Mechanical properties of tau pre-mRNA splice site hairpin and its mutants

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

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CHAPTER II Single-molecule studies of small molecule ligands binding

to tau pre-mRNA splice site hairpin and its mutants

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Mechanical properties of tau pre-mRNA and its mutants

1.1 Abstract: Understanding the behavior of RNA during the translation and other processes becomes more and more important today since knowledge of biological functions of RNA has increased rapidly. In the last few decades the development of technologies makes the single molecules manipulation much more convenient and precise, which makes it possible to investigate the relationship between these bio-functions and conformational dynamics of RNA. In this research we mainly focus on studying tau pre-mRNA splice site hairpin and some of its mutants using optical tweezers. Single tau pre-mRNA molecules are combined with DNA/RNA hybrid handles, which can be stretched with optical tweezers, measuring the tiny force around 20 piconewtons (pN). In this part, we constructed the tau pre-mRNA splice site hairpin flanked by DNA/RNA hybrid handles for single-molecule pulling experiment. Applying Extensible Worm-Like Chain (EWLC) model, we compared the difference of mechanical properties between wild type tau pre-mRNA and some of its mutants, revealing the difference in conformational dynamics between wild type tau pre-mRNA and its mutants.

1.2 Introduction: Microtubule associated protein tau (MAPT) is a microtubule binding protein that promotes microtubule assembly^{1,2}. It plays key roles in microtubule stability, which is abundant in axons of neuron cells of central nervous system.

The human MAPT gene is located in chromosome 17q21³, which is composed of 15 exons⁴, and 11 of which encode the six major isoforms in mature neuron cells by

alternative splicing of exons 2, 3 and 10 (Fig 1.1)⁵. Four domains are encoded by exons, respectively⁶. Alternative splicing of exon 10 leads to tau isoforms with 3 or 4 microtubule binding domains, called 3R (exon 10-) or 4R (exon 10+) tau, respectively^{5, 7}. In the normal human neuron cell, the value of 3R:4R tau is around one. Some disease related mutations are found in coding region of exon 10 and alter the amino acid residue sequence of microtubule-binding domains and reshape the affinity of Tau to microtubules⁸⁻¹⁰. While other disease-related mutations are clustered near the 5' splice site of the exon 10, which are synonymous or located in the intron¹¹⁻¹⁵. The latter mutations alter the 3R to 4R Tau expression ratio, which suggests these mutations affect the alternative splicing mechanism.

Figure 1.1: Tau has six isoforms, including three isoforms 3R and three isoforms 4R. N represents the number of inserts of 29 amino acids at the N-terminal part.

It is important to realize that more than 90 percent human genes are alternatively spliced. In human brain, alternative splicing occurs even more commonly¹⁶. Alternative splicing plays a crucial role in controlling both the development and the differentiation. The

silencer and enhancer in the intronic and exonic regions are involved in recruiting protein factors for the splicing regulation¹⁷⁻¹⁹. Furthermore, the structure of pre-mRNA may be an important part in impacting the event of alternative splicing.

Two hypotheses have been developed to explain how the exon 10 5' splice site mutations alter the splicing²⁰. The linear mode suggests two protein binding sequence following the 5' splice site. The two binding sites recruit proteins silencing or enhancing the spliceosome activity, respectively.

Figure 1.2: the sequences of WT and its mutants. Mutations that can destabilize the RNA are in red (+3A, +13G, +14U, +16 C). Mutations that stabilize the RNA are in blue (+10C, I17T, 19G).

The stem-loop model suggested a hairpin formation (Fig 1.2) in between exon 10 and intron 10 and covers the 5' splice site. The mutations weaken the stability of hairpin increase the chance of recognition of 5' splice site by spliceosome²¹⁻²³, thus increase the inclusion of exon 10; while mutations strengthen the stability of the hairpin decrease the chance of recognition of 5' splice site and decrease the inclusion of exon 10. Moreover, mutations in the loop region do not effect splicing²⁴.

Several studies provide evidence supporting the stem-loop model²⁵. And some even indicate that some diseases like Parkinsonism and frontotemporal dementia are related to the stability of the RNA stem-loop structure²⁶⁻³⁰ (Fig 1.2). The mutations at positions +3, +13, +14 and +16 of the intron can decrease the stability of stem-loop structure²⁸⁻³⁰, and make it easier to increase the inclusion of exon 10, which can lead to the decrease of 3R:4R ratio³¹. Recent study has shown that the increased amount of 4R isoform is associated with Parkinsonism and frontotemporal dementia³². On the contrary, mutations at position +10, I17T and 19G of the intron can increase the stability of RNA hairpin, leads to the increase of 3R:4R ratio^{14, 31}. In normal neuron cells, 3R:4R ratio is approximately one.

Many approaches have been applied in single molecule manipulation³³⁻³⁵. Single molecule studies about RNA folding and unfolding have been realized using atomic force microscopy, magnetic tweezers, nanopore force spectroscopy and optical tweezers³⁵⁻³⁸. In Optical tweezers, highly focused laser beams are used to generate an optical trap which is able to trap a microscopic transparent particle in three dimensions.

Previous studies about single-molecule reveal that mechanical properties of RNA structures play direct and important roles in various biological processes³⁹⁻⁴¹. Structures in RNA may serve as a mechanical sensor against the unfolding forces generated by helicases, and/or provide stalling force against the molecular motors (such as RNA polymerase and ribosome) moving along the RNA.

In order to test the impact of mutations on stability of hairpin and reliability of stem-loop model, several RNA hairpin structures are studied using optical tweezers.

1.3 Experimental and methods:

1.3.1 Tau pre-mRNA and its mutants

The sequences flanking exon 10 5' splice site are inserted into PUC19 plasmid (Fig 1.3).

The plasmids were kindly provided by R. Kierzek lab.

Figure 1.3: Construction of RNA in PUC19. The model RNAs are inserted into PUC19 vector between Pst I and EcoRI restriction site and sequences below are listed fragment marked above with green (intron 9, 39 nt and intron 10, 63 or 64 nt) and blue (exon 10, 93 nt). Red fragment on the scheme mark position of RNA hairpin for our interests. Red nucleotides in sequence mark mutations within RNA.

WT

5'GGGUGGCGUGUCACUCAUCCUUUUUUCUGGCUACCAAAGGUGCAGAUAA
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGCUCAAAG
GAUAAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCUUCACACG
UCCCAUGCGCCGUGCUGUGGCUUGAAUUAUUAGGAAGUGGUGUGA

DD-PAC

GGGUGGCGUGUCACUCAUCCUUUUUUCUGGCUACCAAAGGUGCAGAUAAU
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGCUCAAAGG
AUAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCUUCAUACGU
CCCAUGCGCCGUGCUGUGGCUUGAAUUAUUAGGAAGUGGUGUGA

DD-10C

GGGUGGCGUGUCACUCAUCCUUUUUUCUGGCUACCAAAGGUGCAGAUAAU
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGCUCAAAGG
AUAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCUCAUACGU
CCCAUGCGCCGUGCUGUGGCUUGAAUUAUUAGGAAGUGGUGUGA

DDI-17T

GGGUGGCGUGUCACUCAUCCUUUUUUCUGGCUACCAAAGGUGCAGAUAAU
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGCUCAAAGG
AUAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCUUCAUACUG
UCCCAUGCGCCGUGCUGUGGCUUGAAUUAUUAGGAAGUGGUGUGA

WT-10C

GGGUGGCGUGUCACUCAUCCUUUUUUCUGGCUACCAAAGGUGCAGAUAAU
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGCUCAAAGG
AUAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCUCAACACGU
CCCAUGCGCCGUGCUGUGGCUUGAAUUAUUAGGAAGUGGUGUGA

WTI-17T

GGGUGGCGUGUCACUCAUCCUUUUUUCUGGCUACCAAAGGUGCAGAUAAU
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGCUCAAAGG
AUAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCUUCACACUG
UCCCAUGCGCCGUGCUGUGGCUUGAAUUAUUAGGAAGUGGUGUGA

+11C

GGGUGGCGUGUCACUCAUCCUUUUUUCUGGCUACCAAAGGUGCAGAUAAU
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGCUCAAAGG

AUAAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCU**C**CACACGU
CCCAUGCGCCGUGCUGUGGGCUUGAAUUAUUAGGAAGUGGUGUGA

+12U

GGGUGGCGUGUCACUCAUCCUUUUUUCUGGGCUACCAAAGGUGCAGAUAAU
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGGCUCAAAGG
AUAAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCUU**U**ACACGU
CCCAUGCGCCGUGCUGUGGGCUUGAAUUAUUAGGAAGUGGUGUGA

+13G

GGGUGGCGUGUCACUCAUCCUUUUUUCUGGGCUACCAAAGGUGCAGAUAAU
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGGCUCAAAGG
AUAAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCUUC**C**CACGU
CCCAUGCGCCGUGCUGUGGGCUUGAAUUAUUAGGAAGUGGUGUGA

+16U

GGGUGGCGUGUCACUCAUCCUUUUUUCUGGGCUACCAAAGGUGCAGAUAAU
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGGCUCAAAGG
AUAAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCUUCACA**U**GU
CCCAUGCGCCGUGCUGUGGGCUUGAAUUAUUAGGAAGUGGUGUGA

+19G

GGGUGGCGUGUCACUCAUCCUUUUUUCUGGGCUACCAAAGGUGCAGAUAAU
UAAUAAGAAGCUGGAUCUUAGCAACGUCCAGUCCAAGUGUGGGCUCAAAGG
AUAAUAUCAAACACGUCCCGGGAGGCGGCAGUGUGAGUACCUUCACACGU
GCCAUGCGCCGUGCUGUGGGCUUGAAUUAUUAGGAAGUGGUGUGA

1.3.2 DNA/RNA hybrid handles

Figure 1.5: the process to produce DNA/RNA hybrid handles. Handle A and Handle B are double-stranded DNA before annealing. Sequence in red is RNA.

Handle A (~500 bp) is produced via PCR using primers (PUC19 handle A forward and PUC19 handle A reverse) (Fig 1.4). After PCR, handle A is biotinylated with biotin-16-dUTP and T4 DNA polymerase. Handle B (~500 bp) is produced via PCR using dig-labeled primers (PUC19 handle B forward and PUC19 handle B reverse-Dig). The RNA molecules (~1100 nt) are produced via *in vitro* transcription by T3 RNA polymerase and synthetic DNA templates. And the target sequences of interest are included in RNA products, which is located in the middle of RNA sequence (flanked by ~500 nucleotide sequences). Then these RNA products are annealed with handle A and handle B products

to form DNA/RNA hybrid handle A and handle B. Handle A is labeled at the 3' end of DNA strand which contains the biotin modified dUTP. Handle B is labeled with digoxigenin at its 5' end of DNA strand.

All RNAs and DNAs are purified by QIAquick PCR Purification Kit.

PUC19 handle A forward: 5'-CTTGTCTGTAAGCGGATGCCG-3'

PUC19 handle A reverse: 5'-CCGGGACGTGTTTGATATTATCCTTTC-3'

PUC19 handle B forward: 5'-CCGTGCTGTGGCTTGAATTATTAGG-3'

PUC19 handle B reverse-dig 5'-Dig-CAGGGTCGGAACAGGAGAGC-3'

1.3.3 Optical tweezers

Optical tweezers have been applied in single-molecule force spectroscopy measurement of biopolymer folding/unfolding. Previous work shows that mechanical tension generated by RNA folding/unfolding plays an important role by interacting with molecular machines, such as RNA polymerase and ribosome. Thus mechanical properties of RNA structures are vital in gene regulation. The biological significance of mechanical property of RNA structures suggests new strategies of regulating RNA involved biological processes including transcription, alternative splicing and translation.

In our single molecule experiment, optical tweezers were used to directly load and measure force applied on a polystyrene particle which is trapped in solution by the optical trap. The force measured ranges from 0.1 pN to 150 pN, which is sufficient to break the noncovalent interactions in nucleic acids.

Some characteristics of our optical tweezers are listed in Table 1.1.

Table 1.1: characteristics of minitweezers

Parameter	Sym.	Value	Unit
Operating Power	P_{op}	150	mW
Peak Wavelength	λ_{max}	845.2	nm
FWHM	λ_{FWHM}	1.99	nm
LASER Operating Current	I_{op}	320	mA

Figure 1.6: optical path of the minitweezers

In our optical tweezers system (Fig 1.6), a laser (845nm) beam is collected by fiber, which gives a cone of light that is collimated by collimating lens. The collimated light goes to a polarizing beam splitter (PBS) which reflects the light completely. The quarter

wave plate ($\lambda/4$) converts linearly polarized light to circularly polarized light which is focused by an objective lens to a point within a fluid micro chamber. This light is then collected by a second objective, and it passes through another quarter wave plate where it is now linearly polarized again. The beam is reflected by another polarizing beam splitter to relay lens which images the back focal plane of the objective onto the force position sensitive detector (PSD) which detects optical trap force.

The focused laser beam or optical trap position can be moved by fiber wiggler. For optical trap position detection, laser beam is split by a pellicle before going to the first PBS and collimated onto a position PSD.

A motorized X-Y-Z stage is used to move the chamber in the X-Y-Z coordinates.

Objective lens can be moved to focus in the Z direction. When focus is optimized in X, Y and Z, the ratio PD signal and PSD signal to XYSUM signal is the same for empty optical trap and for trap with bead.

Figure 1.7: Schematic diagram of an optical tweezers measuring the tiny forces generated by RNA hairpin. The biotin group and the digoxigenin group of the construct bind specifically to a streptavidin coated $\sim 1.8 \mu\text{m}$ particle (SA) and an anti-digoxigenin antibody coated $\sim 3 \mu\text{m}$ particle (AD), respectively.

During single-molecule experiment (Fig 1.7), the SA bead (which is linked to handle B-Dig) is stuck at the tip of micropipette by suction. The AD bead (which can link to handle A-Biotin) was trapped by laser beam. The RNA structure of interest is link to handle B-Dig and handle A-Biotin, and these two handles and link to anti-digoxigenin coated polystyrene bead and streptavidin coated polystyrene bead, respectively. The relatively motion between the two beads can generate stretching force on the linked DNA/RNA hybrid handles resulting in the transition between folded state and unfolded state of the

RNA structure. To generate the construct for pulling experiment, the micropipette is stationary during the experiment while optical trap held the anti-digoxigenin coated polystyrene bead can move relatively to the chamber. After obtaining the construct with a single molecule attached to two beads, we can carry out various quantitative force measurements.

1.3.4 Buffer condition:

The buffer used in experiments contains 200 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, 0.1 mM spermine, pH 7.2.

1.3.5 Extensible Worm-Like Chain (EWLC) model:

A Worm-Like Chain (WLC) model is an effective model which can be used to describe the behaviour of polymers, mostly for the polymers with semi flexible properties (such as DNA and RNA). The behaviour of semi-flexible polymers is determined in a WLC model by the following equation:

$$F = \frac{k_B T}{P} \left[\frac{1}{4 \left(1 - \frac{x}{L}\right)^2} + \frac{x}{L} - \frac{1}{4} \right] \quad (1)$$

where k_B is Boltzmann constant, T, P, L stands for absolute temperature, persistence length and contour length, respectively, x is the end-to-end distance at low force region.

For the WLC model described in eq 1, it is assumed that the covalent bonds of polymer chain do not change under force, which may leads to a shorter extension at high force region. If we take the bond enthalpy into consideration, the Extensible Worm-Like Chain model (EWLC) (eq 2) fits better than the WLC model without consideration of the enthalpy (eq 1).

$$F = \frac{k_B T}{P} \left[\frac{1}{4 \left(1 - \frac{x}{L} + \frac{F}{K} \right)^2} + \frac{x}{L} - \frac{F}{K} - \frac{1}{4} \right] \quad (2)$$

Here K is stretch modulus. For single strand RNA, the persistent length P, contour length L, stretch modulus K are 1nm, 0.59 nm/nt, and 1500 pN, respectively.

1.4 Result and discussion:

1.4.1 Force-ramp experiments of tau pre-mRNA and its mutants

In our optical tweezers experiments, all sequences of tau pre-mRNA and its mutants can unfold into single stranded RNA from stem-loop structure, and refold into hairpin structures from single stranded RNA. Almost all of the pulling cycles show a one-step unfolding transition during stretching. The extension changes of one-step unfolding transition are compatible with the prediction of EWLC model when unfolding of stem-loop structure into stretched single stranded RNA.

Figure 1.8 A) Three types of RNA stem-loop structures, WT, 19G and 14U. The red line in each stem-loop structure indicates that only the structure above the red line can form.

B) Schematic diagram of an optical tweezers for measuring the tiny forces required for unfolding hairpins.

Here we use the wild type tau pre-mRNA (WT), mutant +14U (14U), mutant +19G (19G) in our pulling experiment. During the experiment, the force we applied to the samples at the transition point from stem-loop structure to single stranded RNA is called unfolding force, On the analogy of this, force at the transition point from single stranded RNA to stem-loop structure is called refolding force.

During the unfolding process, the base pairs in stem-loop structures of WT, 14U, 19G tend to break due to the external force applied by optical trap. All base pairs are broken cooperatively in one step to form the stretched, single-stranded RNA, with extension increase in the force ramp curve (Fig 1.9). Similarly, during the refolding process, when the external force decreases to a certain value, these stem forms cooperatively with an extension decrease.

Figure 1.9: force ramp curve of WT, 14U and 19G. the red curve represents the force-loading process, and the black curve represents the force-unloading process.

Figure 1.10: distribution of refolding force (black) and unfolding force (red) in force ramp experiment of WT (left), 14U (middle) and 19G (right), respectively.

According to our pulling result, the unfolding process and refolding process of WT are reversible, as the force for disrupting the hairpin to single stranded RNA and the force at which the single stranded RNA refold into stem-loop structure are almost the same. The

unfolding force and refolding force of WT are around 14.2 pN and 14.5 pN, respectively (Fig 1.10, Panel A).

The unfolding force and refolding force of 14U are around 11.5 pN and 11.5 pN, respectively (Fig 1.10 Panel B). These forces are relatively lower than that of WT, consistent with the fact that +14U destabilizes the stem-loop structure. But it should be noticed that during the pulling process, several hopping events can be found. For this mutant (14U), G-C base pair turns into G-U pair, which decreases the stability of the whole hairpin structure. And the position is this G-U pair may also be crucial for the hopping. In our calculation, the hairpin of 14U contains 21 nucleotides, and G-U base pair locates in the middle of the hairpin, and this mutation which destabilizes the whole structure is in the middle of hairpin, which may amplify this effect of destabilization. Although hopping happens, we are still able to obtain the transition point, forces and extension changes at that point.

The unfolding force and refolding force of 19G are around 15.0 pN and 15.3 pN, respectively (Fig 1.10, Panel C). These forces are relatively higher than that of WT, according with the fact that +19G can enhance the stability of the stem-loop structure.

It should also be noticed that the distribution of refolding force and unfolding force are little bit different, the unfolding force is a little bit higher than refolding force.

Figure 1.11: structure of 19G compared with WT. Highlight in red is the mutation base.

We use program RNAstructure⁴² to predict the hairpin structure. For this mutant (19G), it is interesting to notice that the mutation of base C to G results in a change in the hairpin structure (Fig 1.11). The bulged A can be found in hairpin of wild type while disappear in mutant 19G. Instead of easily replacement of base C to base G, the mutant 19G forms a new different hairpin structure containing a bulged G.

This C19G mutation causes the disruption of the base pair in hairpin, which normally will decrease the stability of the hairpin structure. But due to the change of the whole hairpin structure, this mutant becomes more stable than the hairpin of wild type. In our calculation, the hairpin of 19G contains 27 nucleotides. With more base pairs formed in 19G, it is not surprising that 19G has enhanced mechanical stability.

1.4.2 Force-Extension Change Predicted by Using Extensible Worm-Like Chain (EWLC) Model

All the single-stranded RNA stretching work is calculated based on the EWLC model. The total number of nucleotides of hairpin involved in unfolding process is calculated according to the extension change (ΔX) values at the unfolding force using EWLC model, with the length of the initial and terminal state of the transitions taken into consideration, which may be the diameter of hairpin stem (2 nm).

Figure 1.12: Force- ΔX of unfolding process fitting with EWLC model. The left hand side panel is the Force- ΔX of WT, and the EWLC curve is fitted with 21 nucleotides (brown curve) and 27 nucleotides (green curve). ΔX means the extension change from hairpin structure to single-stranded RNA. Each dot represents one transition of unfolding process. Force- ΔX dots of WT (black), 14U (red) and 19G (blue) fit well with EWLC curve.

For different number of nucleotides, the curves of EWLC can be different. For WT and 14U, the number of nucleotides which forms hairpin structure is the same, 21 nucleotides, and 27 nucleotides are involved in forming 19G hairpin.

It is clear that the unfolding force of WT (Fig 1.12 black dots) is significantly higher than that of 14U (Fig 1.12 red dots). And both WT and 14U fit well in EWLC with 21 nucleotides, which indicates 21 nucleotides are involved to form WT and 14U stem-loop structure. The bulged A is formed in both WT and 14U hairpin structures (Fig 8 Panel A).

And for 19G, the number of nucleotides involving the formation of hairpin structure is different. According to our calculation, 27 nucleotides are involved to form 19G hairpin, so the EWLC curve of 19G is different from that of WT and 14U (Fig 1.12).

Among these three hairpin structures, 19G is the most stable one (with the highest unfolding force among three hairpin), followed with WT. And the most unstable hairpin structure is 14U. All pulling data can fit well with EWLC curve, which provides the number of nucleotides in different hairpin structures. And these nucleotides number tally with the prediction from RNAstructure⁴².

1.4.3 Constant Force Mode

We use the constant force mode in optical tweezers experiments to measure the extension changes for both transitions during unfolding and refolding processes, respectively.

Figure 1.13: extension change in constant force mode. Upper trajectory is extension change of WT under the force of 13.5pN. Middle trajectory is extension change of 14U under the force of 11.0 pN. Lower trajectory is extension change of 19G under the force of 14.7pN.

Two levels can be found in each trajectory (Fig 1.13). The lower level stands for the folded state, and the upper one represents for the unfolded state, Y coordinate is extension. The extension change between folded state and unfolded state of WT, 14U and 19G is 7.1 nm, 6.5 nm, 9.8 nm, respectively, which corresponds to the prediction of the EWLC model. The extension of each hairpin indicates that the hairpin structures of WT, 14U and 19G consist of 21 nucleotides, 21 nucleotides and 27 nucleotides, respectively.

And in the relatively unstable hairpin, 14U, we also found transition between folded state and unfolded state much quickly compared with other two RNA hairpins.

In constant force mode, the external force remains constant, and the tether can fold and unfold repeatedly. The transition from unfolded state to folded state can be characterized by a sudden drop in extension. Inversely, the sudden increase in extension indicates the transition from folded state to unfolded state. The period of time when tether remains in the folded/unfolded state is called the lifetime of folded/unfolded state.

Figure 1.14: Plots of the probability of unfolded state (hollow) and folded state (solid) of WT as a function of lifetime (panel A for WT, panel B for 14U, panel C for 19G). $k_{F \rightarrow U}$ and $k_{U \rightarrow F}$ are the rate constant of unfolding and folding processes, respectively.

And the probability-lifetime curve can indicate the distribution of period of time, which should follow the rule of exponential decay. For each panel in Figure 1.14, unfolding and folding processes fit well with exponential decay. In Figure 1.14, we notice that both the unfolding and folding rates in 14U are much higher than those in WT and 19G,

which indicates the extension changes much faster in 14U. This result is compatible with the extension change trajectory in Figure 1.13.

The force dependent rate coefficient is fitted to Bell model³⁹,

$$k(F) = k_m k(0) e^{\left(\frac{FX^\ddagger}{k_B T}\right)}$$

where k_m is the instrumental factors to rate coefficient, $k(0)$ is the rate coefficient at zero force, $X^\ddagger$ is the distance from unfolded or folded state to the transition state, for unfolded state to the transition state, when calculating the number of nucleotides involved in forming the transition state hairpin, the size of the stem-loop structure (2 nm diameter) should be taken into account. k_B is Boltzmann constant, T is absolute temperature.

The critical force F_c is the force at which unfolded and folded state are equally distributed. It can be obtained from the cross point of the Bell fittings for both unfolding and refolding kinetics.

Figure 1.15: Plots of the rate coefficient of unfolded state (hollow) and folded state (solid) as a function of force in passive mode. Panel A, B, C represents rate coefficient-force relationships in WT, 14U, 19G, respectively. Rate coefficient-force relationships of 14U (red), WT (black) and 19G (blue) are in panel D.

The slopes of folding and unfolding rate coefficients in WT, 14U and 19G are -0.85 and 0.90 (WT), -0.84 and 0.87 (14U) and -0.82 and 1.49 (19G), respectively. The critical force F_c is the force at which unfolded and folded state are equally distributed. It can be obtained from the cross point of the Bell fittings for both unfolding and refolding kinetics. According to Bell model, the distance from unfolded state to the transition state $\Delta X_{\ddagger}^{\dagger}(\text{folding})$, the distance from folded state to the transition state $\Delta X_{\ddagger}^{\dagger}(\text{unfolding})$,

critical force F_c and critical rate coefficient $k(F_c)$ in constant force mode are stated in Table 1.2.

Table 1.2: unfolding/folding parameters in constant force mode

Hairpin structure	$\Delta X_{\ddagger}^{\dagger}(\text{folding})$ (nm)	$\Delta X_{\ddagger}^{\dagger}(\text{unfolding})$ (nm)	F_c (pN)	$k(F_c)$ (1/s)
WT	3.46	3.66	14.51	9.97
14U	3.42	3.54	11.17	64.70
19G	3.34	6.07	15.17	6.89

From Table 1.2, we can see for the critical force F_c , $F_c(19G) > F_c(WT) > F_c(14U)$, which is compatible with the folding/unfolding force distribution in Figure 1.10 and result in Figure 1.12. For the critical coefficient rate $k(F_c)$, $k(F_c)$ in 14U is much bigger than that in WT and 19G, indicates the faster extension changes in 14U. This result tally with the extension change trajectories in Figure 1.13 and exponential decay curves in Figure 1.14.

Distance from unfolded state to the transition state $\Delta X_{\ddagger}^{\dagger}(\text{folding})$ and distance from folded state to the transition state $\Delta X_{\ddagger}^{\dagger}(\text{unfolding})$ of three hairpin structure are given in Table 1.2, which makes it possible to calculate the number of nucleotides disrupted at the unfolding transition and the number of nucleotides involved in the folding transition of each hairpin structure with EWLC model.

Figure 1.16: (un)folding transition state of WT (panel A) and 19G (panel B) in constant force mode.

In Figure 1.16, both the unfolding transition state position and the folding transition state position in WT and 19G are the same, respectively. Using $\Delta X_{\ddagger}^{\ddagger}(\text{unfolding})$ with EWLC model, we can obtain that there are 9 nucleotides disrupted during the mechanical unfolding to transition state, and about 12 nucleotides are involved in the folding transition state according to $\Delta X_{\ddagger}^{\ddagger}(\text{folding})$. And in 19G, about 15 nucleotides are disrupted during the mechanical unfolding to transition state, 12 nucleotides are involved in forming the folding transition state. While in 14U, the unfolding transition state

position and folding transition state position are not the same according to our result in Figure 1.17.

Figure 1.17: unfolding and folding transition state of 14U in constant force mode.

The hairpin of unfolding transition state position is one-base pair shorter than that of folding transition state. About 9 nucleotides are disrupted during the mechanical unfolding to transition state, and about 14 nucleotides are involved in the folding transition state.

1.4.4 Passive Mode

Passive mode is used in optical tweezers experiments to measure the force changed as well as the extension changes for both transitions during unfolding and refolding processes, respectively.

Figure 1.18: Plots of the rate coefficient of unfolded state (hollow) and folded state (solid) as a function of force in passive mode. Panel A, B, C represents rate coefficient-force relationships in WT, 14U, 19G, respectively. Rate coefficient-force relationships of 14U (red), WT (black) and 19G (blue) are in panel D.

In the passive experiments, the external force changes when the state of tether changes.

The extension change between folded state and unfolded state of WT, 14U and 19G is 7.0

nm, 6.5 nm, 9.8 nm, respectively, which corresponds to the prediction of the EWLC model.

Under passive mode, the slopes of folding and unfolding rate coefficients in WT, 14U and 19G are -0.83 and 0.87 (WT), -0.92 and 0.85 (14U) and -0.86 and 1.50 (19G), respectively. With Bell model, the distance from unfolded state to the transition state $\Delta X_{\ddagger}^{\dagger}(\text{folding})$, the distance from folded state to the transition state $\Delta X_{\ddagger}^{\dagger}(\text{unfolding})$, critical force F_c and critical rate coefficient $k(F_c)$ in passive mode are stated in Table 1.3.

Table 1.3: unfolding/folding parameters in passive mode

Hairpin structure	$\Delta X_{\ddagger}^{\dagger}(\text{folding})$ (nm)	$\Delta X_{\ddagger}^{\dagger}(\text{unfolding})$ (nm)	F_c (pN)	$k(F_c)$ (1/s)
WT	3.38	3.54	14.48	33.45
14U	3.74	3.46	11.23	68.71
19G	3.50	6.11	14.99	6.66

In Table 1.3, the order of critical force F_c is $F_c(19G) > F_c(WT) > F_c(14U)$, which is compatible with the result in constant force mode. For the critical coefficient rate $k(F_c)$, $k(F_c)$ in 14U is still bigger than that in WT and 19G, indicates the faster extension changes in 14U, which also tally with the result in constant force mode.

Applied $\Delta X_{\ddagger}^{\dagger}(\text{folding})$ and $\Delta X_{\ddagger}^{\dagger}(\text{unfolding})$ with EWLC, we can calculate the (un)folding transition state for each hairpin. The result is the same as Figure 1.16 and Figure 1.17.

1.4.5 Double mutations on Tau pre-mRNA:

Since 14U is a destabilized mutant which has the lower (un)folding force compared with WT, and 19G has the higher (un)folding force compared with WT, it is interesting to investigate the properties of the mutant which contains both the mutations from 14U(14U) and 19G. The hairpin structure of 14U-19G is shown below (predicted by RNAstructure⁴²):

Figure 1.19: Structure of 14U-19G

During the unfolding process, the base pairs in stem-loop structures of 14U-19G tend to break due to the external force applied by optical trap. And all base pairs are broken in low force region with massive hopping, which indicates that mutant 14U-19G is a destabilized RNA compared with WT.

Figure 1.20: Force distribution of 14U19G under force-ramp mode. The unfolding force is around 10 pN (indicated in blue box).

Under the constant force mode, extension change has been recorded:

Figure 1.21: Extension change in constant force mode. The left-hand side panel is the extension change under different constant force. The right-hand side is the extension distribution under different constant force.

The short lifetime of extension change agrees with the massive hopping near the unfolding transition region. Two levels can be found in each trajectory (Fig 1.21), the lower level stands for the folded state, and the upper one represents for the unfolded state. As the constant force changes from 9.5pN to 10.5pN, the extension distribution changes accordingly and near 10.2pN constant force, the folded state and the unfolded state are equally distributed.

Figure 1.22: Force-dependent unfolding/folding kinetics of WT, 14U, 19G and 14U-19G extracted from passive mode experiments

Under passive mode, the slopes of folding and unfolding rate coefficients in 14U-19G are -0.91 and 0.88, respectively. With Bell model, the distance from unfolded state to the transition state $\Delta X_{\ddagger}^{\dagger}(\text{folding})$, the distance from folded state to the transition state $\Delta X_{\ddagger}^{\dagger}(\text{unfolding})$ and critical force F_c in passive mode are stated in Table 1.4.

Table 1.4: unfolding/folding parameters in passive mode

Hairpin structure	$\Delta X_{\ddagger}^{\dagger}(\text{folding})$ (nm)	$\Delta X_{\ddagger}^{\dagger}(\text{unfolding})$ (nm)	F_c (pN)
14U-19G	3.70	3.58	10.02

Compared with 14U ($F_c=11.23\text{pN}$), mutant 14U-19G has relatively lower critical force. While 19G can stabilize the WT RNA hairpin, the mutation 19(C to G) cannot enhance the stability of 14U, but even weaken the stability of 14U, according to our result. The location of mutation 14(C to U) is near the Exon 10, which might be the important factor for the low critical force as well as the alternative splicing.

Figure 1.23: Force-dependent unfolding/folding kinetics of 14U-19G extracted from passive mode experiments

1.5 Conclusions:

The stem-loop structure of tau pre-mRNA and its mutants can be verified by our pulling experiments. Among the wild type tau pre-mRNA and its mutants (14U and 19G), the

mutant 19G is the most stable and mutant 14U is most unstable. In this part, several methods were used to investigate the mechanical properties of target RNAs. With the force-ramp method, RNA folding and unfolding can be detected and the length of RNA related to this process can be measured. With the length measured, we can calculate the number of nucleotides involved in folding/refolding process using Extensible Worm-Like Chain (EWLC) Model. For WT and 14U, the number of nucleotides which forms hairpin structure is 21 nucleotides, and 27 nucleotides are involved in forming 19G hairpin. This can also supports the stem-loop structure model. In constant force mode, the force is kept constant by an electronic feedback, so that the distance between the trap center and the trapped particle is constant. And in passive mode, the trap position is passively kept constant without electronic feedback, so the trapped particles moves towards the trap center when the RNA unfolds. Using those two methods both unfolding transition state position and folding transition state position can be acquired. And the results obtained in constant force mode are compatible with the result from passive mode; also the life time of folded state and unfolded state can be obtained in those two modes, which can subscribe how soft the RNA is. According to our result, both constant mode and passive mode show that 14U softer than WT and 19G, and 19G is the toughest among three.

The mutant 14U has relatively low unfolding force and the mutant14U-19G has lower unfolding force compared with 14U, which may indicates the importance of mutation 14(C to U) which can affect the hairpin structure more efficiently than mutation 19(C to G). Since the location of mutation 14(C to U) is next to the exon 10, this location could be an important factor for Tau pre-mRNA alternative splicing. Our result reveals the

mechanical properties of WT, 14U, 19G and 14U-19G, which may be useful for understanding the neuron diseases cause and provide a way to treat these diseases.

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Single-molecule studies of small molecule ligands binding to tau pre-mRNA splice site hairpin and its mutants

2.1 Abstract: In recent years, small molecules have been used as drugs to alter RNA structures and control RNA functions. In our research, the hairpin structure of tau pre-mRNA at 5' splice site of exon 10 determines the alternative splicing. Some diseases such as Parkinsonism and frontotemporal dementia may be due to the mutation that destabilizes the RNA hairpin structure, leading to an increase in the inclusion of exon 10, and a decrease of 3R:4R ratio. In this proposal, we aim to answer the following questions. Can we alter the alternative splicing activity by stabilizing the disease-causing RNA hairpins? Are we able to cure these terrible diseases? How can we stabilize the hairpins? A recent study shows that mitoxantrone (MTX) can bind to the tau pre-mRNA and stabilize the hairpin structure. In order to test whether MTX can bind to these disease-causing mutants and increase their stability, we will use thermal melting method as well as optical tweezers to probe directly the effect of MTX binding on the hairpin structure. Upon small molecule binding, the hairpins may change their structure or mechanical properties, affecting the inclusion of exon 10, and altering the 3R:4R ratio.

2.2 Introduction: In recent years, development of small molecules binding to RNA becomes increasingly important¹⁻³. More than 80% of human genomic DNA sequences can be transcribed to functional RNAs, but there are only around 2% of that can directly encode protein sequence^{4, 5}. RNA structure is highly hierarchical. The basic structural unit of RNA is ribonucleotide, which is composed of a heterocyclic base (purine or pyrimidine), a five-carbon sugar and a phosphate group. There are four major types of bases in RNA: guanine (G), adenine (A), cytosine (C) and uracil (U). Canonical linear RNA backbone is composed of ribose and phosphate groups, by forming 3', 5'-phosphodiester bonds. Some intronic RNAs may exist as lariat form by forming a 2', 5'-phosphodiester bond between the 5' end nucleotide and an internal adenine nucleotide. Numerous studies indicate that the RNA structural stability may play key roles in its biological functions.⁶⁻¹²

The thermodynamics of RNA structure is successfully predicted by a nearest-neighbor model.¹³⁻¹⁵ However, thermodynamic properties alone is not sufficient to describe the biological behavior of RNA, as recent studies have shown that mRNAs are usually less structured in vivo than in vitro at similar buffer and temperature conditions.¹⁶ It is important to realize that these functional RNAs can be the key to human diseases. As the structure of these functional RNAs regulates their function, they may have the great potential to be drug targets¹⁷⁻¹⁹. Some small molecules based drugs binding to functional RNAs have been reported^{20, 21}. These small ligands binding to functional RNAs rather than disease-linked proteins are more hopeful about the human diseases treatment, because there are only small amount of drugs can target these proteins, while RNAs can fold into three-dimensional structure like proteins, which make it possible for the specific

binding²². However, it is still be a challenge to find specific ligands for a specific RNA sequence.

Figure 2.1: structure of mitoxantrone

So far, among the great number of ligands have been found which can be used to target RNAs, mitoxantrone (MTX) (Fig 2.1) has become a promising ligand and received much attention lately^{23, 24}. Mitoxantrone is used as an anticancer drug. Usually it can intercalate into the gap between DNA bases, interrupt the DNA repair and synthesis, but it is also useful as a promising ligand to bind to our target RNA, Tau pre-mRNA. It is a remarkable fact that some other ligands can also bind to Tau pre-mRNA, such as aminoglycoside antibiotic neomycin²⁵, which can bind to the major groove of Tau pre-mRNA and stabilize the hairpin structure. But due to the lack of selectively of binding, this class of ligands may not be most suitable ones in future therapeutic application. While mitoxantrone is appropriate for further investigation²⁶. According to NMR result²⁷, mitoxantrone can intercalate in the seam-loop structure of Tau pre-mRNA between base

pair G(-1)-C(+16) and base pair C(-3)-G(+17) flanking the bulged A(-2). Besides, the structure of mitoxantrone also plays a key role in this specific binding, since two arms of anthraquinone moiety can help to recognize the binding site.

Figure 2.2: Tau pre-mRNA-MTX interaction in PyMOL(PDB ID:2KGP). MTX (yellow) intercalate in the seam-loop structure of Tau pre-mRNA between base pair G(-1)-C(+16) and base pair C(-3)-G(+17) flanking the bulged A(-2).

Mitoxantrone that bind to the bulged A of Tau pre-mRNA (Fig. 2.2) can stabilize the stem-loop structure and therefore decrease the exon 10 alternative splicing, which may be crucial to counteract the effect of some mutations such as A(+13) to G(+13), C(+14) to U(+14). Although some research indicates that free mitoxantrone can form a dimer²⁷, a

large amount of spectroscopic observations shows that mitoxantrone binds to Tau pre-mRNA as a monomer.

Figure 2.3: mutation from 14C to 14U

Six major isoforms in mature human neuron cells can be produced by alternative splicing from mRNA²⁸. Some mutations of Tau pre-mRNA may be the cause of neuron diseases. Intronic mutations such as C(+14) to U(+14) destabilize the hairpin structure of Tau pre-mRNA, which can increase the inclusion of exon 10, and lead to the overproduction of 4R Tau isoform and decrease of 3R:4R ratio. This overproduction of 4R may cause Parkinsonism linked to chromosome 17²⁹.

Previous single-molecule force spectroscopy research reveals that the mechanical properties of RNA structures play direct and important roles in various biological processes^{30,31}. Structures in RNA may serve as a mechanical sensor against the unfolding forces generated by helicases, and/or provide stalling force against the linear molecular motors (such as RNA polymerase and ribosome) moving along the RNA²⁹.

Temperature and salt condition can affect the mechanical properties of RNAs^{32, 33}, which allow us to have better understanding about how the natural RNAs respond to the environmental and metabolic stimulation by altering their mechanical stabilities and consequently regulate the relevant biological functions.

To understand the principle of increased splicing in exon 10 caused by the mutations, we will use optical tweezers as well as melting experiment to investigate the properties of the mutants. Mitoxantrone will be used as a ligand to bind to Tau pre-mRNA splice site hairpin and its mutants. Mechanical properties of the RNA-MTX complexes will be tested in force ramp mode using optical tweezers. Thermodynamic properties of the RNA-MTX complexes will be determined using melting method. The ultimate goal is to find a possible way to treat these neuron diseases.

2.3 Experimental and methods

2.3.1 Tau pre-mRNA and its mutants in melting experiments

The RNA we used in melting experiment is commercial available in Sigma company.

These sequences of hairpins for thermal melting are listed below:

Table 2.1: RNA used in melting experiment

WT(30mer)	5'-GGCGGCAGUGUGAGUACCUUCACACGUCCC-3'
14U(30mer)	5'-GGCGGCAGUGUGAGUACCUUCAUACGUCCC-3'
19G(30mer)	5'-GGCGGCAGUGUGAGUACCUUCACACGUGCC-3'

11C	5'-GGCGGCAGUGUGAGUACCUCCACACGUCCC-3'
WT-17T(31mer)	5'-GGCGGCAGUGUGAGUACCUUCACACUGUCCC-3'
19G-17T(31mer)	5'-GGCGGCAGUGUGAGUACCUUCACACUGUGCC-3'

A Shimadzu 2550 spectrometer was used in thermal melting experiment. During the melting process, temperature was changed from 15 degree to 95 degree, with a ramp rate 0.5 degree per minute. The absorption at 260 nm was recorded at different temperature. The concentration of hairpins used in thermal melting experiments was 2 μ M, which were then melted at various buffer conditions. The buffers we used are listed below:

Table 2.2: Buffer used in melting experiment

Buffer No.	NaCl/mM	HEPES/ μ M	EDTA/mM	Spermine/ μ M	MgCl ₂ /mM	MTX/ μ M	pH
1	200	10	0.1	0	0	0	7.3
2	200	10	0.1	100	0	0	7.3
3	200	10	0.1	0	5	0	7.3
4	200	10	0.1	100	5	0	7.3
5	200	10	0.1	0	0	2	7.3
6	200	10	0.1	0	0	4	7.3
7	200	10	0.1	0	0	8	7.3
8	200	10	0.1	0	0	16	7.3
9	200	10	0.1	0	0	20	7.3

For each thermal melting experiment, the hairpin was heated to 95°C, then snap-cooled to 4°C in 10 minutes.

For the hairpin melting curve with one transition, MeltWin 3.5 Program³⁴ with algorithm reported^{35, 36} were used to extract the thermodynamic parameters. For the hairpin melting curve with more than one transitions, with the two-state assumption, the equilibrium constant of *i*th transition K_i is determined by Van't Hoff equation,

$$K_i = e^{\frac{\Delta H_i^\circ \left(\frac{1}{T_{M,i}} - \frac{1}{T} \right)}{R}}$$

Here, $T_{M,i}$ is the melting temperature in Kelvin and ΔH_i° is the unfolding enthalpy in kcal/mol. T is thermodynamic temperature and R is the gas constant. For a melting curve with only two transitions, ignoring the baseline effects, the observed absorbance A ,

$$A = \frac{A_1 K_1 + (A_1 + A_2) K_1 K_2}{1 + K_1 + K_1 K_2}$$

Here A_1 and A_2 are the fractional hyperchromicities of each transition.

For two-transition curves, the first derivatives of the absorbance to temperature

$$\begin{aligned} \frac{\partial A_t}{\partial T} = & \frac{A_1(K_1 \Delta H_1^\circ + (\Delta H_1^\circ + \Delta H_2^\circ) K_1 K_2) + A_2(\Delta H_1^\circ + \Delta H_2^\circ) K_1 K_2}{(1 + K_1 + K_1 K_2) T^2 R} \\ & - \frac{(A_1(K_1 + K_1 K_2) + A_2 K_1 K_2)(\Delta H_1^\circ K_1 + (\Delta H_1^\circ + \Delta H_2^\circ) K_1 K_2)}{(1 + K_1 + K_1 K_2)^2 T^2 R} \end{aligned}$$

The parameters A_1 , A_2 , ΔH_1° , ΔH_2° , $T_{M,1}$ and $T_{M,2}$ are extracted using least-square fitting method.

2.3.2 Optical tweezers experiment with MTX

In order to test the binding affinity of MTX with Tau pre-mRNA, we add 0.2 μ M MTX into pulling buffer (200 mM NaCl, 5 mM MgCl₂, 0 mM Tris-HCl, 0.1 mM spermine, 0.2 μ M MTX).

2.4 Result and discussion

2.4.1 Thermal melting results in different buffer conditions

For all six hairpins (WT, 14U, 19G, 11C, WT-17T and 19G-17T) in buffer 1, 2, 3 and 4, the heating and cooling curves almost overlapped. And two thermal melting transitions were observed in some hairpins

Figure 2.4: UV absorptions at 260 nm for WT in buffer 1 (black), 2 (red), 3 (blue) and 4 (purple) during the thermal melting experiment, respectively. Temperature verses absorption trajectories of WT in different buffer conditions are shown in different color curves (left-hand side). First derivations of the heating curves for WT in different buffer conditions are shown in right-hand side.

Figure 2.5: First derivations of the heating curves for 14U, 19G, 11C, WT-17T and 19G-17T in different buffer conditions

Table 2.3 Thermodynamic parameters obtained at 260 nm in buffer 1, 2, 3 and 4 (*data are presented in the form of transition 1/transition 2 for curve with two transitions)

T _m /°C \	WT	14U	19G	11C	WT-17T	19G-17T
Buffer 1	71.5	58.1	75.4	67.6	*60.6/80.8	80.0
Buffer 2	74.6	61.3	*52.0/77.0	70.6	*67.6/84.4	*63.7/81.7
Buffer 3	*47.0/75.4	63.5	*46.1/78.9	73.1	*67.5/85.4	*62.5/83.1
Buffer 4	*50.6/76.4	64.2	*55.4/79.3	74.2	*67.2/86.4	*61.9/84.3

$-\Delta G/\text{kcal/mol}$	WT	14U	19G	11C	WT-17T	19G-17T
Buffer 1	6.7	3.6	9.0	5.7	*2.0/7.4	10.4
Buffer 2	7.5	4.6	*2.4/6.0	6.1	*2.9/8.6	*2.4/9.0
Buffer 3	*1.1/6.1	5.6	*1.7/7.7	7.2	*3.3/7.7	*2.7/8.8
Buffer 4	*1.7/5.9	5.4	*2.3/7.5	7.4	*3.1/8.4	*2.6/8.9
$-\Delta H/\text{kcal/mol}$	WT	14U	19G	11C	WT-17T	19G-17T
Buffer 1	67.3	55.9	81.2	63.8	*28.2/60.4	85.3
Buffer 2	69.9	60.9	*51.1/52.9	63.3	*32.5/65.2	*30.4/71.6
Buffer 3	*36.9/54.8	70.5	*26.1/64.3	68.9	*37.5/64.7	*35.1/67.9
Buffer 4	*41.2/48.0	67.2	*42.3/62.9	69.6	*34.9/68.8	*35.6/60.1

For the hairpin WT, 19G, WT-17T and 19G-17T, two transitions during the heating process were observed. From buffer 1 to buffer 4, different concentration of spermine and MgCl_2 were added. Fitting with both the van't Hoff equation and the two-state model, we extracted the thermodynamic parameters.

Figure 2.6: Tm2 (°C) changes in different buffers. In each buffer, WT-17T has the highest Tm2 and 14U has the lowest Tm. Buffer 1 contains 200mM NaCl, 10mM HEPES, 0.1mM EDTA; Buffer 2 contains 200mM NaCl, 10mM HEPES, 0.1mM EDTA and extra 0.1mM spermine; Buffer 3 contains 200mM NaCl, 10mM HEPES, 0.1mM EDTA and extra 5mM MgCl₂, Buffer 4 contains 200mM NaCl, 10mM HEPES, 0.1mM EDTA with extra 0.1mM spermine and 5mM MgCl₂.

Both spermine and MgCl₂ can increase the thermal stability of these hairpins, according to our thermal melting result. Among these six hairpins, 14U has the lowest Tm, and both 14U and 11C have the lower Tm compared with WT, which suggests that mutations at location 11 (U to C) and location 14 (C to U) can destabilize the thermal stability of Tau

pre-mRNA. While the mutant 19G, WT-17T and 19G-17T have higher melting temperature compared with WT.

Different concentration of MTX was added into buffer to investigate whether MTX can be a ligand which can specific bind to Tau pre-mRNA. According to literature²⁷, MTX is able to bind to the bulged A in Tau pre-mRNA hairpin structure. For all six hairpin constructs, five different concentrations of MTX were used in thermal melting experiments. The concentration of MTX is 2 μ M, 4 μ M, 8 μ M, 16 μ M and 20 μ M in buffer 5, 6, 7, 8 and 9, respectively. The concentration of hairpin was 2 μ M.

Table 2.4 Thermal melting temperature obtained at 260 nm in buffer 5, 6, 7, 8 and 9
(*data are presented in the form of transition 1/transition 2 for curve with two transitions)

T _m /°C \	WT	14U	19G	11C	WT-17T	19G-17T
Buffer 5	72.5	62.6	*54.4/75.8	71.1	*58.5/81.0	80.3
Buffer 6	73.4	63.6	*53.5/77.2	72.9	*64.1/81.1	80.9
Buffer 7	74.0	66.1	*54.5/77.4	74.5	*61.9/82.0	80.4
Buffer 8	75.7	67.8	*55.7/76.9	75.5	*58.0/82.0	80.3
Buffer 9	42.7/76.5	68.3	*55.8/76.8	*46.8/75.5	*60.6/81.8	80.7

Figure 2.7: Tm2 (°C) changes in different buffers. For WT, 14U and 11C, melting temperature increases when the MTX concentration increases. For 19G, WT-17T and 19G-17T, the melting temperature are almost at the same level with different MTX concentrations.

During the thermal melting process, MTX can affect the melting properties of WT, 14U and 11C. According to the hairpin structures predicted, WT, 14U and 11C have bulged A and bulged C in the stem-loop structure, and MTX that bind to the bulged A of Tau pre-mRNA and its mutant with bulged A can stabilize the stem-loop structure and therefore can decrease the exon 10 alternative splicing. While WT-17T have bulged C at the same position in stem-loop structure but the melting temperature remains almost the same at

different concentrations of MTX, which behaves different with WT, 14U and 11C, indicates the bulged C may not be the important factor for the MTX binding.

It is interesting to see that for some RNA in thermal melting experiment, two melting temperatures were observed in certain buffer condition. Such as 19G in Buffers 2, 3, 4, 5, 6, 7, 8 and 9. Spermine and Mg^{2+} can stabilize the hairpin stem structure regions differently. It's likely that the top stem (above the A bulge) can be more stabilized by the cations nonspecifically resulting in the melting of bottom stem before the top stem. Specific binding of MTX to the A bulge can also enhance the stability of the hairpin stems with an A bulge. Addition of MTX does not affect the melting temperature of the top stem of the hairpins without the A bulge (19G, WT-17T, and 19G-17T), but can alter the thermal unfolding the pathway, probably because MTX may have non-specific electrostatic interactions with the hairpins.

Our previous studies also shown that the structure rearranged happened in mutant 19G. According to our pulling experiment, the bulged A in stem-loop structure is missed in 19G because of the structure rearranged and bulged G is formed. The thermal melting experiments give one evidence that there's no bulged A in mutant 19G, which allows us to have a deeper understanding about the structure rearrangement.

Figure 2.8: RNA hairpin constructs predicted by RNAstructure³⁷

For WT-17T and 19G-17T, base U was inserted in position 17 to form A-U base pair, which can affect MTX binding to RNA stem-loop structure. From the thermal melting result, both melting temperatures of WT-17T and 19G-17T are at the same level in various concentrations of MTX, which also suggests that MTX can specific bind to Tau pre-mRNA and its mutant with bulged A.

2.4.2 Mechanical properties of Tau pre-mRNA with MTX binding

To further investigate how the specific binding of MTX affects the mechanical properties of Tau pre-mRNA, single-molecule spectroscopic method using optical tweezers were applied on the MTX-RNA complexes. In the force ramp experiment, the pulling buffer contains MTX (200mM NaCl, 10mM Tris, 5mM $MgCl_2$, 100 μM Spermine, 0.2 μM MTX) was used in this single-molecule binding study. For comparison, another buffer (200mM

NaCl, 10mM Tris, 5 mM $MgCl_2$, 100 Spermine, 1 μM neomycin) with binding ligand neomycin, which is known to bind to the major groove of Tau pre-mRNA exon 10, was used in force ramp experiment.

Figure 2.8: Force ramp (un)folding trajectories of WT. Red trajectory in each panel represents the unfolding process, where the external force applied on RNA increased from 5 pN to 25 pN, and the stem-loop structure stretched into a single stranded RNA in this process. Black trajectory in each panel represents the folding process, where the external force decreased from 25 pN to 5 pN, and the single stranded RNA refold into stem-loop structure in this process. (A) The pulling buffer: 200mM NaCl, 10mM Tris, 5mM $MgCl_2$, 100 μM Spermine. The unfolding force is around 14.5 pN, and the unfolding trajectory and folding trajectory are well overlapped, which suggest that the

mechanical folding and unfolding are in equilibrium. (B) The pulling buffer: 200mM NaCl, 10mM Tris, 5mM $MgCl_2$, 100 μM Spermine, 0.2 μM MTX. Pulling buffer contains MTX affects the unfolding. The unfolding force is around 20 pN. The unfolding trajectory and folding trajectory are not overlapped. (C) The pulling buffer: 200mM NaCl, 10mM Tris, 5 mM $MgCl_2$, 100 Spermine, 1 μM neomycin. Pulling buffer contains neomycin also has the effect on unfolding force. The unfolding force is around 16.2 pN, and the unfolding trajectory and folding trajectory are well overlapped.

Both MTX and neomycin can bind to Tau pre-mRNA exon 10 region. The increase of mechanical stability of MTX-RNA complex consists with the thermal melting results. The folding and unfolding trajectories of WT in pulling buffer contains neomycin show a well overlapped pattern, suggesting that mechanical folding and unfolding are in equilibrium in this buffer condition. While for the pulling buffer contains MTX, the folding and unfolding trajectories are not overlapped, suggesting that folding and unfolding processes are not reversible.

MTX can make on the interaction with A bulge. Binding to A bulge can stabilize the RNA hairpin structure according to our result. The melting results of WT, WT-17T, 19G, 19G-17T show that A bulge can affect MTX binding to RNA. In WT, melting temperature increases when MTX concentration increases, but in WT-17T, melting temperature remains unchanged. Similar results can be obtained in 19G and 19G-17T. Those results indicate that MTX can specifically binding to A bulge in our RNA hairpins. And Figure 2.8(B) shows the irreversible (un)folding processes, which may indicate that

unfolding process and refolding process have different pathways. During the unfolding process, MTX can stabilize the hairpin structure, which can lead to high unfolding force. And in refolding process, the RNA may form into hairpin structure first, and then MTX can binding to A bulge. So in Figure 2.8(B), irreversible (un)folding processes were observed. And neomycin is a non-specific binding ligand which can bind to hairpin structure and increase the stability of RNA hairpin, which will lead to the reversible (un)folding processes in Figure 2.8(C).

Figure 2.9: unfolding force distribution of WT in pulling buffer without MTX (left-hand side) and pulling buffer with MTX (right-hand side). Same RNA tether was measured during the pulling experiment with different buffers. For the left-hand side, the pulling buffer: 200mM NaCl, 10mM Tris, 5mM $MgCl_2$, 100 μ M Spermine. And for the right one,

the pulling buffer: 200mM NaCl, 10mM Tris, 5mM $MgCl_2$, 100 μ M Spermine, 0.2 μ M MTX.

The unfolding force of WT in pulling buffer without MTX is around 14.5 pN, which is consistent with previous pulling result. For the unfolding property of WT in pulling buffer with MTX, two different peaks were appeared, which suggests that two kinds of unfolding paths happened in the buffer with MTX. Lower unfolding force is around 15 pN, which behaves like the trajectories in pulling buffer without MTX. This indicates MTX may not bind to RNA, leading to a relatively low unfolding force in MTX buffer.

2.5 Conclusions

The thermodynamic properties can be revealed by our thermal melting experiment. Among all six hairpins, mutant 14U is the most unstable one, which is consistent with previous single-molecule studies. MTX can specifically bind to Tau pre-mRNA and its mutants with bulged A in the stem-loop structure. Both thermal melting experiments and single-molecule experiments show that MTX can increase the stability of target RNA. Upon small molecule binding, these target RNA hairpins is able to change the thermodynamic properties as well as the mechanical properties, which may affect the inclusion of exon 10, alter the 3R:4R ratio. Our result reveals that small molecule like MTX can bind to some disease-causing RNAs, change the stability of these RNA, which may help to develop a potential treatment dealing with the neuron diseases.

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