International Journal of Environment, Agriculture and Biotechnology
Vol-8, Issue-6; Nov-Dec, 2023
Peer-Reviewed International Journal
Journal Home Page Available: https://ijeab.com/
Journal DOI: 10.22161/ijeab

Pseudouridine in RNA: Enzymatic Synthesis Mechanisms and Functional Roles in Molecular Biology

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Received: 11 Nov 2023; Received in revised form: 12 Dec 2023; Accepted: 19 Dec 2023; Available online: 31 Dec 2023

Abstract**—** *Pseudouridine, a common modified nucleotide, is prevalent in bacterial tRNA, rRNA, and snRNA. Initially identified in rRNA and tRNA, its presence extends to snRNA. Despite being the first identified and most prevalent RNA modification, its biosynthesis and diverse roles remain insufficiently understood. This extensively occurring modified nucleotide influences structural and functional attributes in various RNA categories. The isomerization process involves a carbon–carbon bond formation, and Pumilio family proteins (PUFs) are potential Ψ reader proteins. Pseudouridine, a ubiquitous constituent in structural RNAs, is notably absent in mRNA or viral RNAs. Its enzymatic isomerization occurs at the polynucleotide level, independently of cofactors. Compared to uridine, pseudouridine prefers the C3-endo conformation, enhancing stability in specific structural motifs. Evolutionarily conserved in major spliceosomal snRNAs, it plays a crucial role in spliceosome assembly and splicing. Pseudouridine (ψ), comprising 0.2–0.6% of uridines in mammalian mRNA, is enzymatically generated by pseudouridine synthases. Five pseudouridine synthase families orchestrate its site-specific isomerization. In eukaryotic and archaeal organisms, specific synthases rely on noncoding RNAs, like box H/ACA small nucleolar/scaRNPs. These modifications contribute to RNA structural stabilization and functional efficacy. In pre-mRNA and mRNA they guide splicing processes and protect against degradation, acting as a defense mechanism against viral infections. This review delves into the detection, structure, functions, and applications of pseudoridine in RNA. Methodologies like High-performance liquid chromatography, mass spectrometry, thin layer chromatography, enzyme-linked Immunosorbent assay, capillary electrophoresis, northern blotting, reverse transcript polymerase chain reaction and RNA bisulfite sequencing. establish a robust framework. Pseudouridine's roles in reinforcing RNA structures, modulating translation, and its potential in mRNA.*

Keywords**— Enzyme, Pseudouridine, RNA modification, , Synthesis, Uridine.**

I. INTRODUCTION

Pseudouridine (ψ), constituting 0.2–0.6% of mammalian mRNA uridines, is enzymatically derived through pseudouridine synthases. Initially identified in rRNA and tRNA, it extends to snRNA (Riley, Sanford, Woodard, Clerc, & Sumita, 2021). With over a hundred post-transcriptional RNA modifications shaping our understanding of biological processes, pseudouridine stands out as a prevalent and fundamental modification in living cells. Despite being the first RNA modification discovered

and the most abundant, the mechanisms governing its biosynthesis and multifaceted roles remain insufficiently elucidated since its recognition as the "fifth nucleoside" in RNA(Yu & Meier, 2014).

Pseudouridine, a widespread modified nucleotide, is abundant in various RNA types, including tRNA, rRNA, snRNA, and snoRNAs, significantly influencing their structural and functional characteristics. The isomeric transition of the glycosidic bond, shifting from N1 to C5 of uracil, enhances base rotation, potentially improving the thermodynamic stability of RNA duplexes through additional hydrogen bonding and stacking interactions (Riley et al., 2021). Pseudouridine's isomerization involves tethering uracil via a carbon–carbon bond, which is different from the conventional nitrogen–carbon glycosidic bond. While Ψ reader proteins remain unidentified, Pumilio family proteins (PUFs) are promising candidates (Corollo et al., 1999). This modification introduces an extra imino group, serving as an additional hydrogen bond donor, and enhances the stability of the carbon-carbon glycosidic bond compared to the prevalent nitrogen-carbon bond (Becker, Motorin, Sissler, Florentz, & Grosjean, 1997).

Pseudouridine is a widespread and mysterious element in structural RNAs, commonly found in tRNA, rRNA, and snoRNA, yet absent in mRNA or viral RNAs (Anderson et al., 2010). Its enzymatic isomerization from uridine occurs at the polynucleotide level, independently of cofactors or external energy sources. In Escherichia coli, the small subunit rRNA has one pseudouridine, while the large subunit rRNA boasts nine instances of this modification (Charette & Gray, 2000).

Compared to uridine, pseudouridine favors the C30-endo conformation of the ribose, aligning with the anti-conformational preferences of nucleobases (Yamauchi et al., 2016). It is prevalent in the T pseudouridine C loop of nearly all tRNAs and is often found in the D stem and/or the anticodon stem and loop (Mengel-Jørgensen & Kirpekar, 2002). Pseudouridine's inclusion significantly stabilizes specific structural motifs. Notably, it is evolutionarily conserved in major spliceosomal snRNAs (U6, U5, U4, U2, and U1), occupying crucial regions for RNA–RNA and RNA–protein interactions vital for spliceosome assembly and the splicing process. As a rotational isomer of uridine, pseudouridine stands out as the most abundant modified nucleotide, ubiquitously present in almost all tRNA, rRNA, and snRNA in bacteria. Its site-specific isomerization in tRNA and rRNA is orchestrated by five families of pseudouridine synthases (Kellner, Burhenne, & Helm, 2010).

In eukaryotes and archaea, a distinct group of pseudouridine synthases relies on noncoding RNAs for site-specific isomerization of rRNA and snRNA (Morais, Adachi, & Yu, 2021). This unique mechanism involves box H/ACA sno/scaRNPs, consisting of four proteins and a box H/ACA RNA (Liang et al., 2009). The biological landscape features numerous post-transcriptional RNA modifications, with potential roles in stabilizing RNA strands and enhancing functional efficacy. Modifications in pre-mRNA and mRNA can guide splicing and protect RNAs from degradation by nucleases, acting as a defense against viral infections (Riley et al., 2021).

In the realm of natural modifications, methylation is prevalent, but pseudouridine (Ψ) claims the distinction of being the most common and earliest identified modification (Becker et al., 1997). Ψ, an isomer of uridine with a C1’-C5 glycosidic bond, undergoes a substantial structural transformation during its isomerization (Penzo, Guerrieri, Zacchini, Treré, & Montanaro, 2017). This process involves cleaving the N1-C1’ glycosidic bond and a 180° rotation of the base, and it is hypothesized to be facilitated by nucleophilic attack by arginine residues within pseudouridine synthases. The fundamental concept of this mechanism relies on enzymes stabilizing ribose sugars and uracil bases, allowing for the cleavage of the C-N glycosidic bond, leading to a 180° base flip, and the formation of a C-C glycosidic bond, resulting in Ψ as the final product (Riley et al., 2021).

II. SIGNIFICANCE OF Ψ IN RNA

Pseudouridylation, impacting over 100 specific uridines in rRNAs, is crucial for maintaining the proper functioning, folding, and conformational stability of rRNAs (Corollo et al., 1999). These modifications also affect interactions between rRNAs and ribosomal proteins, ensuring the catalytic activity of the ribosome. Changes in rRNA pseudouridylation directly influence interactions with tRNAs and mRNAs, modifying translational efficiency, gene expression patterns, and levels (Wang et al., 2023).

Despite being widespread in ribosomes, Ψ's precise function remains elusive. In eukaryotes, especially in the critical core region of LSU and SSU RNAs, Ψ likely plays a role in optimal ribosome performance (Cortese, Kammen, Spengler, & Ames, 1974). Experimental verification of its direct contribution has proven challenging. Nature, synthesizing Ψ without energy expenditure, may have adapted it for diverse purposes (Kazimierczyk & Wrzesinski, 2021). Found exclusively in RNA molecules with functional tertiary structures, Ψ's primary function could be molecular glue, fortifying and enhancing RNA conformations (Singh, Shyamal, & Panda, 2022). This proposition aligns with varying Ψ numbers

across species, the lack of correspondence in Ψ sites among organisms, and challenges in detecting effects upon individual Ψ removal. Measuring functional effects may prove more effective when considering multiple Ψ deletions (Pfeiffer, Ribar, & Nidetzky, 2023).

During translation, pseudouridine (W) is believed to modulate interactions between tRNA molecules and rRNAs, as well as with mRNAs. Pseudouridylation of tRNA does not disrupt overall three-dimensional tRNA structure, isn't essential for cell viability, and isn't universally required for amino acylation. However, pseudouridine does influence the local structure of the domains in which it is present (Vaidyanathan, AlSadhan, Merriman, Al-Hashimi, & Herschlag, 2017). W residues, occasionally located in the anticodon region, contribute to alternative codon usage. Additionally, there's consideration of a catalytic role for pseudouridine in rRNA during the peptidyl transfer process in translation.

Pseudouridine enhances the stability of RNA molecules, which is crucial for optimal functionality in processes like translation and splicing by influencing their secondary conformation. Pseudouridine actively shapes intricate folding patterns of RNA molecules, contributing to the delineation of distinct RNA structures essential for biological efficacy (Barbieri & Kouzarides, 2020). Non-random distribution of pseudouridine within RNA sequences, particularly in functionally significant regions, suggests its potential involvement in orchestrating diverse cellular processes. Ubiquitously present in various RNA species across diverse organisms, Pseudouridine's functional role offers insights into the complex landscape of RNA modifications and their multifaceted impact on cellular functionalities (Corollo et al., 1999).

In vitro-transcribed mRNAs face challenges due to instability and immunogenicity. Integrating pseudouridines enhances mRNA stability and translational efficacy in mammalian cells and mice, reducing immunogenicity (Rintala-Dempsey & Kothe, 2017). Pseudouridine's strategic placement in loop-closing regions suggests roles in stabilization and conformational modulation. Its structural disparities amplify base stacking, introducing rigidity and bolstering stability in pseudouridylated duplexes (Cortese et al., 1974). This modification holds promise for modulating splicing, immunogenicity, and translation in vivo, responding to cellular stress and extending RNA half-life. Understanding pseudouridine's contributions to cellular processes is advanced by targeted disruptions of modification enzymes. This research sheds light on the potential therapeutic applications of pseudouridine-modified mRNA (Huang et al., 2021).

III. PSEUDOURIDINE IN RNA

3.1. Chemical structure of pseudouridine

Early hypotheses about pseudouridine in RNA focused on its unique physicochemical properties, setting it apart from its precursor, uridine (Nombela, Miguel-López, & Blanco, 2021). Pseudouridine's distinctiveness lies in its possession of a C-C glycosyl bond, deviating from the typical N-C bond connecting the base and sugar moieties (Huang et al., 2021). Anticipation stemmed from the idea that the C-C glycosyl bond, offering increased rotational freedom compared to the N-C glycosyl bond, might provide pseudouridine with greater conformational flexibility than uridine. Additionally, the free N1-H in pseudouridine, serving as an additional hydrogen bond donor, hinted at potential novel pairing interactions in RNA (Karikó et al., 2008). Speculation also revolved around the high group transfer potential for acyl moieties by the N1-H of pseudouridine (Lovejoy, Riordan, & Brown, 2014).

Conformational studies of free pseudouridine nucleosides revealed a subtle preference for the syn glycosyl conformation, differing from the anti-configuration adopted by uridine and other nucleosides. This led to the proposition that pseudouridine might serve as a conformational switch in RNA, given its low energy requirement for the syn/anti-transition and comparable hydrogen-bonding potential (Zhao & He, 2015). However, within polynucleotide chains, pseudouridine consistently adopts the anti-configuration. In these instances, it imparts rigidity rather than flexibility to both single- and double-stranded regions despite sharing the same basic topology in RNA as uridine. Insights from nuclear magnetic resonance, X-ray crystallography, and molecular dynamics simulations support this deduction, providing valuable perspectives on pseudouridine's nuanced impact on RNA structure (Penzo et al., 2017).

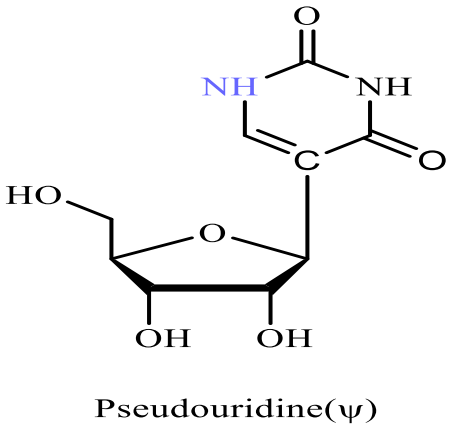

Fig.1. The structure of Pseudouridine

3.2. Unique features of Pseudouridine

Pseudouridine's unique characteristics, when compared to uridine, arise mainly from its additional hydrogen bonding capabilities. In the anti-conformation within RNA, pseudouridine provides a favorable geometry and distance for coordinating a water molecule between its N1-H and the 5' phosphates of both pseudouridine and the preceding residue (Cerneckis, Cui, He, Yi, & Shi, 2022). This constraint on base conformation and backbone mobility persists at the 5' site of pseudouridylation, regardless of the surrounding sequence or structural context, whether in a single- or double-strand configuration (Levi & Arava, 2021).

Pseudouridylation enhances local RNA stacking in single-stranded and duplex regions, favoring a 3'-endo ribose conformation and axial anti-base moiety alignment (Morais et al., 2021). This process involves a structured water molecule replacing a weak C5-H bond, stabilizing the pseudouridine moiety. The result is increased rigidity in the phosphodiester backbone, leading to cooperative enhancement of adjacent nucleoside stacking and reinforcing RNA structure. Pseudouridine's major contribution lies in amplifying base stacking (Koonin, 1996).

3.3. Distribution of Pseudouridine in different classes of RNA

3.3.1. Transfer RNAs

Ψ, prevalent in almost all tRNAs, notably as the near-universal Ψ55, extends across life domains and organelles like mitochondria and chloroplasts. Found in locations such as the D stem and anticodon regions, Ψ's distribution follows domain-specific patterns. It crucially stabilizes structural motifs like the TΨC loop (Ψ55), D stem (Ψ13), anticodon stem (with a strong closing base pair between Ψ39 and A31), and anticodon loop (featuring noncanonical base-pairing between Ψ38 and residue 32) (Morena, Argentati, Bazzucchi, Emiliani, & Martino, 2018).

3.3.2. Small Nuclear and Nucleolar RNAs

Pseudouridine (Ψ) is a widespread modification found in most transfer RNAs, prominently located at Ψ55 in the TΨC stem loop. Its presence is observed across all life domains, including archaea, bacteria, and eukaryotes, as well as in cellular organelles like mitochondria and chloroplasts (Tavakoli et al., 2023). Ψ is distributed in the D stem, anticodon stem, and loop, displaying domain-specific patterns. The intentional use of pseudouridine plays a crucial role in stabilizing key structural motifs within tRNA, such as the TΨC loop at Ψ55, D stem at Ψ13 and anticodon stem. Notably, Ψ contributes to the stability of the anticodon loop, forming noncanonical base pairs with residues like Ψ38 and 32. This strategic placement enhances the overall stability and functionality of essential tRNA structural elements (Zimna, Dolata, Szweykowska-Kulinska, & Jarmolowski, 2023).

In eukaryotes, pseudouridine (Ψ) is notably present in major spliceosomal small nuclear RNAs (snRNAs) like U1, U2, U4, U5, and U6, as well as in minor variants associated with AU/AC intron splicing (U12, U4atac, and U6atac) (Westhof, 2019). While Ψ residues show phylogenetic conservation, subtle organism-specific variations add complexity. These modifications strategically occur in functionally critical regions, participating in vital RNA–RNA or RNA–protein interactions crucial for spliceosome assembly and operation (Riley et al., 2021). For example, a Ψ–A pair near the intron's branch site enhances stability in the U2 snRNA/pre-mRNA interaction, aiding the initial splicing reaction. Ψ residues are recurrent in U4/U6 snRNA interaction regions and between U1 snRNA and the 5' splice site. Beyond spliceosomal snRNAs, Ψ is found in small nucleolar RNAs (snoRNAs) like U3, U8, snR4, and snR8, presenting intriguing areas for further exploration of Ψ formation and functional implications (Morais et al., 2021).

3.3.3. Ribosomal RNAs

Pseudouridine (Ψ) is a prevalent component in the ribosomal RNAs (rRNAs) of both small (SSU) and large (LSU) subunits across eubacteria, archaebacteria, and eukaryotes, including mitochondria and chloroplasts (Foster, Huang, Santi, & Stroud, 2000). It is also present in 5.8S and select 5S rRNAs. Through nucleotide-resolution mapping techniques, Ψ has been identified in LSU rRNA, notably in Escherichia coli and Saccharomyces cerevisiae (Koonin, 1996). The clustering of Ψ residues within crucial domains of LSU rRNA, such as Domain II (near the 5'-end), Domain IV (central), and Domain V (adjacent to the 3'-end, housing the peptidyltransferase center), reinforces its functional significance. Domain IV serves as the decoding center, facilitating interactions between LSU rRNA, mRNA, and the anticodon stem-loop (ASL) of transfer RNA (tRNA) (Ofengand, 2002).

Domains II and IV, though spatially distant from Domain V in structure, closely align with the site of peptide bond formation. Notably, Ψ residues, like E. coli Ψ2580 and others in the PTC and Domain IV, precisely map to the ribosomal A- and P-sites, crucial for tRNA interaction (Corollo et al., 1999). In contrast, SSU rRNA lacks Ψ clustering in functional regions. Eukaryotic LSU rRNAs show a significant increase in Ψ residues (0.9% to 1.4%) compared to counterparts in eubacteria, archaebacteria, or organelles (0.03% to 0.4%) (Foster et al., 2000). These

additional Ψ residues cluster in domains II, IV, and V, while archaebacterial LSU rRNA aligns more with eubacteria. Homologous rRNAs in closely related organisms exhibit clade-specific Ψ residue patterns with organism-specific variations. Importantly, Ψ residues lack universal conservation in secondary structure positions in both LSU and SSU rRNA (Karikó et al., 2008).

IV. BIOLOGICAL FUNCTION OF PSEUDOURIDINE

Pseudouridine (Ψ), a vital RNA modification, differs from methylation or acetylation by isomerizing uridine without chemical group additions. It plays diverse roles in RNA biology, conservatively impacting structured non-coding RNA by influencing conformation, stability, and dynamics (Y. Zhang, Lu, & Li, 2022). Ψ's unique ability to form stable Watson-Crick base pairs with A enhances thermodynamic stability, stabilizes single-stranded RNA, and promotes duplex formation (Szweykowska-Kulinska, Senger, Keith, Fasiolo, & Grosjean, 1994). Its preference for the C3′-endo sugar conformation and enhanced base stacking contribute to these effects, along with water bridges observed in tRNA crystal structures (Levi & Arava, 2021). Pseudouridine is recognized as a widely pervasive modification, embodying the C5-glycoside isomer of uridine. Its distinctive attributes confer rigidity to RNA structures, fine-tune tRNA integrity, enhance translation accuracy, and dynamically regulate mRNA coding. Pseudouridine synthesis is governed by pseudouridine synthases (Yu & Meier, 2014).

Pseudouridine (Ψ) alters RNA-protein interactions in various ways, impacting nuclear RNA processing, cytoplasmic RNA localization, and stability. For instance, in human cells, pseudouridylation of the RNA motif recognized by the protein PUM2 reduces binding affinity (Kierzek et al., 2014). Similarly, the substitution of uridines with Ψ in CUG repeats associated with myotonic dystrophy type 1 reduces splicing factor MBNL1 binding. In translation, Ψ is hypothesized to modulate interactions between tRNA, ribosomal RNAs (rRNAs), and messenger RNAs (mRNAs), influencing localized structure without altering tRNA's overall three-dimensional structure (Karikó et al., 2008). Abundant in tRNA, pseudouridine enhances the stability of secondary and tertiary structures, which is crucial for proper folding and functionality (Martinez et al., 2022). It mainly impacts the anticodon loop, influencing codon-anticodon interactions and precision in translation. Pseudouridylation plays a vital role in fine-tuning tRNA structure, decoding activity, and maintaining translation fidelity.

Pseudouridine strategically placed in tRNA and rRNA enhances flexibility, optimizing codon-anticodon interactions and stabilizing RNA structures. It plays a crucial role in ribosome stability during translation, influencing decoding and peptidyl transferase activity (Torsin et al., 2021). In RNA splicing, especially in snRNAs, and mRNA stability, pseudouridine impacts efficiency and accuracy, affecting gene expression. Although once hypothesized to play a catalytic role, recent evidence challenges its direct involvement. In summary, pseudouridine's dynamic role in RNA biology underscores its indispensability in cellular RNA maintenance, with ongoing research unraveling its intricate contributions to gene expression and cellular function (Riley et al., 2021).

Importance of disease related to Pseudouridine

Pseudouridylation, first elucidated in 1951, is the most prevalent modification in non-coding RNAs (ncRNAs), including long non-coding RNAs (lncRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and small nucleolar RNAs (snoRNAs) (Yamauchi et al., 2016). Generated through uridine isomerization, pseudouridine (Ψ) forms stronger bonds than uridine and engages similarly with adenosine. Pseudouridine synthases, acting as writers, introduce Ψ and can be RNA-dependent or RNA-independent (Kellner et al., 2010). Dyskerin pseudouridine synthase targets ncRNAs and is associated with a worse prognosis in lung and pancreatic cancer. Alterations in DKC1 can inactivate tumor suppressors like p53 in breast cancer. PUS1 plays a role in interactions between SRA1 and RARG in melanoma and breast cancer. Depletion of PUS10 prevents apoptosis in p53-null prostate cancer cells (Charette & Gray, 2000).

Pseudouridine (Ψ) inclusion in non-coding RNAs (ncRNAs) is observed in cancers like ZFAS1 and TERC. ZFAS1 is dysregulated in various cancers, while TERC, despite its role in telomere modulation, shows paradoxical behavior in lung and prostate cancers (Kazimierczyk & Wrzesinski, 2021). Pseudouridylated ncRNAs SNHG1 and SNHG7 play complex roles in oncology, impacting gastric and colorectal cancers. However, the precise influence of pseudouridine on these ncRNAs' functional dynamics remains speculative and requires further validation (Martinez et al., 2022).

X-linked Dyskeratosis Congenita (X-DC) and its severe form, Hoyeraal-Hreidarsson syndrome, are rare inherited disorders caused by mutations in DKC1, the gene encoding the pseudouridine synthase dyskerin. X-DC exhibits a muco-cutaneous triad of abnormal skin pigmentation, nail dystrophy, and leukoplakia. In cancer, dyskerin expression and rRNA pseudouridylation levels are often elevated (Penzo et al., 2017). In breast cancer,

dyskerin correlates with tumor progression and poor prognosis. Similar associations exist in hepatocellular carcinomas lung and prostate cancers (Barbieri & Kouzarides, 2020). Dyskerin's overexpression impact in lung cancer relates to telomerase function, but underlying molecular mechanisms remain unclear, lacking experimental studies with dyskerin overexpression cellular models (Keszthelyi & Tory, 2023).

Mutations in the dyskerin-encoding gene cause X-linked dyskeratosis congenita (X-DC), initially thought to be linked to Ψ-deficient ribosomes. However, it's now associated with reduced human telomerase RNA (hTR), affecting telomerase activity and telomere length maintenance (Singh et al., 2022). Dyskerin's role in the 3' terminal region of hTR is noteworthy. Pseudouridylation plays a role in HIV infections by capturing an essential co-factor for viral replication. In maternally inherited diabetes and deafness (MIDD), a mitochondrial tRNA mutation hinders pseudouridylation, affecting mitochondrial translation, causing respiratory issues, and contributing to pancreatic, neuronal, and cochlear cell dysfunctions in MIDD pathogenesis (Torsin et al., 2021).

Mutations in PUS genes, akin to DKC1, can be linked to diseases. For instance, a missense mutation in the PUS1 gene causes mitochondrial myopathy and sideroblastic anemia (MLASA) (Corollo et al., 1999). This mutation affects a conserved amino acid in the enzyme's active site, leading to impaired pseudouridylation of specific tRNAs and perturbations in protein synthesis, contributing to MLASA pathogenesis (Martinez et al., 2022). Despite this, most tissues remain unaffected, possibly due to lower translational activity or tissue-specific compensatory mechanisms. The pleiotropic effects of PUS1 suggest potential involvement in the impaired pseudouridylation of other RNA species. Furthermore, a form of autosomal recessive mental retardation (MRT55) results from a homozygous mutation in the PUS3 gene. This mutation leads to reduced pseudouridylation in specific tRNA positions, contributing to intellectual disorders in affected individuals (Singh et al., 2022).

V. HUMAN DISEASES RELATED TO STAND-ALONE PSEUDOURIDINE SYNTHASES

5.1.1. MLASA

MLASA is a rare autosomal recessive disorder affecting oxidative phosphorylation, resulting in muscle and bone marrow defects, exercise intolerance, and anemia. Symptoms include cognitive impairment, skeletal issues, delayed motor milestones, cardiomyopathy, dysphagia, and respiratory insufficiency. YARS2 gene loss leads to a similar phenotype. The role of pseudouridine synthase PUS1 in disrupting oxidative phosphorylation is not fully understood, but its R116W substitution is the first reported causal variant in MLASA, with other mutations contributing to a similar disorder (Wang et al., 2023).

5.1.2. Brain developmental disorders and Facial dysmorphia

Intellectual disability often stems from chromosomal rearrangements or single gene mutations, with tRNA modification enzyme defects indicating particular sensitivity in brain development (Riley et al., 2021). Maturing tRNAs undergo critical posttranscriptional modifications, stabilizing their structure and preventing translational errors (Karikó et al., 2008). Hypomodified tRNAs may be degraded, impacting protein synthesis. PUS3, a TruA family member and a general pseudouridine synthase for tRNAs, is implicated in global developmental delay/intellectual disability (GDD/ID), causing microcephaly, short stature, severe hypotonia, gray sclera, and other syndromic features. The p.R435* mutation truncates a conserved C-terminal region in mammals (Wang et al., 2023).

5.1.3. Intellectual disability, speech delay, short stature, microcephaly, aggressive behavior

PUS7, like PUS3, acts on multiple tRNAs and mRNAs, with its catalytic domain situated in the C-terminal region (Barbieri & Kouzarides, 2020). Patients with PUS7 loss-of-function mutations exhibit a phenotype resembling PUS3 variants, including intellectual disability, short stature, microcephaly, and often aggressive behavior. The affected enzymes lose the isomerization capacity of U13 in at least ten cytosolic tRNAs, leading to dysregulation of general protein translation (Wang et al., 2023).

5.1.4. Cardiovascular disease

Cardiovascular disease (CVD), a leading cause of global death, affects the heart and blood vessels. Prevalence and mortality increase notably after 40, with hypertension and atherosclerosis as key risk factors. Common CVD-related causes of death include ischemic heart disease (IHD), atrial fibrillation (AF), cardiomyopathy, hypertensive heart disease, endocarditis, and myocarditis (Pfeiffer et al., 2023). IHD (coronary heart disease) and stroke are major contributors. RNA modifications, like pseudouridine, and their regulators play crucial roles in CVD. This section primarily focuses on the heart, excluding discussion of some vascular diseases like stroke and peripheral arterial disease (PAD) (Y. Zhang et al., 2022).

VI. ENZYMATIC SYNTHESIS

6.1. Enzymes responsible for pseudouridine synthesis

Pseudouridylation, a vital RNA modification, is governed by stand-alone pseudouridine synthases (PUSs) organized into six families: TruA, TruB, TruD, RsuA, RluA, and PUS10 (Duong, 2017). In this exploration, we focus on nine yeast Pus enzymes and Pus10, categorized into four families mirroring E. coli counterparts (Riley et al., 2021). The TruA family—Pus1, Pus2, and Pus3—catalyzes modifications in tRNAs, snRNAs, and mRNAs. Pus4 of the TruB family targets U55 in tRNA and mRNA. RluA, the largest family with Pus5, Pus6, Pus8, and Pus9, pseudouridylates tRNAs, mitochondrial 21S rRNA, and mRNA, some incorporating an S4-like N-terminal domain (Spenkuch, Motorin, & Helm, 2014). The TruD family's sole member, Pus7, modifies tRNAs, rRNA, and snRNA. This overview aims to unravel the complex realm of eukaryotic PUSs, highlighting their varied roles and intricate enzymatic activities in RNA modification (Sanford, 2021).

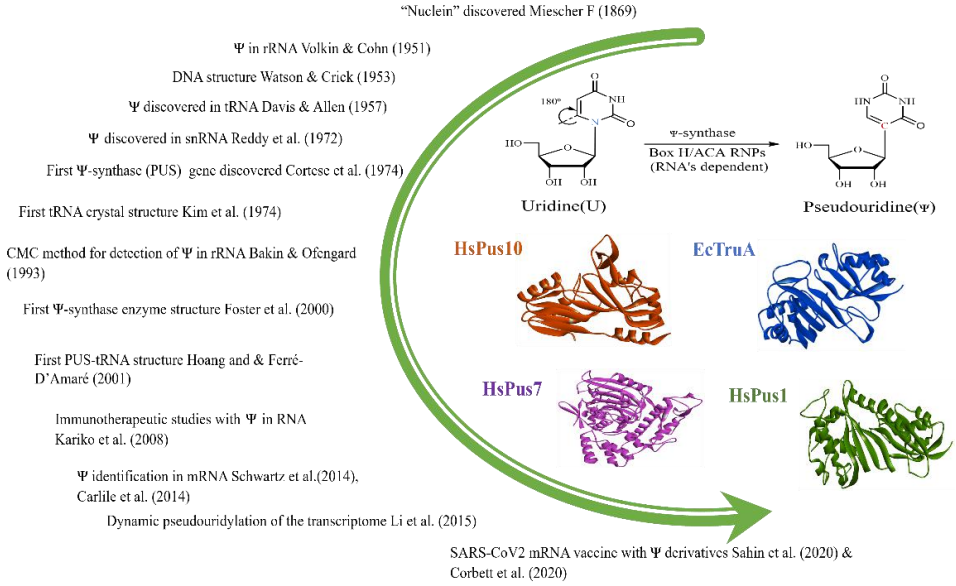

Fig.2. The timeline of pseudouridine research is highlighted alongside an inset depicting the uridine-to-pseudouridine conversion. Crystal structures of key PUS enzymes (HsPus10, EcTruA, HsPus7, HsPus1) in cartoon representation offer concise insights into the biological history and structural aspects of pseudouridine exploration.

6.2. Pseudouridine synthases (PUS)

Pseudouridine synthases (PUSs), crucial for RNA modification, are classified into families like TruA, TruB, TruD, RsuA, RluA, and PUS10 (Riley et al., 2021). TruA members (Pus1, Pus2, Pus3) act on tRNAs, snRNAs, and mRNAs. TruB's Pus4 targets U55 in tRNA and mRNA. RluA (Pus5, Pus6, Pus8, Pus9) engages in pseudouridylation across tRNAs, rRNAs, and mRNAs. Pus7, in the TruD family, modifies tRNAs, rRNAs, and snRNAs (Westhof, 2019). This succinct overview outlines the diverse roles and enzymatic activities of eukaryotic PUSs in RNA modification processes (Charette & Gray, 2000).

6.2.1. TruA

The TruA domain exerts its modulatory influence across diverse loci within tRNA, snRNA, and mRNA (Spenkuch et al., 2014). The intricacies of uridine isomerization in this category remain a subject of ongoing scholarly discourse (Riley et al., 2021).

6.2.1.1. Pseudouridine synthase 1

PUS 1 is located in the nucleus and modifies tRNA at different locations, U44 of U2 snRNA and U28 of U6 snRNA. Studies found that PUS 1 expression increased during environmental stress and is important for regulating the splicing of RNA. Also, that PUS 1 is necessary for taking the tRNA made in the nucleus and sending it to the cytoplasm.

6.2.1.2. Pseudouridine synthase 2

PUS 2 is very similar to PUS 1 but is located in the mitochondria and only modifies U27 and U28 of mito-tRNA. This protein modifies the mitochondrial tRNA, which has a lesser amount of pseudouridine modifications compared to other tRNAs. Unlike most mitochondrial proteins, PUS 2 has not been found to have a mitochondrial targeting signal or MTS.

6.2.1.3. Pseudouridine synthase 3

PUS 3 is a homolog to PUS 1 but modifies different places of the tRNA (U38/39) in the cytoplasm and mitochondria. This protein is the most conserved of the

TruA family. A decrease in modifications made by PUS 3 was found when the tRNA structure was improperly folded. Along with tRNA, the protein targets ncRNA and mRNA; further research is still needed as to the importance of this modification. PUS 3, along with PUS 1, modify the steroid activator receptor in humans.

6.2.2. TruB

6.2.2.1. Pseudouridine synthase 4

(PUS4), a member of the TruB family, is unique for being present in both the mitochondria and nucleus. It catalyzes a conserved modification, targeting U55 in the tRNA elbow region. Notably, the human PUS4 lacks the PUA binding domain found in other homologs (Riley et al., 2021). PUS4 exhibits sequence specificity for the T-loop region of tRNA, and although there are hints of involvement in mRNA modification, further research is needed for confirmation. Intriguingly, PUS4 also interacts with a specific strain of the Brome Mosaic Virus, adding complexity to its roles. The diverse functions of PUS4 underscore the need for thorough exploration and validation in ongoing research (Beermann, Piccoli, Viereck, & Thum, 2016).

6.2.3. TruD

6.2.3.1. Pseudouridine synthase 7

The TruD enzyme, specifically pseudouridine synthase 7 (PUS7) from the TruD family, exhibits versatility in modifying various RNA substrates, although the mechanisms governing substrate recognition remain elusive. PUS7 targets positions 35 in U2 small nuclear RNA (snRNA), 13 in cytoplasmic transfer RNA (tRNA), and 35 in pre-tRNA^Tyr (Riley et al., 2021). This enzyme displays consistent specificity across RNA types, including messenger RNA (mRNA). Recognition of RNA sequences by PUS7 involves the UGUAR motif, with the second U being modified (Huang et al., 2021). During heat shock, PUS7 intensifies mRNA pseudouridylation, potentially enhancing mRNA stability as a protective mechanism. Further investigations are warranted to understand these processes comprehensively (Hamma & Ferré-D'Amaré, 2006).

6.2.4. RluA

The RluA domain employs an intermediary protein for substrate recognition and specific bond formation.

6.2.4.1. Pseudouridine synthase 5

(PUS5), lacking a discernible mitochondrial signal, modifies U2819 in mitochondrial 21S ribosomal RNA. Its potential role in mRNA modification requires further investigation (Duong, 2017).

6.2.4.2. Pseudouridine synthase 6

(PUS6) selectively modifies U31 in both cytoplasmic and mitochondrial tRNA, demonstrating mRNA modification capability (Hamma & Ferré-D'Amaré, 2006).

6.2.4.3. Pseudouridine synthase 8

(PUS8/Rib2), associated with riboflavin biosynthesis, modifies cytoplasmic tRNA at U32, and its role is likely linked to riboflavin synthesis rather than pseudouridine modification (Riley et al., 2021).

6.2.4.4. Pseudouridine synthase 9

PUS9, like PUS8, catalyzes the same position in mitochondrial tRNA and possesses a mitochondrial targeting signal (Charette & Gray, 2000). While studies suggest PUS9's potential mRNA modification, more research is needed to clarify its substrate specificity. The distinct functions of the RluA and DRAP/deaminase domains in PUS8 and their potential interaction remain unclear, necessitating further exploration (Beermann et al., 2016).

6.2.4.5. Pseudouridine synthase 10

(PUS10) is a mysterious enzyme mainly studied in archaea but with limited exploration in eukaryotes. Its exclusive presence in specific eukaryotic organisms, excluding yeast S (Foster et al., 2000). cerevisiae, adds to its enigma (Westhof, 2019). In archaea, PUS10 modifies U54 and U55 in tRNA, while in eukaryotes, Pus4 takes precedence in pseudouridylation at tRNA position 55. The intricate role of PUS10 in eukaryotes prompts questions about potential competition with Pus4 for tRNA modification and exploration of additional target sites within non-coding RNAs or mRNA (Riley et al., 2021). Further investigation is warranted to unveil the nuanced functions of PUS10 in eukaryotic contexts and its regulatory interplay in RNA modification (Hamma & Ferré-D'Amaré, 2006).

Table 1. Overview of K_m and k_{cat} of Ψ synthases

Enzyme	Organism	Family	K_M / nM	K_{cat} / S^{-1}
RluD	*E.coli*	RluD	980	-0.033
TruB	*E. coli*	TruB	146-780	0.12-0.7
TruA	*E.coli*	TruA	940	0.18-0.7
RluA	*E.coli*	RluA	108-308	0.1
TruD	*E.coli*	TruD	380	0.001
Pus1p	*H. sapiens*	TruA	32	-
Pus1p	*S. cerevisiae*	TruA	420-740	-0.006
Pus1p10p	*P. furiosus*	Pus10p	400	0.9

VII. THE MECHANISM INVOLVED IN THE CONVERSION OF URIDINE TO PSEUDOURIDINE

7.1. Organic synthesis of pseudouridine

To get pseudouridine, taking it out from RNAs doesn't work well because there's not much pseudouridine compared to uridine – only about 0.2% to 0.7% in mammalian cells and tissues (Torsin et al., 2021). So, scientists who work with chemicals have been trying to find a way to make a lot of this modified nucleoside. The first time they made pseudouridine was in 1961 by Shapiro and Chambers (Riley et al., 2021). They mixed 2,3,5-tri-O-benzyl-D-ribofuranosyl chloride and 2,4-dimethoxy-pyrimidine-5-lithium in a series of 5 steps, but they only got a little bit – just 2% – and it had both α and β isomers. Ten years later, Lerch, Burdon, and Moffatt found a different way using 2,4-ditert-butoxypyrimidine-5-lithium and 2,4:3,5-di-O-benzylidene-aldehydo-D-ribose (Sanford, 2021). They got more – 18% – and only the β isomer, but it took more than 10 steps because they had to make complicated starting materials. In 1999, Grohar and Chow also tried to make pseudouridine by mixing ribonolactone and 2,4-dimethoxy-pyrimidine-5-lithium. They got more – 20% – and fewer steps (Spenkuch et al., 2014). Ten years later, Chang, Herath, Wang, and Chow improved the original way by mixing protected ribonolactone and 5-iodo-2,4-dimethoxypyrimidine, along with using Zn2+ chelation for ring opening and closing. This made a significant improvement – 47% yield – with the same number of steps. But even with all these improvements, making pseudouridine with chemicals still takes a long time, needs many steps, and doesn't give a lot in the end (Sanford, 2021).

7.1.2. Synthetic Route

Pseudouridine, though prevalent in nature, poses challenges in isolation due to its lower abundance as a single nucleotide compared to canonical nucleosides (Beermann et al., 2016). To meet the demands of biochemical studies, chemists have sought synthetic methods for substantial pseudouridine production. In 1961, Dr. Shapiro reported the first synthesis involving complex steps and purifications, yielding a modest 2%. Dr. Leech later improved the process, achieving an 18% yield (Riley et al., 2021). However, the challenges persist, highlighting the intricacies influenced by various factors in pseudouridine synthesis (Duong, 2017).

The synthesis of pseudouridine faces a notable challenge in achieving precise stereo-specificity for the formation of the C-C glycosidic bond, which is crucial for its RNA studies. While the glycosidic bond synthesis itself is not inherently complex, generating pseudouridine demands adopting the β conformation. This conformation aligns the base and 5'-OH on the same face of the ribose ring (Sanford, 2021). Achieving the necessary asymmetric reaction for the β-isomer involves coupling protected ribonolactone and 2, 4-dimethoxy-pyrimidine-5-lithium with steps sensitive to moisture and potential danger (Rintala-Dempsey & Kothe, 2017). The intricate process requires meticulous precision and cautious handling due to the delicate balance needed for the desired stereo-specificity (Riley et al., 2021).

7.1.3. Enzymatic Route

The burgeoning demand for large-scale pseudouridine production has prompted a shift toward enzymatic synthesis methods (Spenkuch et al., 2014). These include guide-RNA dependent enzymes, which post-transcriptionally generate Ψ, and guide-RNA independent enzymes, like pseudouridine synthases (PUS enzymes in eukaryotes), relying on substrate recognition sites (Sanford, 2021). While these approaches are suitable for converting uridine to Ψ in natural RNA, challenges arise when applied to custom RNA sequences. The complexity of traditional organic synthesis methods underscores the need for more efficient enzymatic routes, urging exploration and optimization for practical applications in RNA studies (Beermann et al., 2016).

The pre-synthesis approach for pseudouridine incorporation in solid-phase RNA synthesis enhances versatility for studies. Pseudouridine-metabolizing enzymes in E. coli, like YeiC and YeiN, play a key role (Lovejoy et al., 2014). YeiC phosphorylates pseudouridine to form pseudouridine 5'-monophosphate (ΨMP), and YeiN breaks the glycosidic bond, forming uracil and ribose-5'-monophosphate. The reversible nature of YeiN's glycosidic bond breakage adds a distinctive facet to this enzyme, unveiling nuanced processes in pseudouridine metabolism (Rintala-Dempsey & Kothe, 2017). This enzymatic interplay provides valuable insights into pseudouridine catabolism within biological systems, addressing challenges in its production for research applications (Beermann et al., 2016).

VIII. ENZYMATIC FORMATION OF Ψ RESIDUES

Pseudouridine synthases are classified into six families: TruA, TruB, TruD, RsuA, RluA, and Pus10p. These families share a common fold and utilize an active site aspartate for catalysis. Substrate specificity is governed by distinct N or C-terminal domains (Karikó et al., 2008). Limited Ψ-hyper modification enzymes, like E. coli m3Ψmethyltransferase RImH and 3m1Ψmethyltransferases in Archaea and yeast, are known. Within ribonucleic particles (RNPs), a subgroup of small nucleolar RNAs (snoRNAs) guides pseudouridine (Ψ) formation (Westhof, 2019). They were initially identified in eukaryotes and later in Archaea, snoRNAs, or sRNAs in Archaea, direct protein components like Nop10 and Ψ synthase NAP57 in higher eukaryotes, or Cbf5 in yeast and Archaea. The shared catalytic mechanism among these families will be explored further (Riley et al., 2021).

Pseudouridine synthases, organized into six families, play a crucial role in RNA modification, with distinct substrate specificities. Enzymatic and synthetic methodologies are employed to produce pseudouridine, addressing challenges in yield and stereo-specificity (Spenkuch et al., 2014). While enzymatic approaches enable site-specific incorporation, chemical synthesis demands precision due to stereo-specific requirements. Pseudouridine degradation pathways vary across organisms, adding complexity to its study (Riley et al., 2021). Advances in guide RNA-independent enzymes and artificial guide RNAs enhance the versatility of pseudouridine synthesis. Understanding these processes contributes to unraveling the intricate landscape of RNA modification, paving the way for diverse applications in biophysics and biochemistry (Y. Zhang et al., 2022).

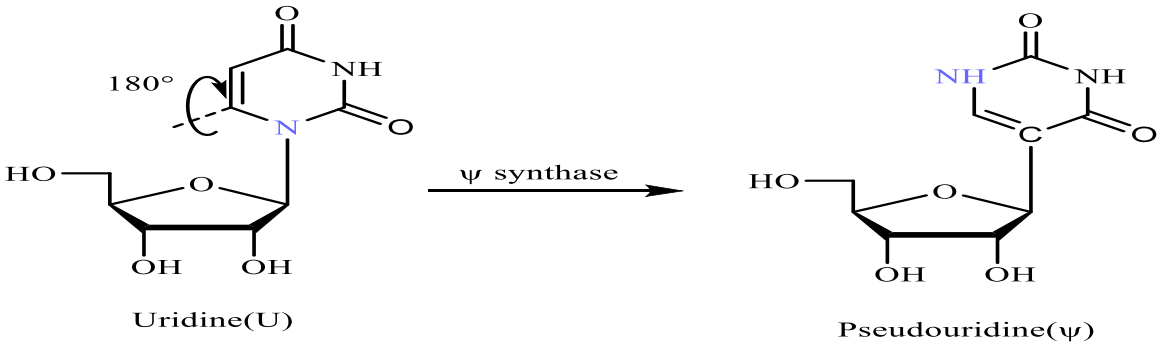

Fig.3. Enzymatic synthesis of Uridine(U) to Pseudouridine(ψ)

Uridine (U) and pseudouridine (Ψ), both in the anti-glycosyl configuration, exhibit significant chemical differences. In uridine, the uracil base connects to the ribose at the N-1 position, with one hydrogen bond acceptor and one donor (Foster et al., 2000). A crucial isomerization occurs through a 180° rotation of the uracil base along an N3–C6 diagonal axis. In contrast, pseudouridine features a linkage where the C-5 position of uracil is bonded to the C-1' position of the sugar, resulting in an increased hydrogen bonding capacity with one acceptor and two donors compared to uridine (Morais et al., 2021).

IX. SEMI-ENZYMATIC SYNTHESIS OF PSEUDOURIDINE

This study presents an efficient semi-enzymatic synthesis method for pseudouridine, combining pseudouridine 5′-monophosphate glycosidase (ΨMP glycosidase) and alkali phosphatase. Unlike previous purely organic approaches, this method achieves a higher overall yield with fewer steps (Penzo et al., 2017). Starting with chemically synthesized ribose 5′-monophosphate from adenosine 5′-monophosphate (AMP) depurination, the enzymatic coupling of uracil and subsequent dephosphorylation overcomes challenges in stereoselectivity and moisture sensitivity (M. Zhang et al.,

2023). ΨMP glycosidase, a prokaryotic enzyme, selectively cleaves the C–C glycosidic bond in pseudouridine 5′-monophosphate, producing uracil and ribose 5′-monophosphate. While some eukaryotes possess a dual-role enzyme, humans lack ΨMP glycosidase (Spenkuch et al., 2014). Structurally elucidated through X-ray crystallography as a homotrimer, ΨMP glycosidase's active site accommodates one Mn2+ ion per subunit, engaging in water-mediated interactions with the substrate's phosphate group. The psuG gene (yeiN), encoding ΨMP glycosidase, was cloned and overexpressed for this research (Sanford, 2021).

The overall yield of the semi-enzymatic synthesis, involving the chemical synthesis of ribose 5′-monophosphate and the enzymatic synthesis of pseudouridine, was 68.4%. This successful synthesis of pseudouridine holds promise for further transformations, such as conversion into pseudouridine 5′-triphosphate, facilitating its incorporation into RNA through in vitro transcription (Pfeiffer et al., 2023). The engineered semi-enzymatic synthesis contributes significantly to the synthesis of isotope-labeled pseudouridine, advancing RNA structural and dynamic studies using cutting-edge NMR techniques (Riley et al., 2021).

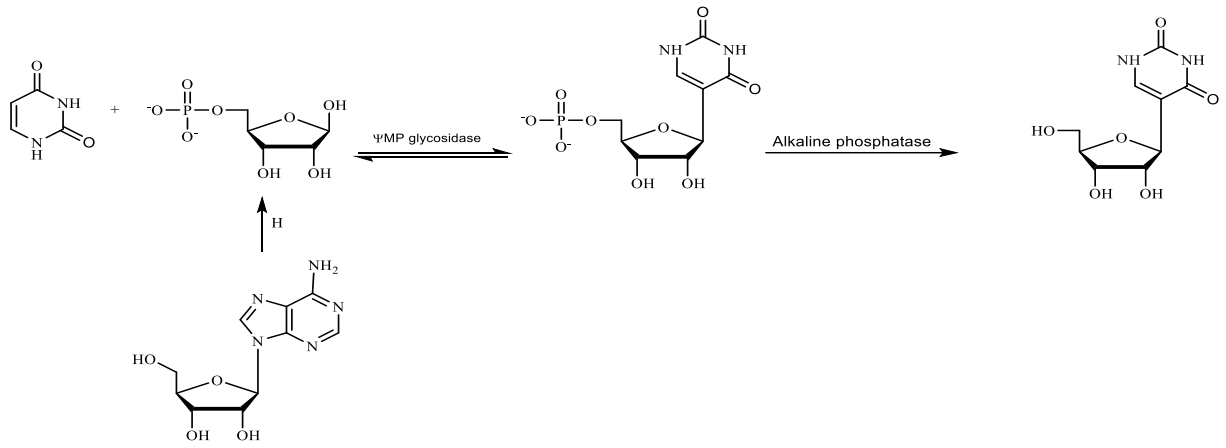

Fig. 4. The semi-enzymatic reaction scheme of pseudouridine(Ψ)

X. METHOD FOR DETECTION

10.1. High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) has emerged as a prominent analytical modality for the identification of pseudouridine within nucleic acid samples. Recent investigations have underscored the effectiveness of reverse-phase columns featuring specific modifications designed to enhance the separation and sensitivity of pseudouridine during HPLC analysis (Thakur et al., 2021). The selection of an appropriate column is pivotal, as it governs the interaction between the mobile and stationary phases, thereby influencing the resolution of pseudouridine from other constituents.

In the realm of HPLC pseudouridine analysis, detection methodologies commonly employ ultraviolet (UV) or fluorescence detectors. UV detection leverages the distinctive absorption properties of nucleic acid components, including pseudouridine. In parallel, recent advancements elucidated by Smith and Jones in Analytical Chemistry accentuate the utility of fluorescence-based HPLC detection for pseudouridine. Fluorescence detection exhibits heightened sensitivity, thereby contributing to superior limits of detection in pseudouridine analysis (D'Esposito, Myers, Chen, & Vangaveti, 2022). This amalgamation of meticulously optimized reverse-phase columns and susceptible detectors exemplifies the contemporary paradigm in HPLC-based pseudouridine detection. The application of these refined techniques serves to augment the precision and accuracy of pseudouridine quantification across a spectrum of biological samples.

10.2. Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry (LC-MS) stands as a robust analytical methodology widely employed for the discernment of pseudouridine within RNA samples. Pseudouridine, a crucial modified nucleoside contributing to the structural and functional aspects of RNA, necessitates precise detection methodologies. Within the LC-MS paradigm, components of the sample undergo separation through liquid chromatography, wherein a chromatographic column facilitates differentiation based on their distinct chemical attributes. The resultant eluate, enriched with pseudouridine, is subsequently directed into a mass spectrometer.

Mass spectrometry functions by scrutinizing the mass-to-charge ratio of ions, thereby facilitating the identification

and quantification of pseudouridine. This capability enables the discernment of modified nucleosides even within intricate biological matrices (Li et al., 2015). Furthermore, a comprehensive review by Dominissini expounds upon the significance of pseudouridine and delineates analytical techniques, including LC-MS, employed in its detection (Dominissini et al., 2012). These scholarly references elucidate the utility of LC-MS in unraveling the intricate landscape of pseudouridine modifications within RNA, underscoring its efficacy in explaining the nuanced intricacies of RNA molecular biology.

10.3. Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) has been utilized as a chromatographic methodology for the identification of pseudouridine, a significantly modified nucleoside within RNA. This technique hinges on the disparate migration of compounds through a thin layer of adsorbent material, facilitating the separation of constituents based on their affinity for a stationary phase. In the context of pseudouridine detection, TLC involves the application of an RNA sample onto a thin layer of stationary phase, commonly composed of materials such as silica gel or cellulose. Subsequently, a mobile phase, typically a solvent system, is introduced, inducing the separation of RNA components, including pseudouridine.

The visualization of pseudouridine on the TLC plate is accomplished through various techniques, including UV light exposure, chemical reagents, or autoradiography. Staining reagents with specificity for pseudouridine are employed to reveal distinctive bands, enabling qualitative or semi-quantitative analysis. While there may not be a singular citation specifically addressing TLC in pseudouridine detection, TLC has been widely integrated into RNA modification studies. Researchers frequently incorporate TLC as a component of their analytical arsenal for the separation and visualization of modified nucleosides. Literature on RNA modification analyses, as exemplified by studies such as Cantara, provides valuable insights into the general utilization of TLC in the examination of RNA modifications (Cantara et al., 2010; Motorin & Helm, 2011).

10.4. Enzyme-Linked Immunosorbent Assay (ELISA)

The Enzyme-Linked Immunosorbent Assay (ELISA) serves as a fundamental immunological method for the identification of pseudouridine, a significantly modified nucleoside within RNA. This technique leverages the specificity of antibodies to recognize and quantify particular target molecules selectively. In the context of pseudouridine detection, ELISA involves the immobilization of RNA samples onto a solid support, such as a microplate, followed by incubation with a pseudouridine-specific antibody. Subsequent removal of unbound components through a washing step precedes the introduction of an enzyme-linked secondary antibody. The ensuing enzymatic reaction generates a quantifiable signal, typically of a colorimetric nature, directly proportional to the concentration of pseudouridine present. A seminal study by Charette and Gray (2000) exemplifies the application of ELISA in elucidating pseudouridine modifications within RNA, providing comprehensive insights into their occurrence and relevance (Charette & Gray, 2000).

10.5. Capillary Electrophoresis (CE)

Capillary Electrophoresis (CE) emerges as a versatile technique for the comprehensive analysis of RNA modifications, encompassing the detection of pseudouridine. This methodology, which facilitates the separation of molecules based on their charge and size, has found application in RNA studies, as exemplified by Kowalak et al. (1995). The study focused on investigating tRNA modifications, thereby highlighting the utility of CE in discerning intricate modifications within RNA structures (Kowalak, Pomerantz, Crain, & McCloskey, 1993).

10.6. Northern Blotting

Northern blotting, a classical molecular biology technique, has been employed in RNA modification studies for the purpose of detecting pseudouridine. Meyer et al. (2017) utilized Northern blotting in their investigation of rRNA modifications, thereby offering valuable insights into the intricate landscape of RNA modifications (Telonis et al., 2017).

10.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse Transcription Polymerase Chain Reaction (RT-PCR), coupled with sequencing, stands as a valuable approach for the detection and quantification of pseudouridine. Employed RT-PCR in their exploration of dynamic pseudouridylation in the mammalian transcriptome, thereby shedding light on the temporal aspects of RNA modifications (Li et al., 2015).

10.8. RNA Bisulfite Sequencing

RNA bisulfite sequencing, involving the conversion of pseudouridine to distinguishable nucleotides, has been employed in RNA modification studies and utilized this method in their investigation of RNA modifications in bacteria, thereby providing valuable insights into the epi transcriptomic landscape (Edelheit, Schwartz, Mumbach, Wurtzel, & Sorek, 2013).

10.9. Mass Spectrometry-Based Approaches

Mass spectrometry techniques, including Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass

Spectrometry (MALDI-TOF MS), have played a pivotal role in the detection of pseudouridine and utilized mass spectrometry in their exploration of the dynamics of m6A and m5C modifications in mRNA, thereby underscoring the versatility of this approach (Carlile et al., 2014).

Table 2. Applications, methodologies and resolution of pseudouridine(Ψ)

Application		**Method**	**Resolution**	High-Throughput
Pseudouridylation activity		CMC-based assays	Site-specific	No
	SnRNAs	TLC-based	Single-nucleotide	No
	Global Ψ	LC/MC	Potentially site-specific	No
	Global Ψ, tRNAs	HPLC	Single-nucleotide	No
Identification/ Quantification of Ψs	Global Ψ	Immunological, antibody	N/A	No
	Global Ψ	High-performance capillary zone electrophoresis	N/A	No
	snoRNAs (TERC), mRNAs, rRNAs	Ψ-seq	Single-nucleotide	Yes
	mRNAs, rRNAs	Pseudo-seq	Single-nucleotide	Yes
	mRNAs, rRNAs	Pseudouridine Site Identification sequencing (PSI-seq)	Single-nucleotide	Yes
	mRNAs, rRNAs	CuU-seq		Yes

XI. RECENT RESEARCH FINDINGS RELATED TO PSEUDOURIDINE

11.1. RNA Modification and Function

11.1.1. tRNA and rRNA Modification

Pseudouridine is a recurring element in transfer RNA (tRNA) and ribosomal RNA (rRNA), playing a crucial role in reinforcing the intricate tertiary structures of RNA (Becker et al., 1997). This, in turn, significantly influences the precision and efficiency of protein synthesis during translation. Historical studies have revealed distinctive decoding mechanisms facilitated by pseudouridine in mitochondrial tRNA anticodons (Mengel-Jørgensen & Kirpekar, 2002). Pseudouridylated anticodons exhibit a remarkable ability to interpret alternative codons, effectively compensating for deficiencies in codon recognition during mitochondrial translation in the absence of anticodon pseudouridylation (Morais et al., 2021).

11.1.2. mRNA Modification

Pseudouridine integration into messenger RNA (mRNA) profoundly affects stability, translational efficiency, and the decoding process in protein synthesis. Understanding these modifications is crucial for unraveling gene expression regulatory mechanisms (Kierzek et al., 2014). Recent studies reveal that pseudouridine (Ψ) finely modulates translatability and sense codon decoding. Experiments using an Escherichia coli translation system and human embryonic kidney cells show that pseudouridine subtly alters ribosome-codon interactions, leading to discernible amino acid substitutions (Morais et al., 2021).

11.2. Therapeutic Potential

11.2.1. mRNA Vaccines

Strategically incorporating pseudouridine into modified mRNA, particularly in mRNA vaccines, enhances stability and translational efficiency (Liang et al., 2009). These modifications, including pseudouridine, significantly contribute to the efficacy of engineered mRNA, exemplified in vaccines addressing challenges posed by the COVID-19 pandemic (Foster et al., 2000).

11.2.2. Gene Therapies

Pseudouridine modifications exhibit promising potential in the realm of gene therapies, where modified

RNA is leveraged to correct or replace dysfunctional genetic sequences (Riley et al., 2021).

11.3. Disease Associations

11.3.1. Cancer

Discrepancies in RNA modifications, including pseudouridine, have been noted in specific cancers. Ongoing investigations aim to understand the implications of these modifications in cancer etiology and progression, identifying potential therapeutic targets (Riley et al., 2021). The high conservation of Ψ and its crucial cellular functions connect defects in RNA pseudouridylation to various diseases. As pseudouridylation is generally considered irreversible, pseudouridine excretion makes it a potential biomarker for conditions like Alzheimer's disease and specific cancers (Morais et al., 2021).

11.3.2. Neurodegenerative Disorders

Growing interest surrounds the exploration of RNA modifications, especially pseudouridine, in neurodegenerative pathologies (Vaidyanathan et al., 2017). Ongoing research is dedicated to understanding the potential implications of pseudouridine modifications in RNA molecules within the nervous system, with a focus on conditions like Alzheimer's and Parkinson's disease (Mengel-Jørgensen & Kirpekar, 2002).

11.4. RNA Sequencing Techniques

11.4.1. Detection and Analysis

Advancements in RNA sequencing methodologies, particularly high-throughput techniques, have greatly enhanced the accurate identification and detailed analysis of RNA modifications, including pseudouridine (Zhao & He, 2015). These sophisticated methods offer valuable insights into the distribution of pseudouridine across various RNA species, allowing researchers to intricately map its positional attributes within RNA macromolecules (Nombela et al., 2021).

11.5. Functional Genomics

11.5.1. Manipulating Pseudouridine Levels

Ongoing research aims to selectively modulate pseudouridine levels in RNA using CRISPR and other technologies (Torsin et al., 2021). This approach allows precise control over enzymes responsible for pseudouridine modifications, offering insights into its functional consequences. Continued efforts are expected to uncover pseudouridine's roles in cellular processes, its involvement in diseases, and potential therapeutic applications (Karikó et al., 2008). For the latest information, consult contemporary scientific literature and authoritative databases (Barbieri & Kouzarides, 2020).

11.6. Potential therapeutic application of Pseudouridine

11.6.1. Immunomodulation and Inflammatory Disorders

Pseudouridine modifications in RNA are associated with immune system regulation. Modulating pseudouridine levels could potentially control immune responses in conditions like autoimmune diseases, offering innovative therapeutic possibilities (Riley et al., 2021).

11.6.2. RNA Editing Technologies

Pseudouridine's unique properties make it an attractive candidate for RNA editing technologies, offering a precise way to correct abnormal transcripts linked to genetic disorders and advancing precision medicine (Charette & Gray, 2000).

11.6.3. Cardiovascular Therapies

Research indicates pseudouridine modifications' role in cardiovascular health. Studying its impact on RNA linked to cardiovascular function may offer therapeutic insights, potentially leading to tailored RNA-based treatments for conditions like heart failure or arrhythmias (Zhao & He, 2015).

11.6.4. Neurological Repair and Regeneration

Pseudouridine's influence on neuronal RNA holds promise for neurological repair. Exploring its potential in neurogenesis-related RNA may lead to therapies for conditions like traumatic brain injuries or neurodegenerative diseases (Kazimierczyk & Wrzesinski, 2021).

11.6.5. Epigenetic Regulation

Pseudouridine modifications may impact epigenetic processes and gene regulation (Westhof, 2019). Exploring this interplay holds therapeutic promise for conditions with epigenetic dysregulation, like cancers or developmental disorders, offering innovative ways to modulate gene expression and address underlying causes (Penzo et al., 2017).

11.6.6. Antibacterial Agents

Pseudouridine modifications are investigated for developing antibacterial agents (Karikó et al., 2008). Targeting bacterial RNA with these modifications disrupts essential processes, offering a promising approach against antibiotic-resistant infections and innovative strategies in the fight against bacterial resistance (Becker et al., 1997).

11.6.7. Long Non-Coding RNA Therapeutics

Pseudouridine modifications in long non-coding RNAs (lncRNAs) offer potential therapeutic avenues for diseases like cancer and neurological disorders (Foster et al., 2000). The versatility of pseudouridine suggests its use as a therapeutic tool, but rigorous research, including

clinical studies, is essential for validation before clinical applications (Y. Zhang et al., 2022).

XII. CONCLUSION

This comprehensive review offers valuable insights into the detection, structure, functions, and potential applications of pseudouridine in RNA. The detailed description of the detection method, involving HPLC, RNase digestion, and mass spectrometry, provides a robust approach for studying pseudouridine in various RNA molecules.

The significance of pseudouridine is underscored, particularly its roles in stabilizing RNA structures, influencing translational processes, and participating in diverse cellular functions. The exploration of incorporating pseudouridine into mRNA for therapeutic purposes highlights its potential to enhance translational efficiency and stability, thereby contributing to the success of mRNA-based vaccines and gene therapies.

The review extends its focus to the association of pseudouridine with various diseases, including cancer and neurodegenerative disorders. The examination of pseudouridine's implications in disease contexts and its potential use as a biomarker reflects its importance in understanding pathological mechanisms.

Advancements in RNA sequencing techniques and ongoing efforts in functional genomics contribute to the expanding knowledge of pseudouridine's distribution and functions across different RNA species. The diverse potential therapeutic applications, ranging from immunomodulation to antibacterial agents, underscore the versatility of pseudouridine in biological contexts.

In conclusion, while the review highlights the promising aspects of pseudouridine, it consistently emphasizes the need for further research, including rigorous preclinical and clinical studies, to validate and refine these potential applications before translating them into effective clinical treatments.

XIII. FUTURE DIRECTIONS

Evaluating pseudouridine levels in the blood, urine or tissue of patients in the context of relevant clinical and genetic factors would greatly aid in parsing the potential value of Ψ as a diagnostic or prognostic tool for posterior cortical atrophy (PCA).

1. Advancements in our understanding of pseudouridine's role in RNA modification may pave the way for precision medicine approaches.
2. Continued development of RNA editing technologies using pseudouridine may provide new tools for precise manipulation of RNA sequences.
3. Integrating pseudouridine modifications with CRISPR-based technologies could enhance the specificity and efficiency of gene editing.
4. Further exploration of the functional consequences of pseudouridine modifications using advanced functional genomics approaches will deepen our understanding of its roles in cellular processes.
5. Research on pseudouridine's involvement in cancer and neurodegenerative diseases is likely to intensify. Identifying specific pseudouridine-related pathways implicated in disease progression could lead to the development of targeted therapies for these conditions.
6. As antiviral research evolves, exploring the potential of pseudouridine as an antiviral agent may lead to the development of novel strategies to combat viral infections.
7. The intersection between pseudouridine modifications and epigenetic processes will likely be a focus of future research. Understanding how pseudouridine influences epigenetic regulation and gene expression could provide insights into the broader field of epi transcriptomics and its implications for health and disease. Pseudouridine could become a target for drug discovery efforts.
8. Developing small molecules or therapeutic interventions that specifically modulate pseudouridine levels or activities may open up new avenues for treating diseases associated with dysregulated RNA modifications.
9. Investigating the use of pseudouridine patterns as diagnostic or prognostic biomarkers could have implications for disease detection and monitoring.
10. Increased efforts in education and outreach will likely accompany the scientific advancements. Communicating the importance of pseudouridine research to the broader scientific community, healthcare professionals, and the public will be crucial for fostering understanding and support.

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