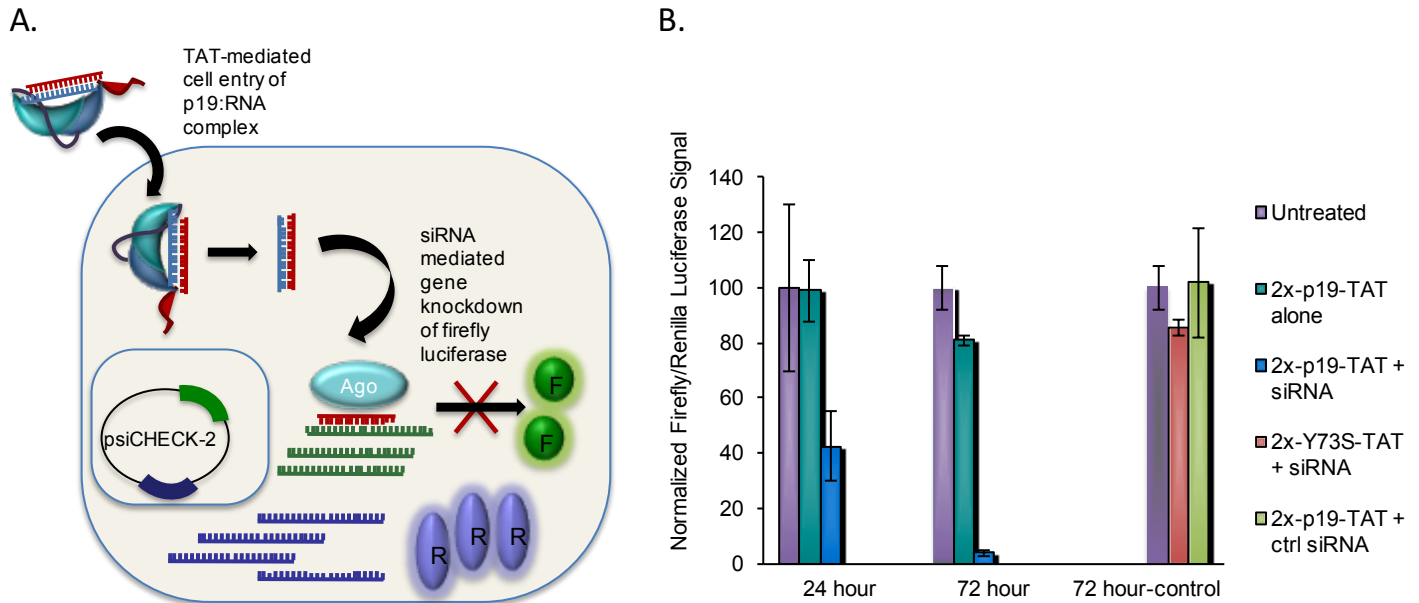


Figure 3.4 2x-p19-TAT allows cell entry and gene knockdown of firefly luciferase reporter gene in Huh7 cells

(A) Schematic depicting the experimental design, where 2x-p19-TAT mediated delivery of siRNA targeting firefly luciferase into Huh7 cells expressing a dual luciferase reporter vector (psiCHECK-2). (B) Treatments of 2x-p19-TAT alone or 2x-p19-TAT:siRNA complexes were applied for 24 or 72 hours until cells were harvested for dual luciferase assays, which assessed the level of siRNA mediated knockdown of firefly luciferase as compared to the renilla control. Treatments indicate potent and sustained gene knockdown activity of 2x-p19-TAT in human cells. Further control treatments assess the specificity of gene knockdown after 72 hours. The wild-type p19 delivery construct, 2x-p19-TAT, is complexed with a non-targeting control siRNA ("ctrl siRNA") and does not show knockdown activity. To delineate the role of p19's high affinity binding for siRNA versus electrostatic interactions between the TAT domain and siRNA, we employ a control fusion protein (2x-Y73S-TAT), where p19 is incapable of binding siRNA but which maintains low affinity interactions between TAT and siRNA. We apply 2x-Y73S-TAT with siRNA targeting firefly luciferase for a 72 hour treatment and do not observe knockdown activity

p19 fusion protein is a non-cytotoxic delivery agent

As cytotoxicity is a problem with many siRNA delivery agents, we sought to investigate whether siRNA delivery using the 2x-p19-TAT fusion protein is associated with any cytotoxicity. We utilized MTT assays to report on cell viability over the course of a 72 hour treatment (**Figure 3.5**). These assays demonstrate that the p19 fusion protein is non-cytotoxic, as the viability of the cells is comparable to the untreated control sample even after 72 hours of treatment. In contrast, transfecting the siRNA using a cationic lipid based transfection reagent RNAiMAX (Invitrogen, Burlington, ON) is associated with substantial cytotoxicity after 72 hours, resulting in nearly 80% decrease in cell viability.

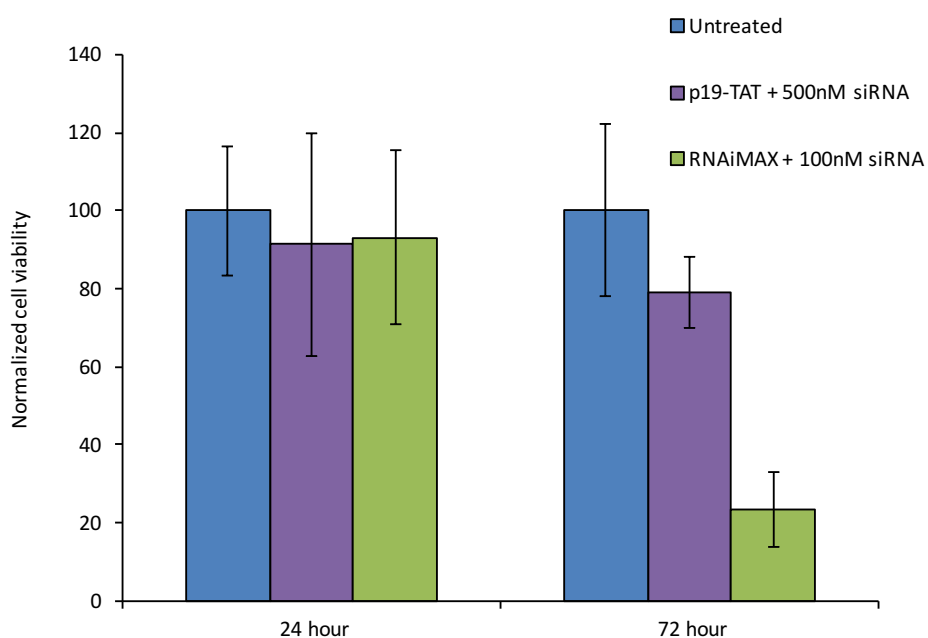


Figure 3.5 Cell viability assays

MTT assays reporting on cell viability following 2x-p19-TAT or RNAiMAX-mediated siRNA delivery for 24 and 72 hours. The 2x-p19-TAT: siRNA treatment does not cause any substantial decreases in cell viability whereas RNAiMAX shows substantially reduced cell viability after 72 hours.

Enhancing delivery efficiency through endosomolysis

It has been reported that cell-penetrating peptide based methods of cell delivery are limited by endosomal entrapment after cellular uptake [120]. Endosomal entrapment can be overcome by applying cell-penetrating, endosomolytic peptides as a co-treatment, which has been applied to enhancing cytoplasmic delivery of proteins to live cells [113,122,123]. In response to the shift to acidic pH which occurs with endosome maturation, peptides derived from the N-terminal twenty residues of influenza hemagglutinin-2 (HA2) undergo a conformational change that allows a central hinge region to become amphipathic and insert into the membrane, thus disrupting the endosomal membrane and allowing pore formation [124]. The E5 peptide is derived from HA2, but has been optimized for solubility and endosomolytic activity [125,126] and the E5-TAT peptide co-treatment has been demonstrated to be an effective and non-toxic cell treatment [123]. Thus we applied the E5-TAT peptide as a co-treatment to our delivery assay to enhance 2x-p19-TAT mediated siRNA delivery to the cytoplasm (**Figure 3.6A**). In this assay we apply 2x-p19-TAT complexed with luc2 siRNA targeting firefly luciferase to cells expressing the dual luciferase reporter vector (psiCHECK-2) as performed previously, but in this assay we add 10 μ M E5-TAT to the cell culture media. After a 24 hour treatment, we assay the gene knockdown activity via dual luciferase assays. With the E5-TAT co-treatment, we observed an enhanced potency of gene knockdown activity of the firefly luciferase reporter relative to the renilla control, after a 24 hour treatment (**Figure 3.6B**). We observe that the 24 hour treatment of 2x-p19-TAT with siRNA displays a range of gene knockdown activity, as observed in the variability of gene knockdown activity at the 24 hour point between **Figure 3.6B** and **Figure 3.3B**. We observe consistent and robust knockdown at the 72 hour time point (**Figure 3.6B**).

We thus observe that the addition of the E5-TAT peptide allows for a more efficient gene knockdown activity, as the extent of knockdown at 24 hours with the E5-TAT peptide is as robust as the 72 hour activity observed in **Figure 3.6B** without the addition of the peptide.

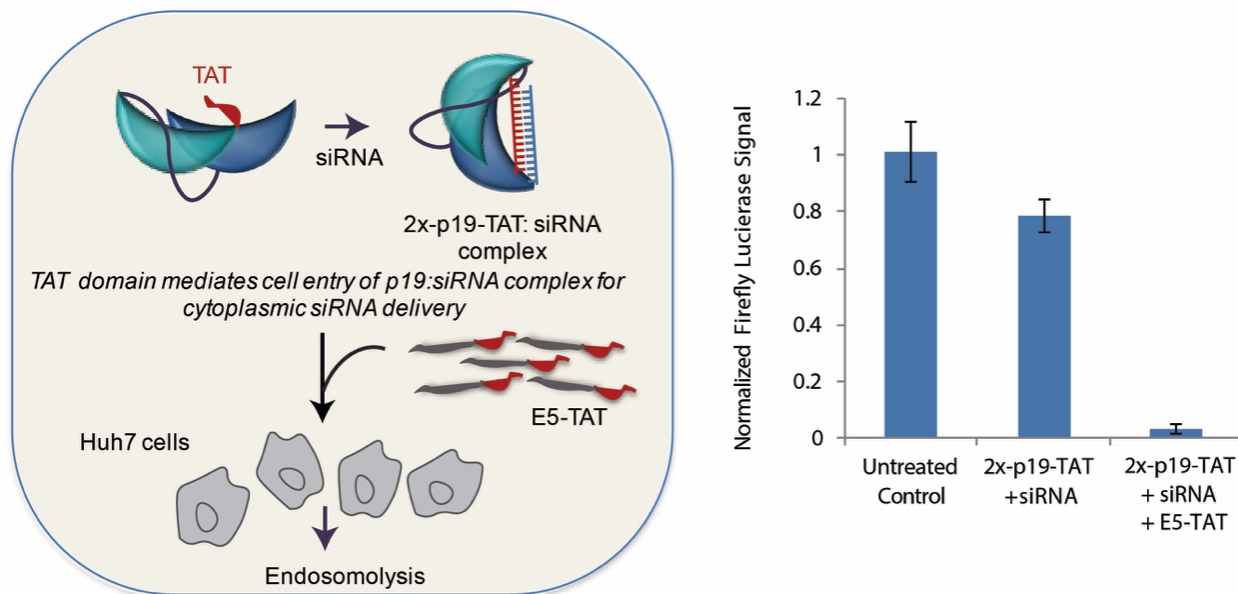


Figure 3.6 Promoting endosomal escape for enhanced gene knockdown

(A) Experimental approach for co-incubating cells with endosomolytic peptides (E5-TAT) and p19:siRNA complexes to enhance cytoplasmic delivery of siRNA (B) Gene knockdown reporter assay via dual luciferase assays assesses the effect of enhanced endosomolysis on the gene knockdown activity of 2x-p19-TAT treatment in Huh7 cells expressing a dual luciferase reporter vector (psiCHECK-2). A 24 hour treatment of 2x-p19-TAT in complex with siRNA targeting the firefly luciferase gene is performed with and without the co-incubation of 10 μ M E5-TAT peptide. The additional treatment with the E5-TAT peptide enhances gene knockdown activity of the 2x-p19-TAT treatment.

Discussion

In this work we report a novel fusion protein between the p19 protein and the TAT peptide for use as a siRNA delivery agent. The addition of the cationic ‘TAT’ peptide to the linked dimer construct, 2x-p19, does not alter the protein’s ability to bind siRNA with high affinity ($K_d = 0.46$ nM, **Figure 3.2A,D**). This dissociation constant is only slightly altered from that of wild-type p19 ($K_d = 0.2$ nM) [14,28,36]. We demonstrate that 2x-p19-TAT is able to deliver siRNA into Huh7 cells through the use of fluorescence microscopy and genetic reporter assays. Imaging the delivery of fluorescently labelled siRNA demonstrates that 2x-p19-TAT facilitates entry of siRNA into the cell (**Figure 3.3**). Furthermore, this fluorescence was observed in all of the cells treated, reflecting that 2x-p19-TAT allows siRNA delivery with high transfection efficiency. Here we demonstrate the effectiveness of 2x-p19-TAT in Huh7 cells, but we hypothesize that it will also be effective in a variety of cell types, including primary cells; just as other TAT-fusion proteins have been demonstrated to be non-cell type dependent [119]. The genetic reporter assays demonstrate 2x-p19-TAT-mediated siRNA delivery results in potent gene knockdown that is sustained over 72 hours (**Figure 3.4**). The level of knockdown observed is comparable to that of standard cationic lipid transfection reagents. Importantly, 2x-p19-TAT achieves this potent gene knockdown activity without toxicity, in contrast to the stark toxicity observed with lipofection (**Figure 3.5**). This observation is in agreement with other approaches for TAT-mediated siRNA delivery that are also non-toxic [127]. We also explore the role of endosome entrapment of the p19:siRNA complex, and observe that knockdown activity is improved through co-incubating the p19:siRNA complex with the E5-TAT endosomolytic peptide. This observation supports previous reports that TAT-mediated protein transduction to the

cytoplasm is limited by endosome entrapment, [120] and so potency and efficiency of this technology would thus greatly benefit from targeting this limiting step in the delivery process.

The p19 protein has been employed in several systems for investigating small RNA biology, including human cells [30,36,42,128]. The utility of p19 has been further enhanced through mutations and protein fusions [36,43,62,63,67]. As reported previously, linking two p19 monomers with a flexible linker gives the protein a higher thermostability and a higher affinity for its bound siRNAs, which are generally beneficial properties for p19's development as a biotechnology tool [62,128]. The linked dimer construct, 2x-p19, gives the opportunity to genetically fuse a single tag or fusion protein to the functional dimer unit, giving unique opportunities for p19-based technology. For example, a cyan-fluorescent protein (CFP) fused to the linked dimer functions as Forster Resonance Energy Transfer (FRET) reporter of Cy3-labeled siRNAs [62]. In the work presented here, we generate a dimer construct with one fused TAT domain on the C-terminus of the linked p19 dimer. We demonstrate that this one domain is sufficient to allow cell entry of p19 with its bound RNA and have thus avoided unnecessarily employing two cell-entry domains. Furthermore, the addition of a single tag to the functional dimer unit avoids potential deleterious effects on protein stability from having two tags per dimer. As described by the available crystal structures, p19 forms a tail-to-tail homodimer, making the C-termini of the monomers closely associated. The addition of a highly charged domain such as the TAT peptide to this terminus of the monomer construct would likely result in electrostatic clashes, which could negatively impact p19 folding or the dimerization activity. Therefore, the base construct 2x-

p19 employed in the work presented here, affords unique opportunities in p19's development as a biotechnology tool and as an siRNA delivery agent.

The first description of a protein-based siRNA delivery approach was a fusion protein between a dsRNA binding domain (DRBD) and three consecutive TAT domains, allowing for potent and non-cytotoxic siRNA delivery *in situ* and *in vivo* [127,129]. The novelty of this approach was that fusing a CPP to a RNA-binding moiety circumvented the charge neutralization that has negatively impacted other CPP-mediated siRNA delivery strategies [121]. Additionally, TAT-DRBD binds siRNAs of any sequence and thus is broadly applicable. Our approach using the p19 protein shares these two assets with TAT-DRBD, and may also benefit from p19's unique binding characteristics for small RNAs. p19's high affinity and its mechanism of binding siRNA are likely assets that will aid in developing p19 as an *in vivo* siRNA delivery agent.

Two other approaches have been described that also apply recombinant p19 fusion proteins for siRNA delivery into human cells. The first employs p19 linked to capsid proteins derived from the Hepatitis B virus, to create a macromolecular siRNA carrier with many p19 molecules within the larger capsid structure. Integrin binding peptides are fused to the capsid proteins, allowing targeting to cancer cells and subsequent cytoplasmic delivery of siRNA [130,131]. A second approach employs p19 fused to a ephrin mimetic peptide (YSA), allowing targeting of p19:siRNA complexes to EphA2 receptors on cancer cells, and subsequent cytoplasmic delivery of siRNA [183]. Our work is the first to employ the linked p19 dimer construct for siRNA delivery, which serves as a useful approach as p19 exists as a functional dimer, and by recombinantly linking the two proteins we observe enhanced properties including higher thermal stability and higher affinity for siRNA [74]. Working

with the recombinantly linked dimer construct affords the ability to make mutations or alterations to a single site of the functional dimer unit. These reports along with the work we present here add to a growing body of applications of p19 for siRNA delivery.

Due to p19's very high affinity for siRNA, the question arises of how it would release the siRNA upon cell entry to allow the siRNA to associate with the RISC complex. This is especially interesting given that p19's endogenous function during viral infection is largely attributed to its ability to sequester siRNAs away from AGO proteins. There are two explanations for the release of siRNA by p19 upon cell entry. Firstly, we hypothesize that in the context of siRNA delivery the equilibrium will be shifted in favour of AGO proteins. During viral infection p19 is expressed at high levels to outcompete AGO, but it cannot deprogram an already loaded RISC [25]. As a siRNA delivery agent, there will eventually be less p19 than AGO proteins in the cytoplasm, allowing AGO proteins to outcompete p19 for siRNA. Furthermore, kinetic studies have demonstrated that p19 exhibits highly reversible binding of siRNA, characterized by rapid binding and marked dissociation [14]. Secondly, TAT-mediated transduction involves an initial trapping of the material in intracellular endosomes that undergo acidification [121]. We have previously reported that p19's binding of siRNAs is pH dependent and is optimal at neutral pH, [132] therefore it is likely that the drop in pH in the endosome promotes dissociation of siRNA from p19.

In this work we have engineered the p19 protein, which has evolved to bind siRNAs with extremely high affinity, for use as a siRNA delivery agent through fusion with a TAT peptide. This novel fusion protein can be applied to deliver any siRNA of interest and has advantages over current strategies for *in situ* gene knockdown and awaits further development for *in vivo* siRNA delivery. Fusion proteins have only begun to be explored for

siRNA delivery agents, however it is clear they offer unique advantages over other delivery technologies including small size of the complex, simple generation, and opportunity for fusions with cell-targeting peptide domains [133]. The technology reported here adds to the growing field of creative strategies for overcoming the largest challenge facing siRNA therapeutics.

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**Chapter 4 - Enhanced specificity of the viral suppressor of RNA
silencing protein p19 toward sequestering of human miRNA-122**

Preface

This chapter consists of data previously published in *Biochemistry* entitled “Enhanced specificity of the viral suppressor of RNA silencing protein p19 towards sequestering of human microRNA-122” PMID: (21819044). It is reproduced in this thesis with permission from the publisher – refer to **Rights and Permissions**. This publication was authored by J. Cheng, D. C. Danielson, N. Nashed, R. Singaravelu and J. P. Pezacki. The individual contributions of each author are detailed below:

As co-first author of this publication, I made significant experimental and intellectual contributions to this article. J.P. Pezacki, J. Cheng and I conceived of the research ideas and experimental plan. I contributed to sample preparation for the biochemical assays. I performed the cell culture and the pull down assays for the *in situ* miR-122 sequestration assay. J. Cheng performed the mutagenesis and the biochemical assays. R. Singaravelu aided in sample preparation for the miR-122 sequestration assay. N. Nashed performed the qRT-PCR for the *in situ* miR-122 sequestration assay. J. Cheng, J.P. Pezacki and I wrote the first draft of the manuscript and editing was performed by all authors.

Abstract

Tombusviruses express a 19-kDa protein (p19) that, as a dimeric protein, suppresses the RNA silencing pathway during infection by binding short-interfering RNA (siRNA) and preventing their association with the RNA-induced silencing complex (RISC). The p19 protein can bind to both endogenous and synthetic siRNAs with a high degree of size selectivity but with little sequence-dependence. It also binds to other endogenous small RNAs such as miRNAs but with lower affinity than to canonical siRNAs. It has become apparent, however, that miRNAs play a large role in gene regulation; their influence extends to expression and processing that affects virtually all eukaryotic processes. In order to develop new tools to study endogenous small RNAs, proteins that suppress specific miRNAs are required. Herein we describe mutational analysis of the p19 binding surface with the aim of creating p19 mutants with increased affinity for miR-122. By site-directed mutagenesis of a single residue, we describe p19 mutants with a nearly 50-fold increased affinity for miR-122 without altering the affinity for siRNA. Upon further mutational analysis of this site, we postulate that the higher affinity relies on hydrogen bonding interactions but can be sterically hindered by residues with bulky side chains. Finally, we demonstrate the effectiveness of a mutant p19, p19-T111S, at sequestering miR-122 in human hepatoma cell lines, as compared to wild type p19. Overall, our results suggest that p19 can be engineered to enhance its affinity towards specific small RNA molecules, particularly non-canonical miRNAs that are distinguishable based on locations of base-pair mismatches. The p19-T111S mutant also represents a new tool for the study of the function of miR-122 in post-transcriptional silencing in the human liver.

Introduction

In the past decade, it has become apparent that small, non-coding RNAs are key regulators of gene expression and influence virtually all eukaryotic cellular processes [12,134]. These small RNAs (~20-30 nt in length) inhibit specific gene expression as determined by their nucleotide sequence. Small RNAs have dsRNA precursors that can be derived transcriptionally or from exogenous sources. The RNA silencing pathway in cells is responsible for excising small dsRNAs of a characteristic length from their precursors, subsequently unwinding the duplex, locating the complementary target RNA sequence, and ultimately inhibiting its expression [12]. The RNA silencing pathway serves a critical role in the cellular innate immune response to dsRNA. Particularly in plants, the pathway serves as an anti-viral response by generating small interfering RNAs (siRNAs) which inhibit replication of the viral genome [134].

Although the RNA silencing response to dsRNA is extremely potent, several RNA viruses have evolved mechanisms of inhibiting the pathway, thereby allowing viral propagation in their host. Tombusviruses, such as TBSV and CIRV, are a family of RNA plant viruses that express a 19-kDa protein, p19, which, as a dimer, binds small dsRNAs with nanomolar affinity, thus preventing their incorporation into the RNA-induced silencing complex (RISC) [24,135]. The binding is based on hydrogen bonding and electrostatic interactions between the β -sheet surface of the homodimer and the sugar-phosphate backbone of the siRNA, thereby allowing sequence-independent binding [28,32]. p19 displays specificity based on size of the siRNA, preferentially binding 21-nt siRNAs with a dramatic drop in affinity for longer species. This size selectivity is determined by two pairs of tryptophan residues which provide end-capping interactions that stabilize a 21-nt siRNA in the binding pocket of the

dimer [28,32,80]. Due to its unique binding properties, p19 has been used as an effective suppressor of RNA silencing in a variety of systems, including plants [30,37], human cells [30,42], *C. elegans* [136], and mouse embryonic stem cells [43]. The p19 protein has also been used to profile miRNA levels as part of an enzyme immunoassay [63], and track viral infection in plants using a p19-GFP fusion reporter protein [25]. Furthermore, we have engineered higher affinity p19 mutants [74] as well as p19 fluorescent fusion constructs which sense p19 binding to small RNAs based on Förster resonance energy transfer (FRET) [62].

It has also been shown that p19 binds another class of small RNA molecules, miRNAs, with high affinity [37,42,80]. miRNAs are transcribed from non-coding regions of the genome and regulate expression of endogenous genes. Specific miRNA expression profiles correlate with tissue type and phases of development. Dysregulation of these profiles is associated with several human pathologies, including cancer [137]. Additionally, viruses modulate host miRNA expression as well as express their own virally-encoded miRNAs in order to facilitate their pathogenesis [138]. miRNA-122 is a liver specific miRNA [139,140] whose expression has been shown to positively regulate the replication, translation and virion production of the Hepatitis C Virus [141-143]. Consequently, means of miR-122 sequestration are presently being investigated as an antiviral strategy [144].

We hypothesize that p19 can be engineered to have high affinity and specificity for miR-122, which would allow it to be used to sequester miR-122 in biological systems and potentially serve as a novel antiviral tool. We have previously reported that p19 has the ability to bind miR-122 with nanomolar affinity, although with substantially lower affinity than for its canonical 21-nt siRNA ligands [80]. The differences in affinity are likely because, unlike

siRNAs, miRNAs are irregularly shaped due to mismatches in their nucleotide sequence, which create bulges in their secondary structure, potentially hindering interactions with p19. In this work, we aim to identify residues in p19 that interact with miR-122 uniquely in order to better understand the interaction and develop p19 mutants with higher affinity for miR-122. We have targeted the residues of the p19 binding site we predict to interact with secondary structure elements of the miR-122 molecule (**Figure 4.1**). Through site-directed mutagenesis, we have identified several residues that affect p19's binding affinity for miR-122 and describe two mutants of p19 which display a nearly 50-fold higher affinity for miR-122 compared to wild type. Furthermore, we demonstrate one of the p19 mutant's effectiveness at sequestering miR-122 in human hepatoma cell lines. This work lays the foundation for the use of mutant p19 proteins for the study of miRNAs, providing a protein-based sequestration tool for miRNAs such as miR-122. This technology will help elucidate the role of miRNAs in regulating biological systems such as liver function as well as studying their interactions with other targets such as viral genomes.

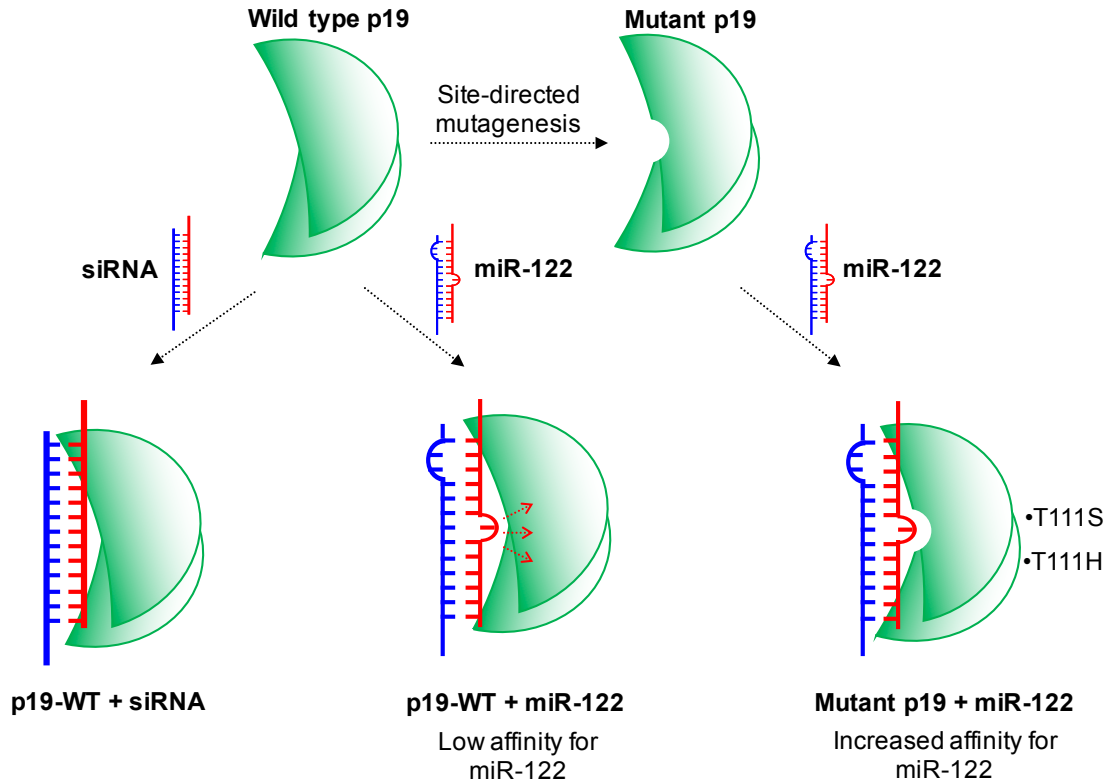


Figure 4.1 Depiction of the site-directed mutagenesis strategy for identifying p19 mutants with enhanced affinity for miR-122.

p19 residues predicted to be interacting with the bulges in the secondary structure of miR-122 were mutated in order to potentially create new, high affinity binding interactions. Substitution of Thr111 with a serine or histidine residue resulted in high affinity binding which enhanced binding to miR-122 without altering its affinity for siRNA.

Materials and Methods

DNA Oligonucleotides and Fluorescently-Labelled siRNAs

All DNA oligos used in the cloning experiments were purchased from Sigma GenoSys (Oakville, ON) and purified by reverse-phase cartridge purification. All RNAs were synthesized by Dharmacon (Lafayette, CO), purified by polyacrylamide gel electrophoresis (PAGE), and desalted using reverse-phase HPLC. The purity was demonstrated to be >95%

according to the manufacturer's specifications. The duplexed siRNAs used in the electrophoretic mobility shift assay have the following sense and antisense sequences respectively: Cy3-CSK siRNA (21-mer) 5'-Cy3-CUA CCG CAU CAU GUA CCA UdTdT-3' and 5'-AUG GUA CAU GAU GCG GUA GdTdT-3'; Cy3-GL2 siRNA (28-mer) 5'-Cy3-ACA UCA CGU ACG CGG AAU ACU UCG AAdT dT-3' and 5'-UUC GAA GUA UUC CGC GUA CGU GAU GUdT dT-3'. The Cy3-labelled miR-122 (Cy3-miR-122) used has the following sense and antisense sequences, respectively: 5'-Cy3-AAA CGC CAU UAU CAC ACU AAA UA-3' and 5'-UGG AGU GUG ACA AUG GUG UUU GU-3'. Both strands were deprotected, annealed, and precipitated according to the manufacturer's protocols. To ensure that all of the labelled strands were duplexed, we used a slight excess of the unlabeled strand during annealing of Cy3-miR-122 duplex. The 21-nt Cy3-labelled single-stranded (ss)RNA (Cy3-ssRNA) has the following sequence: 5'-Cy3-CGU ACG CGG AAU ACU UCG AUU-3'.

Cell Culture

Huh7.5 human hepatoma cells were grown in an incubator at 37 °C with 5% CO₂, in cell culture medium, i.e. Dulbecco's Modified Eagle Medium (DMEM) (Gibco-Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 nM non-essential amino acids (NEAA), 50 U/mL penicillin, and 50 µg/mL streptomycin.

Plasmid Construction and Site-Directed Mutagenesis

Construction of the pTriEx-p19 plasmid encoding the wild type, codon-optimized Carnation Italian Ringspot virus p19 protein (p19-WT) with a C-terminal octa-histidine tag (8xHis-tag) was described previously [145]. Construction of various mutant p19 candidates with

enhanced miR-122 affinity was performed using the QuickChange II site-directed mutagenesis kit (Stratagene) according to manufacturer's protocol. The template DNA for the mutagenesis and the complementary primer pairs used for the mutated constructs are described in **Table S4.1** of Supporting Information. All constructed DNA plasmids were confirmed by DNA sequencing.

Protein Expression and Purification

Bacterial expression of the His-tagged p19-WT and mutant derivatives was carried out as previously described [145] with the following modifications. Briefly, *E. coli* strain BL21 (DE3) cells harbouring the p19 constructs were grown at 37°C until an optical density at 600 nm (OD_{600}) of 0.5-0.6 was achieved. Expression of p19 proteins was induced by IPTG at a final concentration of 1 mM. Cultures were then grown for an additional 3-4 h at 28°C or until OD_{600} reached ~ 1.5. After harvesting, bacterial pellets were resuspended in lysis buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole, 1 mM dithiothreitol (DTT), 1X Complete EDTA-free Protease Inhibitor Cocktail (Roche), pH 8.0) and lysed by sonication on ice-bath. Cell lysate was then centrifuge at 20000 g for 20 min at 4°C. The soluble lysate fraction containing the His-tagged p19 proteins was loaded to HisTrap FF Ni²⁺-affinity column (GE Healthcare, Piscataway, NJ). After protein binding, the resin was washed with 10 column volumes of wash buffer (50 mM Tris, 300 mM NaCl, 50 mM imidazole, pH 8.0). Elution of the His-tagged p19 proteins was carried out using elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0) and 10 mM of DTT was added immediately to the eluate. The pooled eluates were concentrated to 0.5 ml using the Amicon Ultra 10-kDa MWCO centrifugal filter device (Millipore, Concord, MA). The concentrated samples were then injected into Superdex 200 size exclusion column (GE Healthcare, Piscataway, NJ) at a flow

rate of 0.5 ml/min. The p19 proteins eluted as stable dimers and were recalcitrant to denaturation even upon boiling for 15-20 min [74,145,146]. Fractions containing the desired p19 proteins, as determined by SDS-PAGE analyses, were pooled and stored at 4°C for subsequent assays and analyses. Pooled p19 fractions were monitored by SDS-PAGE as a single band and estimated to be >95% pure.

Electrophoretic Mobility Shift Assay (EMSA) and Data Analysis

For EMSA binding experiments, samples were prepared by incubating 2 nM Cy3-labelled small RNAs with various concentrations of purified p19 or its mutant derivatives in buffer containing 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.02% v/v TritonX-100, 2 mM DTT, pH 7 for 1 h at room temperature. Two microliters of 5X TBE sample buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, 15% Ficoll type 400, 0.02% xylene cyanol) was added to 18 µl of binding reaction. Ten microliters of each sample was analyzed by electrophoresis at a constant voltage of 100 V for 50 min through a 6% TBE DNA retardation gel in 0.5X TBE running buffer (Novex, Invitrogen). The gels were imaged with Fluorescent Method Bio Image Analyzer III (Hitachi, Japan). Bands corresponding to bound and unbound fluorescently-labelled small RNAs were quantified with ImageJ software (National Institutes of Health, USA). The fraction of RNA bound by p19 was determined by taking the value of integrated band intensity corresponding to the p19-bound RNA complex over the sum of integrated band intensities from the complex and unbound RNA. The direct binding experiments were analyzed by plotting the fraction bound values against various concentrations of p19 and fitted according to the following equation (eq. 1):

$$\Delta P = \Delta P_{\text{Max}} \left(\frac{K_d + np + x}{2np} - \sqrt{\left(\frac{K_d + np + x}{2np} \right)^2 - \frac{x}{np}} \right) \quad (1)$$

where ΔP denotes the change in fluorescence intensity, ΔP_{Max} is the maximal change in fluorescence intensity, K_d is the dissociation constant, n is the number of equivalent sites on the p19 dimer, p is the concentration of labelled small RNA, and x is the concentration of the p19 dimer.

Circular Dichroism (CD) and Thermal Melt Analysis

CD spectra were recorded on approximately 5 μM p19-WT, p19-T111S, p19-T111H, p19-T111A, p19-T111Y or p19-Y73S in 20 mM sodium phosphate pH 7.2, 25 mM NaCl and 10 mM DTT on a Jasco J-815 CD spectrometer with a 1-mm path length quartz cell at 25°C.

Spectra reflect an average of 8 scans recorded from 250 to 190 nm with a 0.2 nm step resolution, response of 1 s, a speed of 20 nm/min, and a bandwidth of 1 nm. Following CD spectroscopy, the concentration of the samples was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) and used to calculate the molar ellipticity per mean residue. CD data was analyzed using the CD-Pro suite of programs [147]. Thermal denaturation of the p19 proteins was performed using a Jasco thermal control unit (model PTC-423S/15) with a heating rate of 1°C/min from 25 to 95°C. At each step, the molar ellipticity at 222 nm was recorded.

Transient Expression of His-Tagged p19-WT and p19-T111S in Huh7.5

Cells

For transient expression of p19-WT and p19-T111S, Huh7.5 cells were seeded at 5.0×10^5 cells per 60-mm dish culture media. Cells were transfected once they reached 70%

confluency (usually around 24 hours post-seeding). Prior to transfection, cells were washed once with phosphate-buffered saline (PBS; 137mM NaCl, 2.7mM, KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and then with transfection media, i.e. OptiMEM (Invitrogen) while preparing transfection complexes. Transfection complexes were prepared by mixing 7 µg of pTriEx-p19 or pTriEx-p19-T111S DNA plasmid, or no DNA (mock) with 14 µl of Lipofectamine 2000 (Invitrogen) in 2.5 mL transfection media and incubating at room temperature for 20 min. Each transfection complex mixture was then added to the cells and incubated for 4 h at 37°C. After 4 hours, one equivalent volume of recovery media, i.e. antibiotic-free DMEM containing 20% (v/v) FBS and 100 nM minimal non-essential amino acids, were added. Transfected cells were then incubated at 37 °C and 5% CO₂ for 24 h.

Western Blot Analysis of Transient Expression of His-tagged p19-WT and p19-T111S in Huh7.5 cells

Cells were washed twice with PBS and lysed with a lysis buffer consisting of 50 mM Tris-HCl (pH 6.8), 2% SDS and 10% glycerol 24 h post transfection. A protease inhibitor cocktail mix (Roche Diagnostics, Penzberg, Germany) was added to each extract. The protein concentration of the whole cell lysate was quantified by using the Bio-Rad DC Protein Assay according to the manufacturer's protocol. Prior to loading, a final concentration of 100 mM DTT and 0.1 % v/v bromophenol blue was added to each sample. A total of 50 µg protein/well was loaded onto a 12% SDS-PAGE gel. The resolved proteins were transferred to a Hybond-P (Amersham Biosciences, Piscataway, NJ) polyvinylidene difluoride membrane. The membrane was probed against a horse radish peroxidase (HRP)-conjugated monoclonal anti-His-tag antibody (1:5000 dilution; R & D Systems, Inc.,

Minneapolis, MN). As a loading control, membranes were stripped and re-probed using a mouse anti-PTP-1D 1° antibody (1:2500 dilution; Sigma, Saint Louis, MO) followed by a 2° HRP-conjugated goat anti-mouse IgG antibody (1:10,000 dilution; Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA). The blot was developed using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Baie d'urfe, QC) according to the manufacturer's protocol. The band intensities were measured by densitometry using ImageJ software (National Institutes of Health, USA).

Quantification of miRNA Bound by His-tagged p19

Twenty-four hours post-transfection, cells were trypsinized and washed twice with PBS by centrifugation at 1000 rpm, 5 min at room temperature. Cells were then lysed by resuspending the cell pellet with lysis buffer (pH 8) containing 150 mM KCl, 50 mM HEPES, 0.5% v/v NP-40, 0.5 mM DTT, 1X Complete EDTA-Free Protease Inhibitor Cocktail (Roche), and 100U/mL RNaseOUT (Invitrogen), and incubation at 4 °C with rotation for 10 min. The lysate was passed through a 0.2 µm filter unit to remove cell debris. Filtered lysate was loaded onto a 1 mL HisTrap FF column (GE Healthcare, Piscataway, NJ) that was prepared RNase-free by washing with the following RNase-free solution: 10 column volume (CV) 0.1 M NaOH, 0.05 NaCl, 10 CV water, and then equilibrated with 10 CV lysis buffer without DTT, to capture any His-tagged p19 that was transiently expressed in the cell. The His-tagged p19 proteins bound to the column were eluted with buffer containing 50 mM Tris pH 8, 100 mM NaCl, 50 mM EDTA. The elution fraction was collected for subsequent qPCR analysis.

Total RNA was extracted from the eluate using miRVana miRNA Isolation Kit (Ambion) according to the manufacturer's protocol. Ten nanograms of the total RNA were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in combination with the RT stem-loop primers against U6 RNA (endogenous control) (P/N:4427975, Applied Biosystems) and miR-122 (P/N:4427975, Applied Biosystems) according to manufacturer's instructions. miRNA levels were analyzed using Taqman real-time (qRT-PCR) method [148]. Each PCR sample included 1X Universal Taqman PCR Mastermix (Applied Biosystems), 0.2 mM TaqMan probes against either U6 RNA, or miR-122 (Applied Biosystems), 1.5 mM forward primer and the universal reverse primer. Relative miRNA abundance was calculated using the comparative Ct method [149]. The data represent the average of 3 independent experiments each performed in triplicates.

Results & Discussion

Strategy for Mutational Analysis of p19 Binding with miR-122

Previously, we have demonstrated that the p19 protein is able to bind to an irregularly structured miRNA, miR-122, with nanomolar affinity [80]. It was proposed that p19's capability to bind miR-122 with nanomolar affinity is likely due to retention of key interactions observed between p19 and siRNA. Although miR-122 is 23-nt in length, the base-pairing mismatches in the miRNA may slightly compress the length of the molecule, potentially allowing the duplex region to adopt a length closer to the standard 19-bp region in an A-form dsRNA duplex observed in a 21-nt siRNA molecule [32]. This compression would facilitate miR-122 to fit between the two end-capping helices of the p19 dimer;

thereby retaining the interactions that give p19 its size selective affinity for 21-nt siRNAs. In this case, it is likely that the miRNA docks onto the p19 protein in similar fashion to siRNA but with 2 possible docking orientations, due to the presence of asymmetric bulges in the miR-122 molecule. Notably, p19 displays ~100-fold reduction in affinity for miR-122 compared to 21-nt siRNA, likely due to perturbations to the known p19-siRNA interactions as a result of the structural irregularities arising from the base pair mismatches in the miR-122 duplex [80]. Therefore, we performed mutational analysis of residues predicted to interact with the structural irregularities of miR-122, with the aim of creating a more accommodating binding site which would give rise to higher affinity binding. Our initial strategy was to create “pockets” on the binding surface of p19 to accommodate the bulges of miR-122 by mutating residues on p19’s binding surface to residues with smaller side chains. This approach is conceptually similar to the bump-hole approach developed by Shokat and co-workers that have been very successfully applied to the study of kinases [150-152].

The first step in the strategy involved estimating the location of the bulges relative to the residues of the p19 binding site. In order to predict the position of miR-122’s bulges, we used the canonical siRNA as a model to represent their locations relative to p19’s small RNA binding surface according to the crystal structure [28]. In our previous publication, we had approximated the position of the nucleotide bases of the “compressed” miR-122 molecule in both docking orientations relative to the bases of the siRNA [80]. The first bulge is due to a 1-bp mismatch in the middle of the miR-122 sequence, which we estimate to be aligned with the siRNA base pair C10-G10’, in both possible docking orientations (**Figure 4.2**). Another 2-bp mismatch exists at the side of the miR-122 molecule, which we estimate to be aligned with the siRNA base pairs G2-C18’ and U3-A17’ in docking orientation 1 and with base

pairs A17-U3' and C18-G2' of the siRNA in docking orientation 2 (**Figure 4.2**). These bases of the siRNA were then used to estimate which residues of the p19 binding surface are in proximity to the bulge in the miR-122 molecule.

The small RNA binding surface of p19 consists of an 8-stranded β -sheet as a result of p19 dimerization [28]. Using a molecular viewer program, we identified residues on the β -sheet with side chains facing the interface which displayed no direct interaction with siRNA according to the crystal structure [28]. Substitution of these residues minimizes the chance of disrupting key interactions between p19 and the rest of the miR-122 molecule since the majority of interactions that p19 has with siRNA are thought to be retained with miR-122 [80]. Out of these candidates, we identified residues that are also proximal to the hypothesized location of the miR-122 bulges (see **Figure 4.2A**). This approach resulted in 4 potential sites for mutagenesis experiments at residues Val69, Tyr73, Thr111, and Thr122 of p19.

The side bulge of the miR-122 molecule caused by a 2-nt mismatch is parallel to G2 and U3 in the siRNA molecule in docking orientation 1, or G2' and U3' in docking orientation 2 (**Figure 4.2**). The Tyr73 residues in both p19 monomers are predicted to be in proximity to the side bulge of the miR-122 molecule in each of the 2 possible docking orientations respectively, due to the asymmetry in the miR-122 molecule (**Figure 4.2A**). Assuming the side bulge of miR-122 pushes back against the p19 surface close to Tyr73 (**Figure 4.2B**), we hypothesized that mutating this tyrosine to a smaller residue could reduce any potentially unfavourable steric interactions and allow for a better fit for the miR-122 into the p19 dimer. Therefore, substituting the Tyr73 residues in both p19 monomers to a serine residue would eliminate the bulky benzene ring while preserving the hydroxyl group for any potential new

interactions, such as hydrogen bonding with the phosphate backbone of the miR-122 molecule. Residues Val69, Thr111 and Thr122 in both p19 monomers are predicted to be in proximity to the middle bulge of the miR-122 molecule in both docking orientations (**Figure 4.2A**). The middle bulge, as a result of 1-nt mismatch is represented as C10 and G10' in the siRNA molecule (**Figure 4.2A**). Assuming the mismatch causes C10 and G10' to push away from each other, they may possibly be forced against Val69, Thr111 and/or Thr122 in both p19 monomers (**Figure 4.2C**). Hence, mutating Val69, Thr111 and Thr122 to residues with smaller side chains may also aid in accommodating the middle bulge. For Thr111 and Thr122, substitution to a serine residue will reduce the size of the side chain by a methyl group while retaining the hydroxyl group for any potential new interactions with the miR-122 phosphate backbone. For Val69, we decided to substitute to an alanine instead of a glycine residue since glycine is thought to be destabilizing for β -sheet structure [153-156] which could have been detrimental to the global structure and stability of p19.

Therefore, the first set of 4 his-tagged p19 mutants included p19-V69A, p19-Y73S, p19-T111S and p19-T122S, as suggested above, were created by site-directed mutagenesis. The mutant constructs were overexpressed in *E. coli*, purified by Ni²⁺-affinity chromatography and then with size exclusion chromatography to >95% purity as determined by SDS-PAGE analysis (**Figure S4.1** of Supporting Information). All p19 mutants were shown to have similar size exclusion elution profiles as wild type p19 (data not shown), suggesting that the introduced mutation did not disrupt p19 dimerization, which is essential for its binding to small RNAs [28,32]. The purified samples were subjected to subsequent analyses.

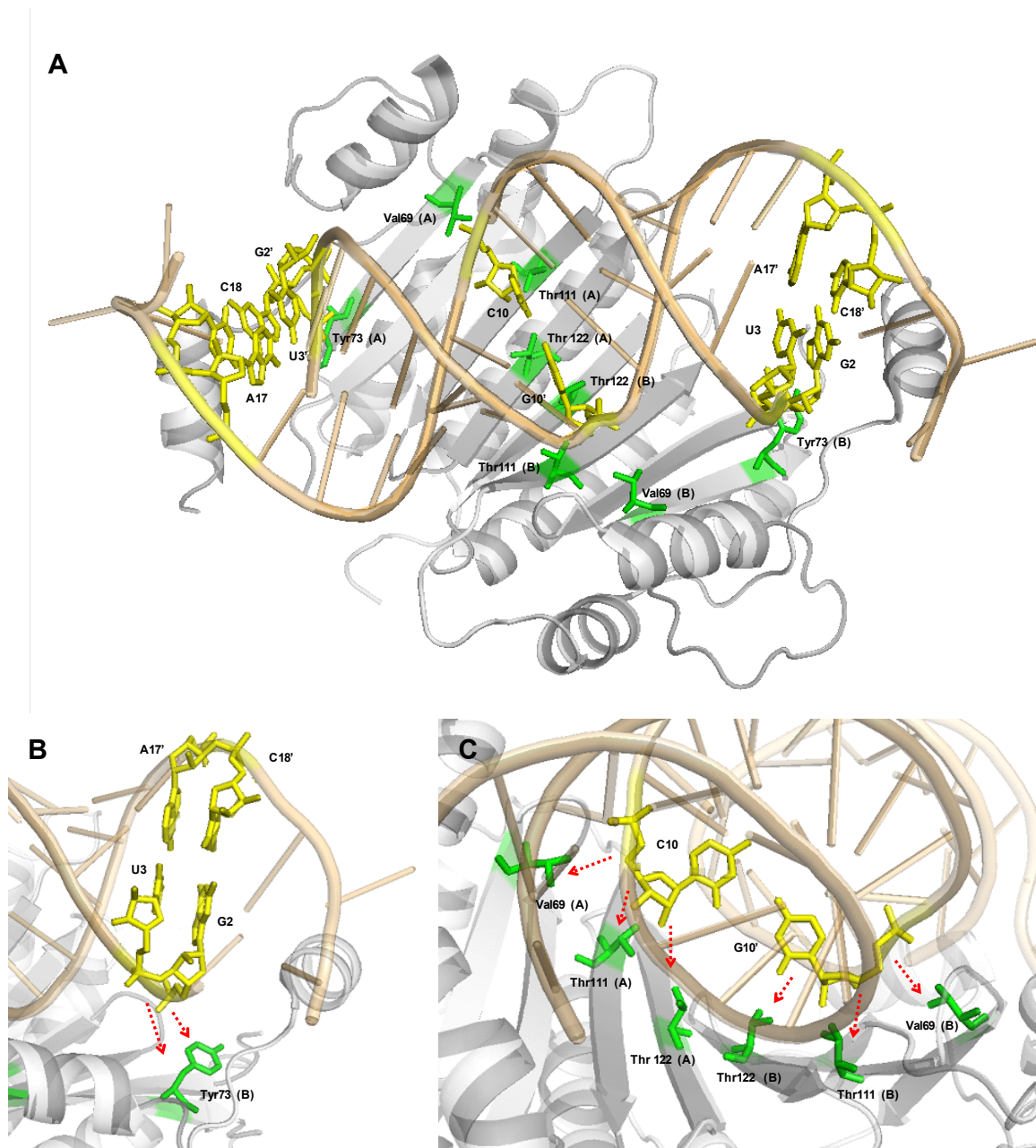


Figure 4.2: Mutagenesis strategy for altering the p19 binding site based on structural predictions

(A) A ribbon diagram of CIRV p19 crystal structure in complex with siRNA (5) (PDB code: 1RPU). Nucleotide bases of the siRNA molecule that are hypothesized to be in similar location as the bulges in the miR-122 molecule (from both docking orientations) are highlighted in yellow. The residues of the p19 binding surface which are predicted to be in close proximity to the bulges in miR-122 are highlighted in green. These residues (Val69, Tyr73, Thr111 and Thr122) are located on the RNA-binding surface of p19 but do not display any direct interaction with the siRNA. The p19 amino acid residues from each monomer subunit are distinguished by designating them A and B. The siRNA bases from each strand are distinguished by number priming. (B) Base-pairing mismatch of the side

bulge in miR-122 represented by base pairs G2'-C18 and U3'-A17 in the siRNA molecule. The mismatch may cause both G2' and U3'' to push back in the direction as indicated by the red arrows against the residue in green. (C) Base-pairing mismatch of the middle bulge in miR-122 as represented by base pair C10-G10' in the siRNA molecule. The mismatch may cause both C10 and G10' to push back in the direction as indicated by the red arrows against the p19 residues in green.

Enhanced Affinity of p19-T111S for miR-122 *in vitro*

To investigate whether the p19 mutants have enhanced affinity for miR-122 and how their binding behaviours to other small RNAs compare to wild type p19, electrophoretic mobility shift assays (EMSA) were performed. This technique is commonly used to assess protein-nucleic acid interactions, including the interactions between p19 or p19-GST fusion protein with small RNAs [24,25,28,32,75,80]. In our previous study, we analyzed the binding affinity and specificity of p19 with various small RNAs using fluorescence-based EMSAs, and the results were consistent with values measured by fluorescence polarization, a method that gives the true equilibrium of the molecular interactions [157,158].

The EMSA experiments were performed by complexing Cy3-labeled RNA with various concentrations of p19 in solution. **Figure 4.3A** illustrates a representative EMSA experiment showing the interaction between the p19 and the Cy3-labeled CSK siRNA (21-mer). There is a gradual increase in the fluorescence intensity of the slower migrating bands with an increase in p19 concentration, corresponding to an increase in the labelled small RNA population in complex with p19. In contrast, there was a gradual decrease in intensity of the faster migrating bands corresponding to unbound labelled miRNA. The band intensities corresponding to the p19-small RNA complex and the unbound RNA were measured by densitometry and plotted as a function of p19 concentration. The curves of the direct binding

experiment also show a dose-response relationship between the small RNAs and p19 (**Figure 4.3B**).

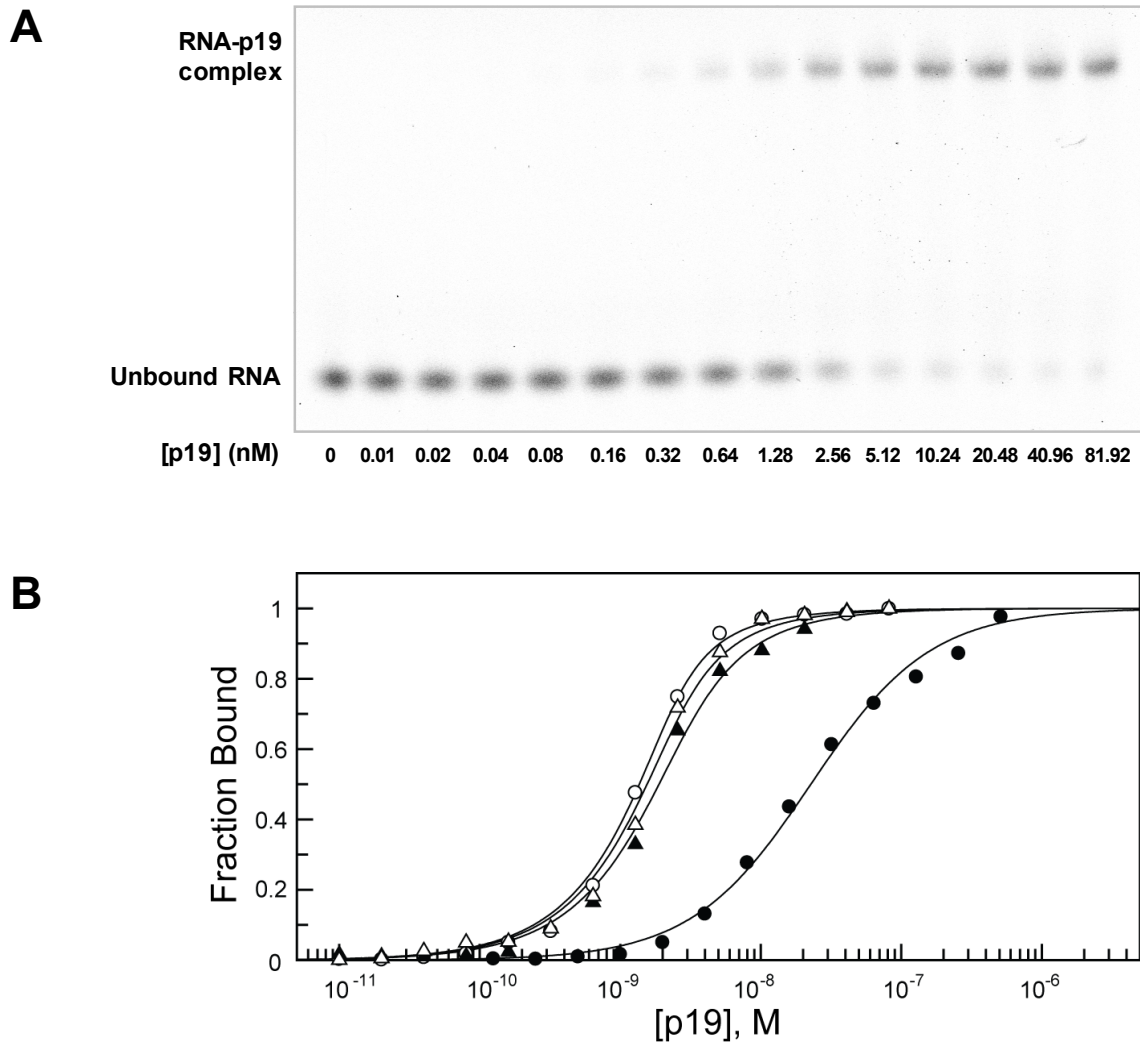


Figure 4.3: Electrophoretic mobility shift assay of p19-small RNA interactions

(A) A representative gel obtained from an electrophoretic mobility shift assay performed with 2 nM Cy3-labeled CSK-siRNA (21-mer) and various concentrations of p19 protein. (B) Representative analysis of direct binding between p19-WT with 21-mer CSK-siRNA (open circle), p19-WT with miR-122 (closed circle), p19-T111S with CSK-siRNA (open triangle), and p19-T111S with miR-122 (closed triangle). The fraction of RNA bound by p19 (the band intensity corresponding to the band shift of p19-RNA complex formation over the sum of band intensities from the complex and unbound RNA) is plotted against various concentrations of p19.

Out of the initial four p19 mutants generated, p19-T111S was the sole mutant to display enhanced affinity for miR-122 relative to wild type. Mutants p19-V69A and p19-T122S both show significantly reduced ability to bind miR-122, therefore we did not pursue those constructs for further analysis (**Table S4.2** of Supporting Information). In the more extreme case, p19-Y73S shows no observable binding (**Table S2** of Supporting Information). The dissociation constant of p19-T111S with Cy3-miR-122 obtained by EMSA (0.4 ± 0.1 nM) was found to be almost 50-fold enhancement compared to wild type (19 ± 4 nM) (**Table 4.1**). Notably, T111S displayed minimal difference in binding affinity for the canonical ligand, 21-nt siRNA (0.27 ± 0.06 nM), compared to wild type p19 (0.21 ± 0.05 nM) (**Table 4.1**).

To ensure that the p19-T111S retained the selective binding properties of wild type p19, we analyzed its binding to a longer siRNA (28-mer) and a 21-nt single-stranded (ss) RNA by EMSA. We were unable to reach saturation using the 28-mer siRNA due to solubility limitation of the p19 protein but the dissociation constant was estimated to be in the 10 μ M range (**Table S4.2** of Supporting Information). Similarly, p19-T111S displayed no binding for the 21-nt ssRNA (**Table S4.2** of Supporting Information) as no slower migrating band corresponding to p19-small RNA complex was observed. The results demonstrate that, along with the enhanced affinity to miR-122, the p19-T111S mutant retains the size-selectivity and specificity to 21-nt siRNA duplex which is consistent with the known binding behaviour of wild type p19 from previous studies [28,74,80].

Table 4.1 Dissociation constants (K_d) of p19-WT and T111 mutants with small RNAs

	K_d (nM)	
	Cy3-miR-122	Cy3-CSK siRNA (21-mer)
p19-WT	19 ± 4	0.21 ± 0.05
p19-T111S	0.4 ± 0.1	0.27 ± 0.06
p19-T111H	0.4 ± 0.1	0.21 ± 0.02
p19-T111Y	6 ± 1	0.38 ± 0.08
p19-T111A	>100 ^a	0.24 ± 0.06

^a Saturation of binding was not achieved at concentration up to 1 μ M of the p19 protein.

Mutational Analyses of the Thr111 p19 Residue

To further investigate the high-affinity binding resulting from mutating T111S, we created three other mutants at this position: T111H, T111A and T111Y. As mentioned, our initial strategy was based on creating a ‘pocket’ in the p19 binding site, to accommodate the bulges in miR-122. We chose to expand the mutational analysis at this site in order to delineate whether the high affinity binding of p19-T111S was due to a decrease in size of the side chain and/or the formation of new hydrogen bonding interactions. By comparing the binding affinities of all the T111 mutants for miR-122 using EMSA, we have deduced that retaining the hydrogen bonding capacity of the residue at the 111 position is more critical for high-affinity binding of miR-122 than simply creating a ‘pocket’ with a smaller residue. The T111H mutant, which possesses a slightly bulkier side chain but retains hydrogen-bonding capacity, displays the same high affinity for miR-122 (0.4 ± 0.1 nM) as T111S, whereas T111A, with a smaller side chain but no hydrogen bonding capacity displays a dramatically reduced affinity for miR-122 (>100 nM) compared to wild type p19. The T111Y mutant,

with a bulky, aromatic side chain but also hydrogen bonding capacity did not bind as well as the T111S mutant, although still exhibits a modest (~3-fold) increase in affinity for miR-122 than wild type p19. Overall, the data suggest that the increase in affinity of the p19-T111S and p19-T111H mutants for miR-122 rely on forming new hydrogen bonding interactions which are not available with threonine. The differences between the interactions provided by threonine and the mutated residues suggest that the new, high affinity interactions between residue 111 and miR-122 require specific orientations of the side chain. Additionally, these new interactions appear somewhat susceptible to steric interference, where bulkier side chains capable of hydrogen bonding do not provide high affinity interactions with miR-122. Notably, the mutations at the T111 residue do not result in significant changes to the affinity of p19 for its canonical ligand, 21-nt siRNA (**Table 4.1**). This is consistent with the X-ray crystal structure that no observable interaction is formed between Thr111 and siRNA [28]. Our data suggests that, as hypothesized, p19 binding miR-122 retains the majority of the interactions which allow siRNA binding. Importantly, the data suggests that there are unique interactions between p19 and miR-122 which are not involved in p19 binding siRNA. This observation substantiates the possibility of engineering p19 mutants with high affinity and selectivity for miR-122.

Structural and Thermal Analysis of p19-T111 Mutants with Circular

Dichroism

In order to determine whether there were any large structural changes associated with the p19-T111 mutants, circular dichroism (CD) spectra were acquired and compared to wild type p19. As shown in **Figure 4.4A**, the far-UV CD spectra of wild type p19 and all of the p19

T111 mutants overlap well with each other, and all possessed minima at 208 and 222 nm suggesting significant α -helical content. This was confirmed by secondary structure deconvolution using the suite of programs provided by CDPro where wild type p19 displays an average of $\sim 24\%$ α -helical and $\sim 25\%$ β -sheet content, and the mutants display $\sim 23\text{-}25\%$ α -helical content and $\sim 25\%$ β -sheet content, except for T111Y which displays $\sim 27\%$ β -sheet content. The similarity in CD spectra indicates that the secondary structure of wild type p19 and the p19-T111 mutants are very similar and that the mutation did not introduce significant changes to the global structure of the protein.

Thermal denaturation analysis was also performed on the p19-T111 mutants and compared to that of wild type p19 by monitoring ellipticity at 222 nm, in order to investigate whether any structural changes could be associated with differing affinities of the p19-T111 mutants for miR-122 versus wild type p19 (**Figure 4.4B**). All of the p19-T111 mutants and wild type p19 showed a significant loss of CD signal intensity over a narrow range of temperatures, with ellipticity values being close to zero at 90 °C. For wild type p19, the thermal unfolding transition temperature or melting temperature (T_m) is ~ 61 °C. All of the p19-T111 mutants show a slightly decreased T_m value. The two mutants with increased affinity for miR-122, T111S and T111H, both show the largest decrease in T_m , at ~ 55 °C and ~ 57 °C, respectively. The difference in the denaturation temperatures indicates that the p19-T111 mutants are less thermally stable than the wild type p19, although the extent of the change does not reflect any decrease in overall viability of the mutants, as can be seen from their binding affinities and size exclusion profiles. The thermostability of proteins has been inversely correlated with protein flexibility [159,160]. The reduced thermostability and thus increased structural flexibility of p19-T111S and p19-T111H may potentially contribute to the enhanced affinity

for miR-122. One of our previous rationale for p19's ability to bind miR-122 at nanomolar affinity maybe linked to its inherent flexibility [80]. Conformational flexibility is thought to be important and widely recognized in protein-nucleic acid interactions [161-163].

Specifically in protein-RNA complexes, this is seen in the binding of Ro protein to it's RNA target [163]. In addition, the flexibility in the hinge region of Dicer enzyme is crucial for its dsRNA binding and processing [162]. Similarly, the increased flexibility of the p19-T111S protein may help ease the fit of the bulky secondary structures inherent in the miR-122 molecule and further enhance the association of the p19-miR-122 complex.

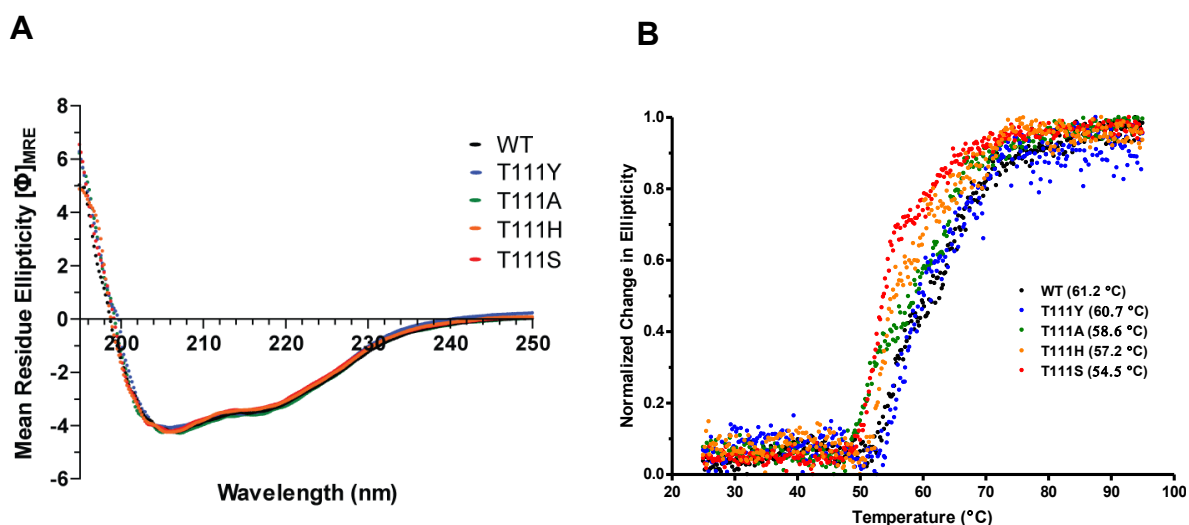


Figure 4.4: Circular dichroism of p19-WT and p19-T111S

(A) Far UV spectra and (B) thermal denaturation profiles monitored at 222nm.

p19-Y73S Mutant Incapable of Binding Small RNA

The p19-Y73S mutant showed no observable binding to siRNA or miR-122. In order to understand why this mutant is incapable of binding, we acquired CD spectra to look for

alterations in protein structure. In comparing the CD spectra of p19-Y73S and wild type p19, we observe a clear difference in the spectra below 210 nm (**Figure S2A** of Supporting Information). This suggests that the Y73S mutation induces a conformational change in p19. Interestingly, the thermal denaturation analysis of p19-Y73S shows that this mutant has slightly higher thermostability than wild type p19 (**Figure S2B** of Supporting Information). It is possible these data reflect that p19-Y73S has adopted a more rigid conformation which is not conducive to ligand binding. From the crystal structure data, it can be seen that there are several basic residues in this region of the C-terminus (R72, R75, R85), which do not contact the siRNA directly but rather form salt bridges to residues on the N-terminus (Glu17, Glu35, Glu41) [28]. These salt bridges have been hypothesized to be important for positioning the α -helix of the N-terminus which carries the end-capping tryptophan residues [28]. Previous studies examining the role of p19 in viral infection, symptom induction, and spread in host plants have created mutants of Tomato Bushy Stunt virus (TBSV) p19, where several of the basic residues from this C-terminal region were mutated to glycine (p19-R72G, p19-RR75-78GG), which resulted in p19 losing all of its *in vivo* functionality as a viral suppressor of RNA silencing [164,165]. The R72G mutation, however, likely resulted in buried charged residues which could have altered the global structure of the protein [28]. Further functional studies have used the p19-RR75-78GG mutant to demonstrate the link between siRNA binding and the *in vivo* functionality and symptom induction by p19, since it was demonstrated that p19-RR75-78GG forms dimers that are incapable of capturing siRNA *in vivo* [29,146]. Another residue near to Y73, K71, forms salt bridges with the siRNA sugar phosphate backbone [28]. The null mutant, p19-Y73S is interesting because, from the crystal structure data, the Y73 residue does not appear to be directly involved in siRNA

binding or stabilizing the end-capping helices. Thus it is possible that either the mutation p19-Y73S alters the salt bridge interactions between nearby residues which have been shown to be crucial for siRNA binding *in vivo*, or the Y73 residue is directly responsible for currently unknown interactions critical for binding siRNA. It is possible that the p19-Y73S mutant may be a more appropriate null p19 mutant with potentially less conformational changes that may be associated with the rather drastic mutations of p19-RR75-78GG or p19-R72G. Further mutational analysis of this residue, as well as structural studies are currently being pursued in our laboratory.

Enhanced Affinity of p19-T111S for miR-122 *in situ*

To demonstrate that p19-T111S can also bind miR-122 with higher affinity than p19-WT in a cellular environment, we have established a method to quantify the relative level of miR-122 captured by p19-T111S compared to p19-WT in Huh7.5 cells, a hepatoma cell line that expresses high levels of miR-122 [166] as summarized in the schematic diagram in **Figure 4.5**. Briefly, Huh7.5 cells transiently expressing His-tagged p19-WT or p19-T111S proteins are harvested and lysed. The His-tagged p19 proteins in the lysate are then captured on a Ni²⁺-affinity column, with the flow-through and the eluate collected for subsequent qPCR analysis. The RNA extracted from the eluate fraction, containing p19 proteins eluted from the column, represents the RNA which was bound to p19. After total RNA extractions, the amount of miR-122 in these fractions was quantified by qPCR using probes against the sequence of miR-122 and subsequently normalized to the expression levels of a reference gene, RNU6B (**Figure 4.5**). The results showed that there is a 2.5 ± 0.5 fold enrichment of miR-122 in the eluate fraction from cells expressing p19-T111S compared to wild type. The flow-through fraction also displayed the expected trend, with more miR-122 present in the

p19-WT flow through than the p19-T111S flow through. This *in situ* miRNA sequestration assay suggests that there is a ~2.5-fold enhancement in the sequestration of miR-122 by p19-T111S relative to the wild type protein in Huh7.5 cells.

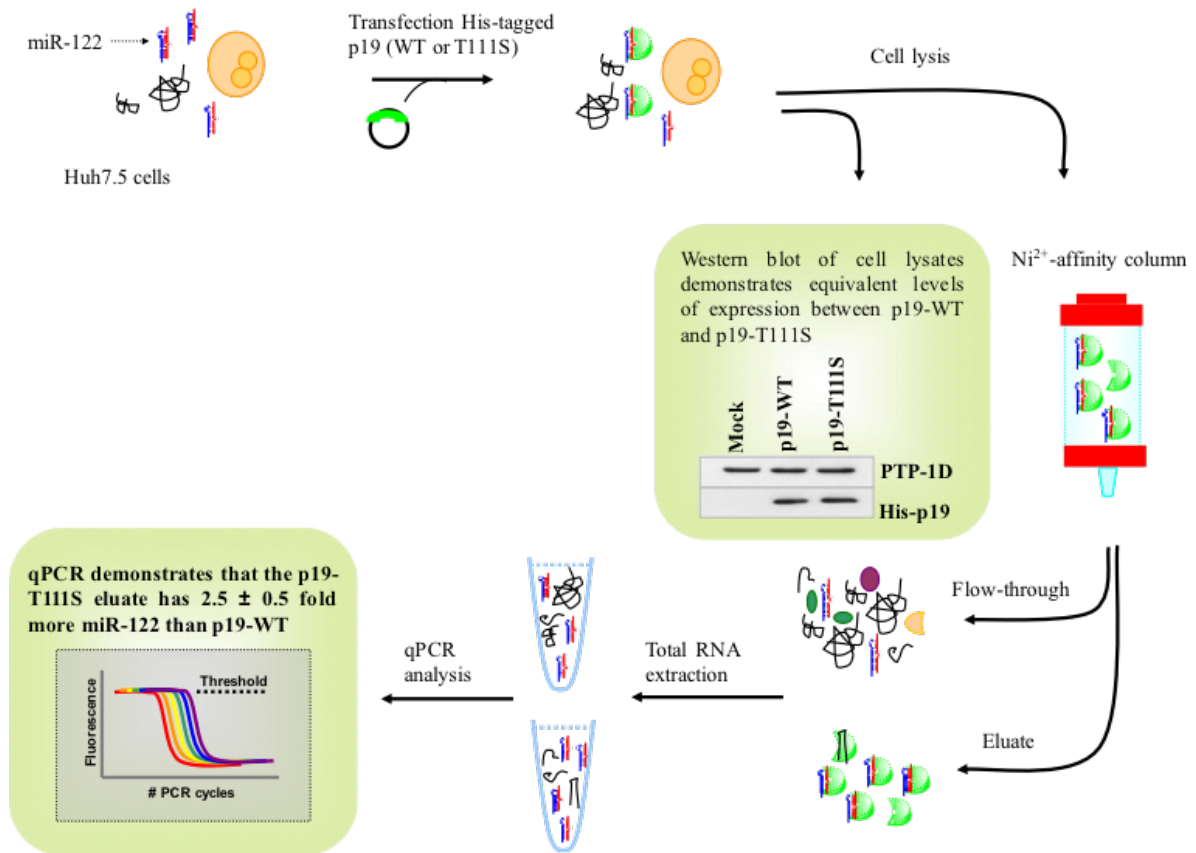


Figure 4.5: Schematic of the *in situ* miR-122 capture experiment

Huh7.5 cells are transiently transfected with either wild type p19 or the T111S mutant. After 24 hours, cells are lysed and p19 expression is analyzed by Western blot. Cells expressing wild type p19 and p19-T111S show very similar expression levels, indicated by the relative (%) density of the p19 band to the PTP-1D loading control. The His-tagged p19 proteins in the lysate are then captured on a Ni²⁺-affinity column. The flow-through fraction and eluate from the column are collected and subject to total RNA extraction. The miR-122 content of the samples is then quantified via qPCR. The high-affinity p19-T111S mutant captured 2.5 ± 0.5 fold more miR-122 than wild type p19.

Conclusion

In this work, we describe our mutational analysis of the p19 protein in binding a non-canonical ligand, miR-122, with the aim of creating high-affinity mutants. In our previous studies, we determined p19 as capable of binding this non-canonical ligand, but with lower affinity than for its canonical 21-nt siRNA ligand. By mutating the residues which we have predicted to be interacting with the bulges in the secondary structure of miR-122, we have created two mutants capable of binding miR-122 with nearly ~50-fold higher affinity than wild type p19. This work shows that it is possible to engineer RBPs such as p19 for accommodating secondary structure of non-canonical ligands. The higher affinity appears to be due to new interactions which rely on hydrogen bonding but can be sterically hindered by bulky residues. Furthermore, the new interactions created through mutagenesis which affect binding affinity are unique to miR-122, and do not affect the affinity of p19 for siRNA. This observation corroborates our hypothesis that the binding surface of p19 interacts differently with miRNAs than siRNAs, thereby allowing the possibility that p19 may be engineered to bind miR-122 with high affinity and specificity. Our aim to alter p19 to preferentially bind specific small RNAs is motivated by possible applications in interrogating the role of specific small RNAs in infectious model systems. Our focus was to identify mutants of p19 with a higher affinity for miR-122, for use in human cells with potential application in inhibiting hepatitis C viral replication. Theoretically, our approach described here could be used to identify mutants with high affinity for other small RNAs in order to understand their roles in other diseases, infections and cellular development.

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Chapter 5 - General Discussion and Future Directions

Modulating the RNA silencing pathway

The previous three chapters centre on our progress developing the p19 protein for novel applications in biotechnology; specifically, for sequestering and delivering small RNAs and engineering the p19 binding site for altered ligand specificity. This work contributes to the larger field of developing small RNAs for biotechnology and human therapy. The ability to modulate the activity of the RNA silencing pathway in biological systems offers immense opportunities for our understanding of biological systems and host-pathogen interactions, and in the treatment of human disease.

VSRS have evolved diverse mechanisms for modulating the RNA silencing pathway [78]. By exploring the function of these proteins outside the context of endogenous viral infection but rather as recombinant protein-based tools, we gain a structurally and functionally diverse ‘tool-kit’ with which to modulate the components of the RNA silencing pathway both in living systems and *in vitro*. The work presented in this thesis explores novel applications of the tombusviral p19 protein, which is the most well studied VSRS. Biochemical annotation of other VSRS is steadily increasing but there remains a lack of structural information for these proteins, as only three VSRS have reliable crystal structure information available (**Figure 1.2**). The gains made in this thesis work in developing the p19 protein for applications in sequestering, detecting and delivering small RNAs stem from detailed understanding of the structure and biochemical function of the p19 protein, thus further exploration of the biochemical properties and biological function of other VSRS will allow both gains in understanding for host-pathogen interactions and will provide novel RNA binding platforms for biotechnology tools aimed at probing RNA function both *in vitro* and in living systems.

Applying p19 in human cells

In **Chapter 2** we present a chemical biology approach for inhibiting the RNA silencing pathway by applying the p19 protein in conjunction with a small molecule, suramin, for potent inhibition of the pathway in Huh7 cells. In this study, the expression of p19 in Huh7 cells, without the suramin treatment, shows only modest inhibition of the activity of an exogenously transfected siRNA, demonstrating that p19 does not have consistently robust activity in human cells [167]. Future work in developing p19 as an effective probe and inhibitor of RNA silencing in human cells should aim to characterize the factors that influence its activity, including expression level and cell-type specific interactions, which should lend insight into the differences in p19's activity between plant and human cell systems.

As discussed in **Chapter 1**, p19's ability to suppress RNA silencing in plants is well established and is finding substantial applications in biotechnology, where ectopic expression of p19 is allowing enhanced foreign gene expression in plants and is being applied for therapeutic antibody production [47-50]. From plant studies, it is well established that p19 expression levels need to be maintained at a high level, as is observed in viral infection, for it to effectively suppress the pathway [13,168]. Recently this observation has been extended to the activity of p19 in human cells, whereby expressing p19 at high levels in human embryonic kidney (B6) cells as part of an engineered adenovirus allowed 100-fold increase in adenoviral copy number and thereby increased the oncolytic activity of the engineered virus [53]. Thus future work applying p19 to inhibit RNA silencing in human cells should express p19 from an adenoviral vector in order to sustain higher expression levels than was

possible with transient expression from plasmid DNA, which would likely allow for higher suppression activity.

Future work should thoroughly examine p19's *in vivo* protein interacting partners in human cells in order to better understand and optimize its activity as a tool to inhibit RNA silencing. Protein interactions may be examined through immunoprecipitation studies, where p19 would be expressed in a range of human cell lines and interacting partners are identified through pull-down and subsequent analysis by mass spectrometry and western blotting. These experiments would help elucidate if p19 is interacting with human proteins, and if there are cell-type specific interactions which could be linked to differences in its activity. Further, by performing these experiments expressing p19 at different levels through plasmid or viral vectors the impact of expression level on its interactions could be examined.

Understanding p19's interactions with and impact on the endogenous small RNA population in human cells will be critical to its development as a tool in human cell systems. RNA interacting partners should be identified through applying crosslinking immunoprecipitation (CLIP) assays, where p19 is covalently crosslinked to RNA interacting partners through exposing cells to UV light, isolating the p19-RNA complexes through immunoprecipitation, and subsequent identification of the bound RNAs through high-throughput sequencing [169,170]. CLIP assays will help elucidate whether differences in the small RNA population of different cell types is impacting p19's activity in these cell systems. Understanding the impact of p19 on the endogenous miRNA expression profile of human cells should be performed through microarray analysis or next-generation sequencing of the small RNA population of cells expressing p19. Both the CLIP assays and RNA profiling studies would compare p19 expressing cells to mock transfected cells, and could also examine p19

mutants with enhanced affinity for human miRNAs (p19-T111S/T111H) and p19 mutants incapable of binding small RNAs (p19-Y73S) [36]. These analyses will help characterize p19's activity in human cells and examine cell type or expression level specific patterns which could be linked to differences in p19's suppressor activity. Further, understanding p19's impact on miRNA mediated gene regulation will be essential to its viability as a biotechnology tool for human systems.

Small molecule modulators of RNA silencing

In **Chapter 2**, we investigate the activity of three small molecules, suramin, ATA, and oxydopamine for their impact on p19:RNA interactions. These small molecules have been reported as inhibitors of human AGO-2 and a range of other RBPs [90,91,94]. Our observations that both ATA and suramin reversibly inhibit p19:RNA interactions is consistent with previous observations that these molecules exhibit electrostatic interactions with the positively charged residues of the binding site, effectively blocking the protein from binding RNA. The differences in suramin's potency for inhibiting p19 versus AGO-2 may be explained by the differences in the domain structure of their RNA binding sites, as observed in the crystal structures of these proteins in complex with RNA ligands [28,105].

Future work should clarify the mechanisms of how these small molecules are inhibiting both p19 and AGO-2 through methods such as isothermal calorimetry as is performed for characterizing ATA-mediated inhibition of the HCV NS3 helicase [101]. The lack of specificity of these compounds may preclude their broader application as an inhibitors of RNA silencing. Our experiments with suramin, however, suggest that a level of specificity of suramin is achieved due to differences in potency for the inhibition of AGO-2 and p19 *in*

vitro and the compatibility of their treatment *in vivo*. Therefore, depending on the biological context, absolute specificity of the compounds may not be necessary. Through optimizing the applied concentration of the compounds, the relative impact on cellular RBPs could be tuned. Profiling the impact of suramin on biological processes such as transcription, translation and the activity of noncoding RNAs should be pursued at a range of concentrations in order to more deeply understand its activity as an inhibitor of RNA silencing. Broadly inhibiting the RNA silencing pathway is desirable under certain circumstances; as discussed in **Chapter 1**, there are extensive applications of p19 in biotechnology for increasing foreign gene expression in plants [47-50], baculovirus titers for protein expression in insect cells [52], and adenoviral titers for oncolytic treatments [53,54]. Thus a chemical biology approach similar to what is presented in **Chapter 2** could be applied in these contexts to further enhance the activity of p19.

The general field of small molecule targeting of protein-RNA interactions or specific RNA sequences and structures is rapidly expanding and future work stemming from this thesis could apply a similar approach as reported in **Chapter 2** to an expanded repertoire of small molecule inhibitors of the pathway [171-173]. In a therapeutic context, it is more common that the aim is to modulate the activity of specific small RNA sequences rather than instigating broad-spectrum inhibition of the pathway. There have been reports of a small molecule inhibiting the action of a specific human miRNAs, such as oncogenic miR-21, through blocking its interaction with human AGO-2, with specificity being garnered from its secondary structures [87]. High throughput cell-based screening has resulted in the discovery of inhibitors of miR-122 which function to suppress miR-122 transcription without impacting other miRNA sequences and which reduced replication of HCV [88]. Another

miR-122 specific inhibitor has been reported which consists of a short oligonucleotide that targets the miRNA seed sequence conjugated to a small molecule which has been rationally designed to bind the AGO-2 binding site [174]. These are a few examples of the many inhibitors which have recently been described which have altered mechanisms and enhanced specificity from the three small molecules tested in **Chapter 2** for miRNA inhibition [171-173]. The enhanced properties of these compounds may make them more attractive for a combination approach with p19 expression for inhibiting the pathway. Given that we are also examining the opportunities for engineering p19 to bind with high affinity to specific human miRNAs, future experiments could examine the impact of p19 mutants with enhanced affinity for human miR-122 (p19-T111S/T111H) in conjunction with suramin treatment or other small molecules which specifically modulate the activity of miR-122 [88] on the activity of miR-122 in human hepatoma cells. The impact of the co-treatment on the both miR-122 expression levels and its downstream gene regulatory targets would be examined, and it would be pertinent to investigate whether the combination treatment would have a synergistic effect and possibly lowering the effective concentration of the inhibitors needed to inhibit miRNA function.

Engineering protein based siRNA delivery agents

In **Chapter 3** we describe engineering the p19 protein as an siRNA delivery agent through fusing a linked p19 dimer with a CPP to generate the fusion protein 2x-p19-TAT. We observe that this fusion protein effectively translocates bound siRNAs across the cell membrane to effect gene knockdown in human cells in culture. Future work for this study should extend the analysis of the activity of 2x-p19-TAT to a range of cell types, including primary cells. In **Chapter 3**, cellular imaging experiments of the delivery of fluorescently

labeled siRNA were performed and we observed that all the treated cells displayed fluorescence. To gain a more detailed understanding of the proportion of cells transfected via the 2x-p19-TAT treatment, future work should include flow cytometry analysis of treated cells. In these experiments, 2x-p19-TAT would deliver fluorescently labeled siRNA and flow cytometry would report on the proportion of cells successfully transfected. Furthermore, by applying this treatment to cells constitutively expressing a fluorescent reporter protein and delivering an siRNA which targets the mRNA of the reporter gene, flow cytometry experiments could further report on the extent of gene knockdown in the transfected cells. By performing these experiments, we will gain a more accurate understanding of the gene knockdown potential of the 2x-p19-TAT treatment which was not possible through luciferase assays, which report on an overall average of the gene knockdown activity across the entire treated cell population. The treatments should be optimized through testing a range of ratios of protein:RNA delivery complexes, which should allow improved potency of the treatment.

The work presented in **Chapter 3** show that p19-mediated delivery of therapeutic RNAs is a promising strategy, however more work is needed to develop the biotechnology further for applicability for *in vivo* siRNA delivery. It will be critical for this technology to address the issue of endosome entrapment in order to enhance its potency and efficiency of siRNA delivery. Future experiments should explore other methods of enhancing endosome escape which do not involve a peptide co-treatment. One option is to engineer fusion proteins of p19 with endosomolytic peptides, which is a strategy we have been exploring to enhance the efficiency of cytoplasmic siRNA delivery. Future work could further build on this technology by generating other p19 fusion proteins with cell type specific targeting peptides,

in lieu of the general cell entry peptide TAT, to explore tissue specific delivery of siRNA mediated by p19 fusion proteins.

In moving towards *in vivo* siRNA delivery, it will be critical to assess the immunogenicity profile of the 2x-p19-TAT:siRNA treatment. A major barrier to the wider application of siRNA therapeutics is the engagement of the innate immune response in response to the administered siRNA or to the delivery platform itself, which is undesirable because of the toxicity associated with excessive cytokine release [175]. Specifically, administration of siRNA molecules are associated with increased production of inflammatory cytokines tumor necrosis factor alpha (TNF α), interleukin 6, and interferons, which are induced either through toll-like receptors (TLRs) or non-TLR mediated pathways such as retinoic acid inducible gene 1 (RIG1) mediated responses to foreign RNA. Preliminary experiments should assess the engagement of the innate immune response in response to 2x-p19-TAT treatment by measuring the interferon and TNF α response from treating cultured primary human peripheral blood mononuclear cells, as is the standard protocol for similar technology [127,176]. After the immunogenicity profile is assessed, 2x-p19-TAT should be applied for *in vivo* siRNA delivery. The 2x-p19-TAT treatment could be administered to mice intranasally as performed with TAT-DRBD [127] or could be administered subcutaneously to the bloodstream as performed with other protein-mediated siRNA delivery agents [177]. Both protein and siRNA therapeutics may induce inflammatory syndromes after systemic administration through induction of the adaptive immune response [175,178,179]. Thus it will be critical to examine whether systemic administration of the 2x-p19-TAT treatment generates neutralizing antibodies, which would be undesirable as an adaptive immune response could decrease the effectiveness of the treatment by promoting clearance and

potentially causing harm to the animal if there is cross-reactivity with endogenous antigens [176,179].

In the development of therapeutic RNAs, chemical modifications of synthetic RNAs increase their serum stability, their ability to cross the cellular membrane, and decrease their immunogenicity [179]. For example, backbone modifications such as methylating the 2' hydroxyls of the ribose (2' O-Methyl) dramatically enhances the stability and decreases the immunogenicity of the siRNA [180]. As these chemically modified RNAs are currently being heavily researched for therapeutic prospects, it would be pertinent to investigate p19's ability to bind chemically modified RNAs and the ability of 2x-p19-TAT to deliver chemically modified RNAs across the cellular membrane. Given that the p19 binding surface engages the hydroxyls of the backbone, it is possible that p19 would have reduced affinity for backbone modified RNAs. Future experiments should examine the available crystal structures of p19 in complex with siRNA, in order to identify positions in the siRNA which would accommodate backbone modified nucleosides in the RNA without perturbing interactions with the p19 binding surface. Given that p19 only covers one half of the siRNA duplex surface, there are several locations which would likely be amenable to modification without disrupting p19's ability to engage the RNA. With the extension of the technology reported in **Chapter 3** to incorporate chemically modified RNAs, it would further enhance p19's applicability as an *in vivo* siRNA delivery agent.

Efficient delivery across the cell membrane and into the cytoplasm remains the biggest challenge facing siRNA therapeutics. A range of materials and platforms are being investigated as siRNA delivery agents for application in research settings and human therapy. Lipid nanoparticles remain the most widely researched, but these approaches

exhibit challenges in biodistribution and bioavailability, often only allowing delivery to the liver and difficulty penetrating solid tumors due to their large size [181,182]. Protein based methods, because of their smaller size and biochemical properties, can offer novel solutions to this problem. Furthermore, the amenability of proteins to genetic fusions allows the facile conjugation of CPPs to allow uptake across the cell membrane or cell targeting peptides to offer cell-type specific targeting. Engineered RBPs are therefore increasingly being investigated as delivery platforms [127,130,131,177,183-185].

Future work stemming from this thesis should seek to understand the relative advantages of the biochemical and structural properties of p19 as a platform for siRNA delivery, in comparison to other RBPs which are either previously reported as siRNA delivery platforms (DRBD, TAV 2b) or amenable to development as siRNA delivery agents, such as other VSRS. These alternate RBP-based siRNA delivery platforms (DRBD, TAV 2b, and p19) all exhibit differences in their size, structural motifs, and properties of binding siRNA ligands; a direct comparison is required to determine which properties of RBPs provide specific advantages as platforms for siRNA delivery. A direct comparison of RBP delivery platforms would require their modification with the same cell penetration moiety (TAT) as described in the work reported here (2x-p19-TAT), and characterizing their siRNA delivery activities in parallel under the same conditions. A direct comparison would bring clarity to the larger field of protein-based siRNA delivery agents, as it would lend mechanistic insights into the relative assets of specific structural motifs and biochemical properties required for efficient cell penetration activity and cytoplasmic delivery of siRNA. For example, it remains unclear to what extent high affinity interaction between the RBP:siRNA provides an advantage for siRNA delivery efficiency. In **Chapter 3**, we hypothesize that p19's high affinity for siRNA

and its unique structure are likely assets for enhancing the stability of the complex and increasing its potential activity as an *in vivo* delivery agent compared to other available methods. p19's high affinity would limit the amount of disassociation before cell penetration of the complex, and p19's ability to cap the ends of the bound RNA duplex effectively protects its bound RNA from RNase enzymes, which are prevalent in the endosome and in the bloodstream and which would decrease the efficiency of siRNA delivery [183]. It remains to be determined, however, to what extent high affinity is an asset to the delivery platform once the complex crosses the cell membrane, given that this stage requires the release of the siRNA to the RNA silencing machinery to effect gene knockdown. Therefore, a comprehensive comparison of different RBP-based siRNA delivery agents with different affinities and structural properties will allow us to identify the optimal properties of a RBP-based delivery agent. Furthermore, rational mutagenesis strategies of p19 and other RBPs, could be applied to explore the optimal affinity of the RBP delivery platform for its bound siRNA. RBP mutants with partially disrupted or enhanced binding affinities, such as those reported in **Chapter 4** could be compared for their siRNA delivery efficiency, in order to dissect the role of affinity and also optimize the siRNA delivery activity. This opportunity for 'tuning' the biochemical properties through protein engineering represents one of the benefits of a protein-mediated delivery agents which is not as readily available to other delivery technologies such as lipid nanoparticles.

Some of the biggest current advancements in biomedical and biotechnological research are due to a detailed biochemical and biophysical understanding of protein-RNA interactions, and further engineering their properties. There is a revolution in genetic engineering in the last five years with the application of a prokaryotic RNA-guided DNA cleavage system,

known as the clustered, regularly interspaced, short palindromic repeat (CRISPR)-associated (Cas) system [186]. The power of these gene editing tools is immense and applications are rapidly increasing, eclipsing the applications of RNA silencing technologies for certain applications [187]. The knowledge gained from the RNA silencing field, especially in how to deliver protein:RNA complexes into human cells, is directly contributing the advancements in Cas technologies. CPP-mediated delivery of Cas9 in complex with its guide RNA is a promising method for genetic reprogramming of human cells in culture with distinct advantages to plasmid or viral-vector based delivery; the transient nature of the protein-based delivery aids the specificity of the gene editing activity, avoiding the off-target effects and spurious genome integration which remains a large limitation in application of these gene editing technologies [188-192]. Direct delivery of the gene editing protein:RNA complexes, either through CPP-mediated delivery or peptides with cell specific tropisms [192] are rapidly finding applications both both in academic and industry settings.

Further opportunities for ‘tuning’ the activity of engineered RBPs for modulating gene expression in biological systems will come from integrating recent advancements in small molecule interactions with RNA and RBPs, as was developed in **Chapter 2**. Recent efforts have resulted in small molecule control of the Cas9 protein which allows conditional activation and enhanced specificity of gene editing [193]. Other opportunities in developing engineered RBPs for applications in biotechnology will come from the genetic incorporation of unnatural amino acids (UAAs) into these proteins which have chemical properties not available to the canonical 20 amino acids [194]. We are currently expanding the scope of recombinant p19 through incorporating UAAs which serve as bioorthogonal handles for protein labeling and conjugation, which will provide novel opportunities in tracking p19’s

localization in living systems, as well as photo-reactive UAAs which allow photo-controlled activity. Thus future work developing engineered p19 proteins with expanded functionality through UAA technology will afford novel applications of p19 for detecting RNA *in vitro* and for modulating RNA activity in living systems.

Engineering p19 for enhanced affinity for human microRNAs

In **Chapter 4**, we present progress engineering p19's RNA binding site to bind with higher affinity to a human miRNA, miR-122. We observe that there are unique interactions between residue 111 of p19 and its bound RNA, and by mutating the protein at this site (T111S/T111H) we form new high affinity interactions with the miRNA without altering its affinity for a canonical fully base paired siRNA. Future biochemical work should assess whether the p19-T111S/H mutants display enhanced affinity for other miRNA sequences and secondary structures, or if the observed enhanced affinity is specific for miR-122. We have pursued structural studies via x-ray crystallography of the WT and mutant proteins in complex with RNA and our results support the modeling presented in **Chapter 4**, where the enhanced affinity from mutating the T111 site is likely due to contacts with the central mismatch of the miR-122 sequence (*manuscript in preparation*). Central mismatches are common to most human miRNAs and are fundamental to their gene regulatory activity; central mismatches in miRNAs are critical to their function as it impacts their interactions with AGO proteins and thereby alters the mechanism of their gene regulatory activity [195,196]. Enhanced affinity for specific patterns in miRNA secondary structure would be useful for p19s applicability for *in vivo* miRNA sequestration.

Future work performing saturation mutagenesis at the T111 site and examining the resulting impact on binding with siRNA and miR-122 is currently being pursued. Future work could further extend the structure-based mutagenesis screen of the binding site presented in **Chapter 4** to other residues of the binding site. It would be further interesting to expand the mutagenesis screen with a high throughput, directed evolution approach to randomizing the residues of the binding site and selecting high affinity mutants through phage display or cell-free *in vitro* selection methodology [197,198]. Applying these strategies would likely result in new mutants with differential affinity profiles for specific RNA secondary structures than those currently described. Broadly exploring the sequence space would be particularly interesting for p19, given that its evolution has been limited by being encoded within another ORF for a cell-to-cell movement protein within the tombusviral genome. As this genetic structure is true for several VSRS, it is likely that randomization of the amino acids in their RNA binding sites and high throughput screening for specific ligand interactions would further expand the applicability of these RBPs in biotechnology.

p19 mutants with enhanced binding affinity for miRNAs, either for specific patterns of secondary structure or for longer duplexes will find applications in detecting and sequestering miRNAs both *in vitro* and *in vivo*. Targeting improved miRNA detection *in vitro* would benefit from engineering p19 to having enhanced affinity for longer duplexes, given that miRNAs are longer (19-25-bp) than p19's canonical siRNA ligands. Engineering strategies could be applied which extend the flexible linker region which position the helical 'reading heads' on the end of the RNA duplex, to allow greater accommodation of longer duplexes. The wide array of p19-based nanotechnology surrounding miRNA detection would benefit from engineered p19s with enhanced properties [68-73].

Expression of p19 offers a unique approach for sequestering miRNAs in living systems in order to repress their function and alter gene expression profiles. We demonstrate here that the T111S mutant shows enhanced ability to sequester miR-122 in Huh7 cells. Future work should extend this strategy to living systems, including mouse models. Expression of p19 from a liver-targeting adenoviral vector in mice has been shown cause a dramatic decrease in miR-122 levels [54]. It is likely that if the mutants described here were applied in a similar approach, miR-122 levels would decrease even further due to a stronger interaction between p19 and miR-122. As discussed earlier, future work applying p19 in living systems will require that the immunogenicity and toxicity associated with p19 expression will need to be assessed, as well as the overall impact on the small RNA profile.

Applying RBPs as a strategy for altering miRNA expression profiles *in vivo* remains a very new field of exploration. Recently, structure based design of the eukaryotic RRM domain allowed engineering specificity for a human miRNA precursor (pre-miR-21), which then was then applied to altering the specificity of the *Giardia* Dicer enzyme through replacing its PAZ domain. Expression of the engineered enzyme allowed degradation of miR-21, thereby decreasing the viability of human cancer cells in culture [199]. With further research, protein-based approaches for modulating miRNA activity *in vivo* may offer an alternative to current methods either in a biotechnology or therapeutic setting. Current methods predominantly involve the application of sequence-specific antisense oligonucleotides, whose development for therapeutics is hindered by difficulty in cellular delivery and the high cost of production [200]. A transient treatment with an engineered RBP would also offer alternatives to permanent modifications being explored by new gene editing technologies [186]. miRNAs are now known to play a multitude of roles in a wide array of host-pathogen

interactions, and modulating the activity of miR-122 remains an important therapeutic option for treatment of HCV [201]. The levels of circulating or tissue-specific miRNAs can also serve as biomarkers for a range of human disease, such as cancer and cardiovascular injury [202,203]. An increased repertoire of tools for detecting and modulating the activity of miRNAs is necessary and new and exciting opportunities are offered by engineered RBPs.

General Conclusion

The thesis work presented here describes novel approaches and technology based on recombinant p19 proteins towards applications in detecting, sequestering and delivering small RNAs both *in vitro* and in living systems. p19's unique binding properties have made it an important platform for biotechnology development surrounding small RNAs.

Furthermore, p19's amenability to modification have allowed us novel opportunities; altering its binding specificity for human miRNAs has allowed enhanced activity of miRNA sequestration in human cells, and generating 2x-p19-TAT fusion proteins have allowed cell penetration of p19:siRNA complexes. Expanded opportunities have also come from combining p19 with small molecule modulators of RNA silencing which have enhanced the ability to suppress RNA silencing in human cells.

This work contributes to the larger field of engineering RBPs for modulating gene expression [198]. With the discovery and engineering of CRISPR-Cas9 systems, there are now widespread applications of gene editing at the DNA level; however, modulating gene expression transiently through the RNA silencing pathway remains critical for applications where genome editing is not warranted or feasible [187]. The evolutionary arms race between hosts and their pathogens has given us a wealth of RBPs with diverse structures and mechanisms of action such as VSRS and bacterial Cas proteins, which can now be explored for a range of biotechnological and biomedical applications.

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Contribution of Collaborators

Contributions of all collaborators are delineated in the preface sections of Chapters 2-4.

Appendices

Supplemental Information for Chapter 2

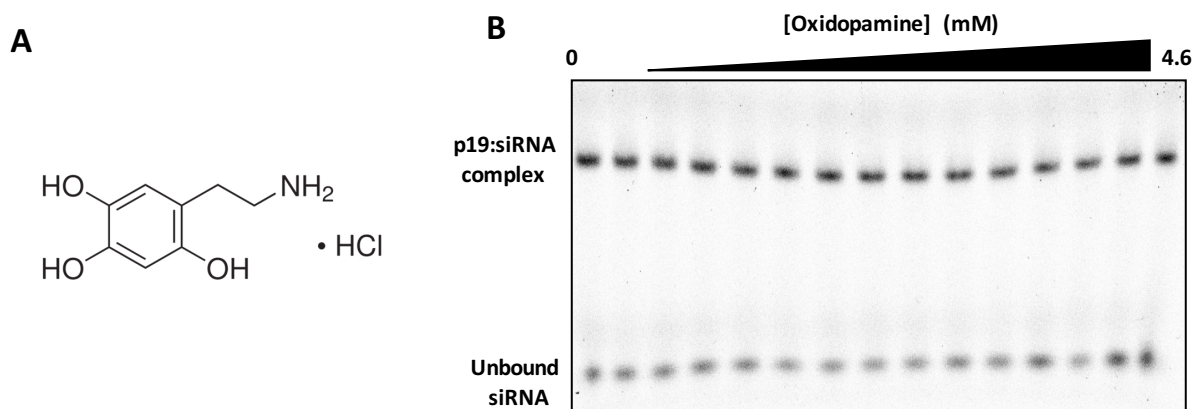


Figure S2.1 (A) The chemical structure of oxydopamine; (B) A representative gel (in gel fluorescence) examining the effect of oxydopamine on p19:siRNA interactions by electrophoretic mobility shift assay. Samples of 10 nM p19 dimer incubated with a range of oxydopamine concentrations (0 to 4.6 mM) and 2 nM Cy3-labeled siRNA. We do not observe any appreciable effect of oxydopamine on p19:siRNA interactions at the concentrations tested.

Supplemental Information for Chapter 4

Table S4.1 p19 mutant constructs and respective mutagenic primer pair used in site-directed mutagenesis.

p19 Mutant Constructs^a	Mutagenic Primer (sense strand sequence)^b
p19-V69A ^c	5'- ggg ttt cgg gaa agt <u>tg</u> c ctt taa gcg cta tct gc -3'
p19-Y73S ^c	5'- gaa agt tgt ctt taa gcg <u>ct</u> c tct gcg cta cga c -3'
p19-T111A ^d	5'- gcc aac cag gtc ggc tgt <u>gc</u> c tat agc att cg -3'
p19-T111H ^c	5'- gcc aac cag gtc ggc tgt <u>ca</u> c tat agc att cg -3'
p19-T111S ^c	5'- gcc aac cag gtc ggc tgt <u>tc</u> c tat agc att cg -3'
p19-T111Y ^d	5'- caa cca ggt cgg ctg <u>ta</u> a cta tag cattcgctttc-3'
p19-T122S ^c	5'- cgg cgt tag cgt <u>ct</u> c cat ttc tgg cgg -3'

^a Letters and numbers after the dash denote the amino acid substitution.
^b Mutated nucleotides are bolded and underlined.
^c The p19-WT DNA plasmid was used as the template for the mutagenesis.
^d The p19-T111S DNA plasmid was used as the template for the mutagenesis.

Table S4.2 Dissociation constants (K_d) of p19-WT and mutants with small RNAs

p19 constructs	Small RNAs	K_d (nM)
p19-WT	Cy3-CSK siRNA (28-mer)	>20000 ^{a,b}
	Cy3-ssRNA (21-nt)	No binding observed ^c
p19-T111S	Cy3-CSK siRNA (28-mer)	>10000 ^b
	Cy3-ssRNA (21-nt)	No binding observed ^c
p19-Y73S	Cy3-miR-122	No binding observed ^c
	Cy3-CSK siRNA (21-mer)	No binding observed ^c
p19-V69A	Cy3-miR-122	>100 ^b
p19-T122S	Cy3-miR-122	>300 ^b

^a Value obtained from previously published result (15).
^b Saturation of binding was not achieved at concentration up to 1 μ M p19 protein.
^c No binding of Cy3-labeled small RNAs observed up to 1 μ M p19 protein used after fluorescence scanning.

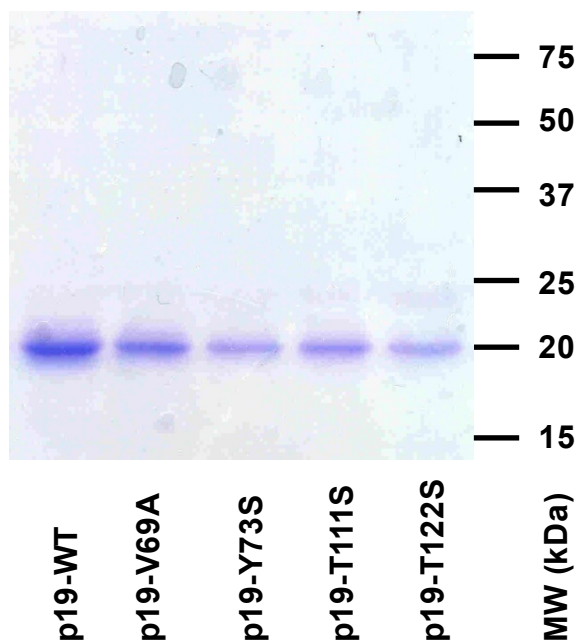


Figure S4.1: 12% SDS-PAGE analysis of purified p19-WT and various mutants

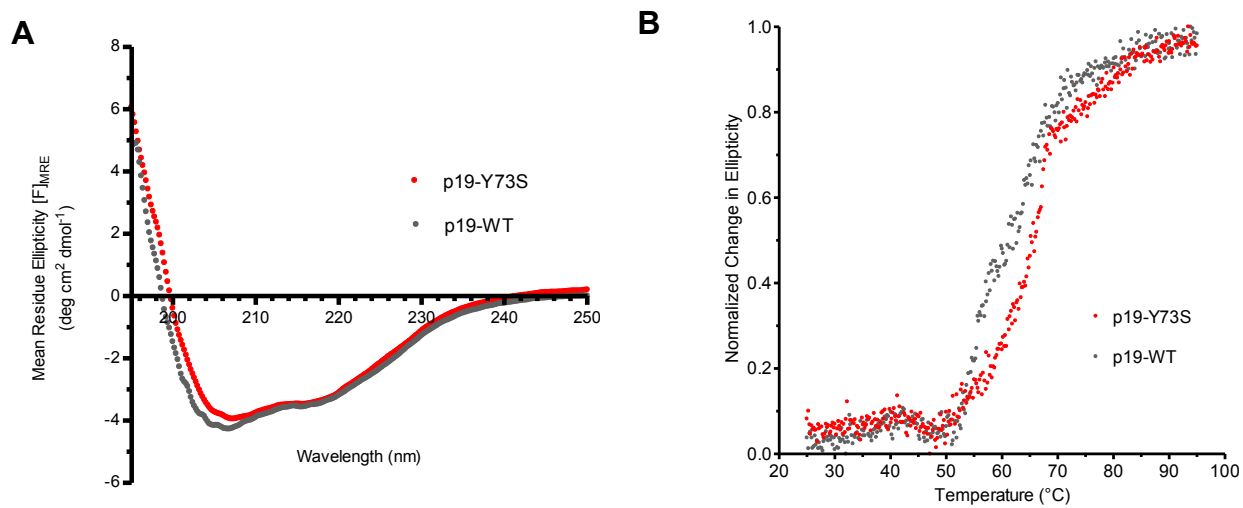


Figure S4.2: Circular dichroism of p19-WT and p19-Y73S.

(A) Far UV spectra and (B) thermal denaturation profiles monitored at 222nm.

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Chapter 1

Danielson, D.C., Pezacki J.P. (2013) Studying the RNA silencing pathway with the p19 protein. *FEBS Lett.* 587(8); 1198-205.

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Chapter 2

Danielson, D.C., Filip, R. Powdrill, M.H., O'Hara, S., Pezacki J.P. (2015) Suppressing RNA silencing with small molecules and the viral suppressor of RNA silencing protein p19. *Biochem. Biophys. Res. Commun.* 463(4):1135-40.

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Chapter 3

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Curriculum Vitae

Dana Vera Foss

formerly Dana C. Danielson

Education

PhD, Biochemistry University of Ottawa Mentor: Prof. John P. Pezacki	2017 Ottawa, Canada
BSc., Hon., Biochemistry University of Winnipeg Graduated as Student of Highest Distinction	2009 Winnipeg, Canada

Research Experience:

University of Tokyo JSPS Research Fellow, Summer Program Institute of Molecular and Cellular Biosciences Mentor: Prof. Yukihide Tomari	2015 Tokyo, Japan
The Scripps Research Institute Visiting Research Scholar Department of Integrative Structural and Computational Biology Mentor: Prof. Ian J. MacRae	2014-2015 La Jolla, USA
University of Winnipeg Honours Research Project Mentor: Prof. Michael Eze	2008 Winnipeg, Canada
St. Boniface Research Centre NSERC Summer researcher Mentor: Prof. Paul Fernyhough	2006, 2007 Winnipeg, Canada

Teaching Experience

Professor, Molecular Evolution, BIO3102 (150 students) University of Ottawa	2017
Guest Lecturer, Advanced Topics in Chemical Biology BPS4129 University of Ottawa	2013-2016
Laboratory Teaching Assistant, Biochemistry 2333 University of Ottawa	2016
Laboratory Teaching Assistant, Biochemistry 3346 University of Ottawa	2012
Mentor/Supervisor of Undergraduate Honours Research Projects (3) University of Ottawa, Laboratory of Dr. John Pezacki	2010-2013
Laboratory Manual & Protocols Development, Biochemistry University of Winnipeg	2009
Tutorial Lecture Teaching Assistant, General Chemistry University of Winnipeg	2009
Tutor, Tutoring Centre, University of Winnipeg	2007-2009

Awards and Honours

Japan Society for the Promotion of Science Fellowship, Summer Program	2015
Ontario Graduate Scholarship	2014-2015
NSERC Vanier Graduate Scholarship	2011-2014
Excellence Scholarship, University of Ottawa	2009-2014
Best Poster Award, Genetic Code Expansion Conference	2016
Keystone Symposium Travel Scholarship	2016
University of Ottawa Mobility Scholarship	2014
NSERC Michael Smith Foreign Study Supplement	2013
Best Oral Presentation award, Departmental Seminar, University of Ottawa	2013
Best Poster Presentation award, 9 th Methods in Protein Structure Analysis	2012
Canada Graduate Scholarship, NSERC	2009-2010
Merit Award, Society of Chemical Industry	2009
NSERC Undergraduate Research Award	2007
Bill Evans Scholarship in Biology, University of Winnipeg	2006
Canada Iceland Foundation Scholarship	2006
Student of Highest Distinction, University of Winnipeg	2005-2009
Board of Regents Special Entrance Scholarship, University of Winnipeg	2004
Academic Proficiency Scholarship, University of Winnipeg	2004-2009
Alumni Scholarship Research Award, St. John's Ravenscourt	2004

Academic Community Engagement

Organizer, Student-Invited Seminar Series, University of Ottawa	2013, 2015, 2016
Mentor, Women in Science in Engineering (WISE), STEM leaders Program	2015, 2016
Seminar leader, Network for Women in Science, Scripps Research Institute	2014
Science Policy Seminar Organizer, Partnership Group for Science and Engineering	2013-2014

Community Engagement

Mentor - Ashbury College – SMART Protein modeling	2015-2016
Extensive mentorship and tutoring of 20 advanced high school students over a 8-month period in protein crystallography and macromolecular structure through designing a 3-D printed model of protein: RNA complex for presentation at an international science fair	
Mentor - Aboriginal Mentorship Program, Let's Talk Science	2013, 2014
Direct mentorship of small groups of high school students in rural areas, aiding their preparation and execution of experiments and research projects. Organized and hosted the students to the University of Ottawa for science fair participation.	
Mentor - Wabano Center for Aboriginal Health, Let's Talk Science	2013
Chemistry demonstrations for youth at local afterschool drop-in center	

Languages

English (fluent)
French (proficient)

Publications

1. Danielson, D.C., Sachrajda, N., Wang, W., Filip, R., Pezacki, J.P. (2016) A novel p19 fusion protein as a delivery agent for short-interfering RNAs. *Mol. Ther. Nucleic Acids* **5**, e303.
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4. Bertrand, R., Danielson, D., Gong, V., Olynik, B., Eze, M.O. (2012) Sodium nitroprusside may modulate *Escherichia coli* antioxidant enzyme expression by interacting with the ferric uptake regulator. *Med. Hypotheses.* 78(1):130-3.
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7. Cheng, J.,* Danielson, D.C.,* Nasheri, N., Singaravelu, R., and Pezacki, J.P. (2011) Enhanced specificity of the viral suppressor of RNA silencing protein p19 towards sequestering of human microRNA-122. *Biochemistry.* 50 (36); 7745-7755.
8. Pezacki, J.P., Blake, J.A., Danielson, D.C., Kennedy, D.C., Lyn, R.K., Singaravelu, R. (2011) Chemical contrast for imaging living systems: molecular vibrations drive CARS microscopy. *Nature Chemical Biology.* 7; 137-145.

Manuscripts in Preparation

9. Danielson, D.C., Schirle, N.S., MacRae, I.J., Pezacki, J.P. Structural examination of p19 mutants with high affinity for human miRNA-122..
10. Danielson, D.C., Powdrill, M.H., Delcorde, J., Rouleau, Y., O'Hara, S., Schultz, P.G. Pezacki, J.P. Unnatural amino acid incorporation into the p19 protein for covalent crosslinking to bound siRNA.

Oral Presentations at Scientific Conferences

1. Danielson, D.C., Pezacki, J.P. Structure based design and unnatural amino acid incorporation into the p19 protein for covalent photo-crosslinking RNA ligands. *Chemical and Synthetic Biology Day*, University of Ottawa. Ottawa, Canada
2. Danielson, D.C., Powdrill, M., Pezacki, J.P. Studying RNA silencing with the suppressor protein p19, small Molecules, and expanded genetic codes. *Canadian Society for Chemistry Conference 2016*. Halifax, Canada.
3. Danielson, D.C., Sachrajda, N., Pezacki, J.P. A novel p19 fusion protein as a delivery agent for short-interfering RNAs. *RNAi2014: Short and Long Non-coding RNAs*. Oxford, England.
4. Danielson, D.C., Sachrajda, N., Pezacki, J.P. A novel p19 fusion protein as a delivery agent for short-interfering RNAs. *Cold Spring Harbor: RNA and Oligonucleotide Therapeutics 2013*. Cold Spring Harbor, NY, USA.
5. Danielson, D.C., Sachrajda, N., Cheng, J.C., Pezacki, J.P. Engineering the p19 protein as a delivery agent for short interfering RNAs. *26th Annual Meeting of the Protein Society*, 2012, San Diego, California.
6. Danielson, D.C., Cheng, J., Koukiekolo, R., Pezacki, J.P. Modifying p19 viral suppressors of RNA Silencing: developing FRET-based probes for detecting and quantifying protein-small RNA interactions. *RNAi 2010, 5th International Conference & Exhibition*. Oxford, England.

Poster Presentations at Scientific Conferences

1. Danielson, D.C., Powdrill, M., Delcorde, J., MacRae, I.J., Schultz, P.G., Pezacki, J.P. Structure based design and unnatural amino acid incorporation into the p19 protein for covalent photo-crosslinking RNA ligands. *Genetic Code Expansion Conference*. August 2016, Corvallis, Oregon.
2. Danielson, D.C., Powdrill, M., Delcorde, J., MacRae, I.J., Schultz, P.G., Pezacki, J.P. Engineering the p19 protein as a tool for studying small RNAs. *Small RNA Silencing: Little Guides, Big Biology*. January, 2016. Keystone, Colorado.

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4. Danielson, D.C., Filip, R., Pezacki, J.P. Inhibiting the RNA silencing pathway using the p19 protein and small molecules. *Keystone Symposium: RNA silencing*. January, 2014. Seattle, Washington.
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9. Danielson, D.C., Koukikolo, R., Cheng, J., Pezacki, J.P. Engineering the viral suppressor of RNA silencing protein p19 as FRET-based probes for imaging protein-small RNA interactions in vivo. *Canadian Society for Chemistry Conference 2011*. Toronto, Canada.

References

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