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Content

CHAPTER I

Mitochondrial Genome Editing: Innovations and Therapeutic Potential

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Fixing the Engine of Life: The Therapeutic Potential of Mitochondrial Editing

Mitochondria, often described as the "powerhouses of the cell," are organelles critical for generating cellular energy in the form of adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS) (Wallace, 2010). This energy production is particularly essential for high-energy-demand tissues such as the brain, muscles, and heart, which rely heavily on ATP for their physiological functions (Nicholls & Budd, 2000; Bers, 2001).

Uniquely, mitochondria contain their own maternally inherited genome known as mitochondrial DNA (mtDNA) (Anderson et al., 1981). The mitochondrial genome encodes 37

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essential genes, including 13 proteins integral to the OXPHOS pathway, 22 transfer RNAs, and two ribosomal RNAs required for mitochondrial protein synthesis (Lightowlers et al., 1997; Balaban et al., 2005). Unlike nuclear DNA (nDNA), which is linear and histone-protected within the nucleus, mtDNA exists as a circular, histone-free molecule in the mitochondrial matrix. This structural difference exposes mtDNA to reactive oxygen species generated during OXPHOS, leading to a significantly higher mutation rate (Wallace, 2005). Additionally, mtDNA primarily relies on base excision repair, lacking the robust DNA repair pathways found in nuclear DNA (Alexeyev et al., 2013).

Another unique feature of mtDNA is its compact, intron-free structure, where even minor mutations can disrupt essential functions. The presence of multiple mtDNA copies per mitochondrion gives rise to heteroplasmy, a condition where wild-type and mutated mtDNA coexist within a single cell. This phenomenon further complicates mitochondrial disease progression and therapeutic strategies.

Mutations in mtDNA are associated with a spectrum of mitochondrial diseases, including Leber's Hereditary Optic Neuropathy (LHON), Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS), Alpers-Huttenlocher syndrome, and Leigh syndrome (DiMauro & Schon, 2003; Saneto et al., 2013). These diseases are often characterized by neuromuscular dysfunction, neurodegeneration, and cardiovascular abnormalities, leading to severe clinical consequences (Gorman et al., 2016). The maternal inheritance pattern of mtDNA mutations complicates disease transmission and treatment approaches (Taylor & Turnbull, 2005).

Advancements in genetic technologies offer promising avenues for treating genetic disorders at their source (Doudna & Charpentier, 2014). Tools such as CRISPR-Cas9 have revolutionized nuclear genome editing by enabling precise genetic modifications (Jinek et al., 2012). However, applying these technologies to mtDNA remains challenging. Mitochondrial DNA is confined within the mitochondrial matrix, protected by two membranes, and lacks a homologous recombination-based repair system, precluding conventional gene-editing approaches (Yoon et al., 1991; Gammage et al., 2018). Additionally, the inability to import guide RNAs into mitochondria further limits the applicability of CRISPR-based tools (Moraes et al., 2018).

Recent breakthroughs, however, have circumvented some of these barriers. Technologies like DdCBEs (DddA-derived cytosine base editors) enable precise mtDNA base editing without the need for guide RNAs, overcoming a fundamental limitation of CRISPR-based systems (Mok et al., 2020). Similarly, mitochondria-targeted TALENs (mitoTALENs) provide a protein-based approach to selectively target and cleave mutant mtDNA, facilitating a heteroplasmic shift toward wild-type mtDNA and restoring mitochondrial function (Gammage et al., 2018; Reddy et al., 2015). These innovative tools represent significant advancements in the field, offering new hope for the treatment of mitochondrial diseases at the genetic level.

Current Technologies and Innovations

mtDNA disorders remain incurable and are associated with severe health impacts, often leading to significant morbidity and premature mortality. These disorders are further complicated by variations in mtDNA that contribute to the development of multifactorial, age-related diseases, such as neurodegenerative

conditions, metabolic syndromes, heart failure, and certain cancers (Toader et al., 2024; Cardona, 2024). The growing prevalence of such conditions, particularly in aging populations, underscores the urgent need for innovative therapeutic strategies.

Gene editing technologies, which allow precise modifications to DNA, have successfully corrected pathogenic mutations in the nuclear genome. However, applying these tools to mtDNA has presented unique challenges, given the structural and functional complexities of mitochondria. Mitochondrial gene therapy is a relatively novel approach that has progressed significant in recent years. These technologies aim to manipulate mtDNA by either reducing the load of mutant variants or correcting specific pathogenic mutations. This can shift a heteroplasmic state toward a healthier, wild-type mtDNA population or even address mutations in homoplasmic states (Sainero-Alcolado et al., 2025). Mitochondrial genome editing tools are generally classified into two categories: nucleases and base editors.

1. mtDNA Nucleases :

Mitochondrial genome editing represents a powerful approach for addressing mtDNA mutations, offering an alternative to traditional nuclease-based strategies that rely on inducing double-strand breaks (DSBs). Targeted nuclease systems, including mitochondrially targeted restriction endonucleases (mitoREs), zinc-finger nucleases (mitoZFNs), mitoARCUS, and mitoTALENs, have transformed the ability to selectively eliminate pathogenic mtDNA (Keshavan et al., 2024; Silva-Pinheiro et al., 2022; Falabella et al., 2022).

mitoREs were the first generation of tools, employing restriction endonucleases fused to mitochondrial targeting sequences (MTS) to cleave mtDNA sequences containing disease-associated

mutations. However, their reliance on fixed recognition sites limits their versatility to specific sequences (Tanaka et al., 2002).

Advancements such as mtZFNs and mitoTALENs have overcome this limitation. mtZFNs combine zinc-finger DNA-binding domains with a FokI nuclease to induce site-specific DSBs in mtDNA. This selective cleavage degrades mutant mtDNA, allowing wild-type mtDNA to replicate, shifting heteroplasmy and restoring cellular function (Gammage et al., 2014). Similarly, mitoTALENs utilize transcription activator-like effector (TALE) proteins fused to nucleases to achieve sequence-specific cleavage. These tools have demonstrated efficacy in various disease models by selectively depleting mutant mtDNA and restoring respiratory chain function (Bacman et al., 2018).

mitoARCUS, derived from the homing endonuclease I-CreI, represents a compact and efficient alternative to mtZFNs and mitoTALENs. Its smaller size enhances mitochondrial delivery, an advantage over bulkier editing tools. mitoARCUS can be programmed to target specific mtDNA sequences, facilitating precise cleavage of mutant mtDNA while sparing wild-type genomes. This selective action shifts heteroplasmy, alleviates mitochondrial dysfunction, and restores cellular respiration, making it a promising tool for therapeutic applications in mitochondrial diseases (Zekonyte et al., 2021).

Beyond medical applications, mitochondrial genome editing tools like mitoARCUS have also demonstrated versatility in agricultural systems. For example, targeted mtDNA editing in Nicotiana tabacum successfully induced cytoplasmic male sterility and abortive seed traits through the allotopic expression of specific mitochondrial genes, highlighting the broader applicability of these technologies (Smith et al., 2023).

To enable clinical translation, further improvements in delivery mechanisms, such as adeno-associated viruses (AAVs) or nanoparticle-based systems, are essential. Enhanced delivery efficiency and precision will facilitate the application of mitoARCUS and related tools in treating mitochondrial diseases. By addressing heteroplasmy and reducing the mutant mtDNA load, these advancements hold significant promise for improving patient outcomes in disorders associated with mitochondrial dysfunction (Gammage et al., 2018; Bacman et al., 2018).

2. mtDNA Base Editors

These editors are categorized into two main groups based on their chemical mechanisms: Cytosine Base Editors (CBEs) and Adenine Base Editors (ABEs). Each group enables distinct base pair conversions, offering unique advantages for treating genetic disorders associated with mitochondrial DNA. This classification highlights both the biochemical foundation and the therapeutic potential of these technologies.

2.1. Cytosine Base Editing

2.1.1 DddA-derived Cytosine Base Editors (DdCBEs)

The development of DdCBEs represents a significant breakthrough in mitochondrial genome editing. These innovative tools, derived from a cytidine deaminase toxin found in Burkholderia cenocepacia, enable precise cytosine-to-thymine (C→T) base conversions without requiring guide RNAs (gRNAs) or DSBs. DdCBEs utilize TALE to guide the editing enzyme to specific target sites within the mitochondrial genome, ensuring high specificity and efficiency (Qiu et al., 2024).

Applications of DdCBEs have been demonstrated across various model organisms, including mice, zebrafish, and mammalian

cell lines. For example, in mouse models, DdCBEs have successfully corrected pathogenic mutations associated with mitochondrial diseases, reducing mutant mtDNA levels below the threshold required for disease manifestation. Furthermore, studies have shown that combining DdCBEs with mitoTALENs enhances editing efficiency and expands the range of targetable mutations (Xie et al., 2024).

The crystal structure of DddA in complex with dsDNA containing its preferred 5'-TC target motif revealed that DddA binds to the minor groove of sharply bent dsDNA, interacting specifically with the 5'-TC motif. The cytosine targeted for deamination is flipped out of the DNA double helix through a "domino effect" mechanism initiated by the displacement of the neighboring thymine. This thymine forms a non-canonical T-G base pair with guanine, stabilizing the transition state. Key residues, such as Phe1375 and His1345, intercalate into the DNA and coordinate the target base within the enzyme's active site (Aihara et al., 2022).

Recent advancements in DdCBE technology include the development of modified variants, such as DddAtox mutants and strand-selective base editors, which offer improved editing efficiency, specificity, and safety. These innovations address some inherent limitations of existing mitochondrial editing tools, broadening the potential for therapeutic applications (Lim K., 2024).

In a groundbreaking study, Mok et al. (2020) introduced a novel RNA-free and CRISPR-independent method for mitochondrial base editing utilizing the DddA toxin. DddA’s unique ability to catalyze C-to-T conversions in dsDNA circumvented the challenges associated with delivering RNA-based editing systems into mitochondria. To mitigate toxicity, split-DddA was engineered into two inactive halves, which reactivated only when bound

adjacently to the target DNA by programmable DNA-binding proteins such as TALE arrays. By fusing split DddA with mitochondrial targeting sequences and uracil glycosylase inhibitors, researchers created DdCBEs capable of precise and efficient C-to-T edits in mtDNA.

This approach avoids cleavage-induced heteroplasmic shifts typical of earlier mitochondrial editing techniques, enabling direct and homoplasmic base editing without depleting mitochondrial DNA copies. Experimental results demonstrated the high specificity and efficiency of DdCBEs in editing various mitochondrial genes, with no evidence of large-scale deletions or significant off-target effects. Furthermore, disease-associated mtDNA mutations were modeled, and mitochondrial functions, such as oxidative phosphorylation, were successfully altered. This study underscored the therapeutic potential of DdCBEs for correcting pathogenic mtDNA mutations, particularly in conditions such as LHON and mitochondrial myopathies.

2.1.2. Monomeric DddA-TALE cytosine base editors (mDdCBEs)

The development of mDdCBEs represents an innovative approach to enhancing mtDNA editing efficiency in human cells. Traditional CRISPR-based systems face challenges in mtDNA editing due to difficulties in delivering guide RNAs to mitochondria. By addressing the limitations of dimeric DdCBEs, mDdCBEs offer a streamlined and efficient solution. Non-toxic, full-length DddAtox variants were engineered to reduce cytotoxicity while maintaining high editing efficiency. Unlike dual-protein systems, the monomeric structure is more compatible with gene therapy vectors with limited capacities, such as AAVs. Through structure-based directed mutagenesis and random mutagenesis, non-toxic DddAtox variants

like AAAA and GSVG were developed, retaining catalytic activity and enabling fusion with TALE proteins or Cas9 proteins to create functional base editors (Mok et al., 2022).

Editing efficiencies of up to 50% have been demonstrated in HEK293T and NIH3T3 cell lines, highlighting the efficacy of mDdCBEs. These editors expanded the targetable scope of base editing by relaxing design constraints for TALE-binding sequences, particularly within the editing window spanning positions 4–11 downstream of TALE-binding sites. Delivery as mRNA significantly reduced off-target editing of mtDNA, with rates lowered by up to 3.7-fold compared to plasmid-based delivery. Unique mutation patterns, inaccessible to dimeric systems, were facilitated by mDdCBEs, underscoring their complementary utility. The ability to achieve precise base editing using a single TALE protein creates new therapeutic opportunities. These advancements hold great promise for addressing mitochondrial genetic disorders, including mitochondrial myopathy and Leber's hereditary optic neuropathy. Furthermore, the compatibility of monomeric structures with single vectors provides a significant advantage in clinical applications (Mok et al., 2022).

The potential applications of mDdCBEs extend beyond mtDNA editing. These tools show promise for nuclear DNA editing and organellar genome engineering, such as targeting chloroplast DNA in plants. Combining mDdCBEs with adenine base editors could further expand their utility. By overcoming the limitations of existing systems, mDdCBEs offer a transformative platform for both research and therapeutic applications in mitochondrial biology.

In addition to monomeric editors, evolved DdCBEs have been developed as CRISPR-free tools for precise C-to-T base editing in mitochondrial and nuclear DNA. These tools address challenges

associated with traditional mitochondrial genome editing methods, such as unreliable delivery of guide RNAs to mitochondria. Utilizing split DddA cytosine deaminases paired with programmable TALE proteins, evolved DdCBEs achieve enhanced editing efficiency and broaden their targeting scope through phage-assisted evolution techniques. Variants like DddA6 and DddA11 exhibit improved performance, with DddA6 showing a 3.3-fold increase in editing efficiency at TC target sequences and DddA11 demonstrating broader compatibility for AC and CC contexts. Editing efficiencies of 15–30% have been reported for these expanded targets, significantly surpassing the capabilities of canonical DdCBEs (Mok et al., 2022).

The utility of evolved DdCBEs extends to modeling disease-associated mtDNA mutations, such as those linked to LHON and renal oncocytoma. For instance, DddA11 enables efficient installation of pathogenic mutations at non-TC sites, achieving editing efficiencies of up to 43% in sorted cell populations. These precise edits resulted in functional alterations in mitochondrial respiration, emphasizing their potential for disease modeling and therapeutic development. Additionally, evolved DdCBEs demonstrated utility in nDNA editing, achieving efficiencies of up to 35% at AC and CC targets. Despite their enhanced activity, these tools maintain off-target editing rates consistent with canonical DdCBEs, ensuring their suitability for precise genetic interventions (Mok et al., 2022).

2.1.3. Zinc finger deaminases (ZFDs)

The development of ZFDs represents a novel platform for precise base editing in nuclear and mtDNA in human cells. Unlike traditional CRISPR-based editors, which rely on DSBs and are associated with undesired mutations, insertions, and deletions, ZFDs

circumvent such complications. This system employs zinc finger DNA-binding proteins fused with split DddAtox, an interbacterial cytidine deaminase, and uracil glycosylase inhibitors (UGI), enabling precise cytosine-to-thymine (C→T) base conversions in target DNA sequences. Importantly, ZFDs do not require external guide RNAs or DNA repair templates, offering high editing accuracy and minimal off-target effects.

To optimize ZFD performance, linker lengths and spacer configurations between zinc finger arrays were evaluated, with a 24-amino acid linker identified as the most efficient. ZFDs demonstrated editing efficiencies of up to 60% in nuclear DNA and 30% in mtDNA, establishing their capability for precise editing at endogenous sites across multiple genes. Furthermore, direct delivery of ZFDs as purified recombinant proteins into human cells was achieved, eliminating reliance on plasmid-based delivery systems and reducing risks associated with genomic integration of foreign DNA.

For mitochondrial applications, mitoZFDs were engineered with specific targeting signals, enabling efficient editing across several mtDNA loci. These mitoZFDs facilitated the creation of heteroplasmic or homoplasmic mutations that were stably maintained in clonal populations. Comparative analyses with TALE-based DdCBEs revealed distinct mutation patterns generated by mitoZFDs, indicating complementary applications for these tools. Additionally, hybrid approaches combining ZFDs with DdCBEs were employed to achieve unique editing outcomes, expanding the versatility of mitochondrial genome engineering. The modular design of ZFDs allows further customization, including reduced off-target effects through modifications in zinc finger specificity and the DddAtox catalytic domain. With compact sizes suitable for packaging into AAV vectors, ZFDs hold significant promise for in

vivo applications, particularly in gene therapies targeting mitochondrial diseases. These findings underscore the transformative potential of ZFDs for precise genome editing in basic research, biotechnology, and therapeutic interventions (Lim et al., 2022).

2.1.4. High-fidelity DddA-derived cytosine base editors (HiFi-DdCBEs)

HiFi-DdCBEs have been developed to address the limitations of conventional DdCBEs for precise mtDNA editing. Traditional DdCBEs enable targeted C-to-T base conversions but are limited by significant off-target activities, often caused by the spontaneous assembly of split DddAtox enzyme halves. To overcome this, interface-engineered variants of DddAtox were designed to minimize off-target effects by preventing unintended reconstitution of the functional deaminase in the absence of target DNA (Lee et al., 2022).

These high-fidelity variants achieve improved specificity without compromising efficiency. This was accomplished by systematically modifying amino acid residues at the dimerization interfaces of split DddAtox proteins. Using structural modeling, specific residues were replaced with alanine to disrupt spontaneous assembly while preserving enzymatic activity when bound to target DNA. Variants such as K1389A, T1391A, and V1411A demonstrated an optimal balance between reduced off-target activity and maintained on-target efficiency. Whole mitochondrial genome sequencing confirmed a significant reduction in off-target C-to-T conversions across various mtDNA loci, underscoring the enhanced precision of HiFi-DdCBEs (Lee et al., 2022).

The utility of HiFi-DdCBEs extends to therapeutic and research applications. Precise and efficient edits were successfully

achieved in mitochondrial genes associated with human diseases, such as MT-ND1 and MT-ND4, without inducing collateral mutations. Moreover, the compatibility of HiFi-DdCBEs with evolved DddA variants like DddA6 and DddA11 expanded their targeting range and efficiency. By mitigating off-target risks, these editors provide a safer and more effective platform for mitochondrial genome editing, paving the way for therapeutic correction of pathogenic mtDNA mutations. This advancement marks a critical step toward clinical applications of base editing for mitochondrial genetic disorders, offering both enhanced specificity and safety in genome engineering (Lee et al., 2022).

2.1.5. Compact zinc finger DddA-derived cytosine base editors (ZF-DdCBEs)

ZF-DdCBEs offer a novel approach for cytosine-to-thymine base editing using all-protein systems composed of zinc finger arrays and split DddA deaminase. This innovative design overcomes the challenges of TALE-based DdCBEs, which are limited by their large size and repetitive sequences that complicate construction and delivery. The compact zinc finger arrays enable the use of single AAV vectors for in vivo delivery, making them particularly advantageous for therapeutic applications.

Extensive optimizations have significantly improved the performance of ZF-DdCBEs. Adjustments to linker regions enhanced flexibility, while modifications to nuclear export signals facilitated effective mitochondrial targeting. Scaffolds derived from human transcription factors were incorporated to increase DNA-binding affinity and reduce immunogenicity. Furthermore, evolved DddA variants were integrated into the system to boost editing efficiency and expand sequence compatibility. To enhance specificity, dimerization properties of DddA were modified, and

catalytically inactive competitive inhibitors were added to minimize off-target effects. These advancements resulted in editors with exceptional precision and efficiency, achieving robust on-target editing in both cell culture models and in vivo systems(Willis et al., 2022).

ZF-DdCBEs successfully introduced disease-associated mutations in mitochondrial and nuclear genes with minimal off-target effects. For example, pathogenic mutations related to mitochondrial myopathy and LHON were accurately modeled. The compact design enabled efficient delivery and editing in tissues such as the heart, liver, and skeletal muscle in mice, demonstrating their potential for in vivo applications(Willis et al., 2022).

Despite these achievements, some residual off-target activity remains, limiting their immediate therapeutic use. However, the modular and adaptable nature of ZF-DdCBEs positions them as highly promising tools for ongoing research and refinement. By mediating precise editing without inducing double-strand breaks, this platform offers a safer and more versatile alternative to existing genome editing technologies. With their compact design and efficiency, ZF-DdCBEs represent a major advancement in genome engineering and hold substantial potential for both research and therapeutic applications (Willis et al., 2022).

2.2. Adenine Base Editing

2.2.1 TALE-linked Adenine Deaminases (TALEDs)

TALEDs represent a major breakthrough in mitochondrial genome editing, enabling precise adenine-to-guanine base editing in human mtDNA. Unlike existing mitochondrial base editors, which primarily facilitate cytosine-to-thymine conversions and are constrained by sequence context, TALEDs expand the scope of editable mtDNA mutations. By combining TALE arrays with

engineered adenine deaminase variants derived from Escherichia coli TadA, TALEDs achieve targeted adenine deamination with editing frequencies reaching up to 49% across multiple mitochondrial genes(Cho et al., 2022).

To enhance specificity and minimize off-target effects, both split and monomeric configurations of TALEDs have been developed. Split TALEDs, constructed using split DddAtox halves, demonstrated high editing activity and precise A-to-G conversions without inducing significant off-target effects. Meanwhile, monomeric TALEDs, incorporating catalytically inactive full-length DddAtox variants, extended editing capabilities to previously inaccessible non-canonical mitochondrial sequences. These configurations underline the adaptability of TALEDs for precise mtDNA editing in diverse cellular and animal models(Cho et al., 2022).

The applications of TALEDs are extensive, including the correction of pathogenic mtDNA mutations. In vitro and in vivo models of mitochondrial dysfunction have been developed using TALEDs, offering powerful tools for drug discovery and therapeutic development. With the ability to correct inherited mtDNA disorders and generate advanced mitochondrial disease models, TALEDs hold transformative potential for mitochondrial biology and genetic medicine. Their contributions mark a critical step toward innovative therapies and research advancements in the field of genetic medicine (Cho et al., 2022).

The Double-Edged Sword: Challenges in Gene Transfer and Editing

mtDNA editing presents unique challenges due to the structural and functional differences between mitochondrial and nuclear genomes. Unlike nDNA, the mitochondrial matrix lacks

efficient RNA import mechanisms, precluding the use of RNA-guided editing tools like CRISPR-Cas9. Additionally, the double-membrane structure of mitochondria acts as a physical barrier, complicating the delivery of editing tools. Furthermore, mitochondria lack homologous recombination pathways, making traditional genome-editing strategies involving DSBs inapplicable to mtDNA.

1. Challenges and Improvements in mtDNA Nuclease Editing

Mitochondrial nuclease-based editing tools, including mitoZFNs, mitoTALENs, and mitoARCUS, represent promising technologies. By selectively introducing DSBs in mutant mtDNA, these tools enable the depletion of defective mitochondrial genomes, resulting in an increased proportion of wild-type mtDNA and functional recovery. However, despite their therapeutic potential, nuclease-based editing faces several challenges related to specificity, delivery, and limitations in editing homoplasmic mtDNA mutations, which require further improvements to enhance their clinical applicability.

1.1. Off-Target Effects and Specificity

One of the major challenges of nuclease-based editing tools is ensuring sequence specificity to avoid off-target cleavage. mtZFNs and mitoTALENs rely on customizable DNA-binding domains fused to the FokI nuclease to introduce DSBs at target loci. However, the requirement for FokI dimerization increases the risk of off-target cleavage at sequences with partial homology, particularly in regions of mtDNA that share similarities with the target site (Gammage et al., 2014; Bacman et al., 2013). Off-target cleavage can result in the unintended depletion of wild-type mtDNA, exacerbating mitochondrial dysfunction. To address this, high-fidelity nucleases have been engineered to enhance the precision of

DSB formation. For example, obligate heterodimeric FokI domains and optimized DNA-binding interfaces in mitoTALENs have been shown to minimize off-target effects while maintaining editing efficiency (Bacman et al., 2018).

Additionally, mitoARCUS, a compact nuclease derived from the homing endonuclease I-CreI, offers enhanced specificity due to its natural ability to recognize distinct DNA sequences. mitoARCUS exhibits reduced off-target activity compared to ZFNs and TALENs, making it a promising alternative for therapeutic applications (Zekonyte et al., 2021). Further advancements in protein engineering, such as optimizing DNA recognition domains and incorporating specificity-enhancing mutations, are critical for reducing off-target editing.

1.2. Delivery Challenges

Effective delivery of nuclease-based tools to mitochondria remains a significant challenge due to the organelle's double-membrane structure and the absence of RNA import mechanisms. mtZFNs, mitoTALENs, and mitoARCUS are typically delivered as protein-based systems fused with a MTS to facilitate localization within the mitochondrial matrix. However, the delivery efficiency of these nucleases varies depending on the cell type, tissue, and delivery platform.

Non-viral delivery systems, such as liposomes and lipid nanoparticles, have been used for mitochondrial targeting in cultured cells, but their in vivo application is limited by poor tissue penetration and mitochondrial uptake efficiency (Paunovska et al., 2022). Viral vectors, particularly AAVs, have demonstrated promise for delivering nuclease-based tools to mitochondria in vivo. For example, AAV9-mediated delivery of mitoTALENs has been shown to successfully reduce mutant mtDNA levels in mouse models,

leading to improved mitochondrial function (Bacman et al., 2018). However, the size constraints of AAVs (~4.8 kb) pose challenges for delivering large constructs such as TALEN pairs. To overcome this, dual-AAV systems and miniaturized nucleases like mitoARCUS have been developed to enable efficient delivery of compact editing tools.

1.3. Homoplasmic Mutations and Selective Editing

Nuclease-based tools are highly effective for heteroplasmic mtDNA mutations. By selectively cleaving mutant mtDNA, nucleases enable heteroplasmic shifting, reducing the proportion of mutant mtDNA below the pathogenic threshold and alleviating disease symptoms. However, in cases of homoplasmic mutations, where all mtDNA copies harbor the same mutation, nuclease-based approaches face a significant limitation. Depleting mtDNA in such cases can lead to severe mitochondrial depletion and cellular dysfunction (Reddy et al., 2015).

To address this, nuclease strategies have been combined with mtDNA rescue systems, where donor wild-type mtDNA is introduced to replace the depleted mutant population. While this approach remains experimental, it highlights the need for alternative strategies, such as base editing or mitochondrial genome transplantation, to correct homoplasmic mutations effectively.

1.4. Mitochondrial DNA Repair and Persistence of Edits

mtDNA lacks robust repair mechanisms, particularly for DSBs. Upon nuclease-induced cleavage, damaged mtDNA is typically degraded, triggering replication of the remaining wild-type mtDNA to maintain copy number (Peeva et al., 2018). However, the persistence of DSBs can lead to incomplete mtDNA elimination and accumulation of defective genomes. This creates variability in

editing outcomes and raises concerns about long-term mitochondrial genome stability.

To improve editing outcomes, strategies such as dose-controlled nuclease delivery and iterative editing cycles have been employed to ensure complete mutant mtDNA depletion and consistent replication of wild-type genomes (Gammage et al., 2016). Moreover, understanding the mechanisms governing mtDNA replication and turnover will be essential for enhancing the precision and persistence of nuclease-based edits.

1.5. Immunogenicity and Toxicity

The immunogenicity and toxicity of nuclease-based tools represent critical concerns for clinical applications. FokI nucleases and other foreign proteins used in mitoTALENs and mitoARCUS can elicit immune responses, particularly when delivered via viral vectors. Additionally, prolonged expression of nucleases may result in off-target cleavage and cellular toxicity. Strategies to mitigate immunogenicity include using humanized protein scaffolds, minimizing nuclease expression duration through inducible systems, and employing transient delivery methods such as mRNA-based approaches (Bacman et al., 2013; Silva-Pinheiro et al., 2022).

While mtDNA nuclease-based editing tools, including mtZFNs, mitoTALENs, and mitoARCUS, have demonstrated remarkable potential for treating mitochondrial disorders, significant challenges remain in terms of specificity, delivery efficiency, and applicability to homoplasmic mutations. Innovations such as high-fidelity nucleases, compact protein designs, and enhanced delivery systems are crucial for overcoming these obstacles. Continued advancements in mitochondrial genome biology and protein engineering will pave the way for the clinical translation of nuclease-

based editing, offering hope for patients with debilitating mitochondrial diseases.

2. Challenges and Improvements in mtDNA Base Editing

Mitochondrial base editing technologies, such as DdCBEs and TALEDs, have emerged as promising tools for precise modification of mtDNA. However, these tools face significant challenges that hinder their efficiency, specificity, and delivery. Addressing these challenges is critical for advancing the therapeutic potential of mitochondrial genome editing.

2.1. Sequence Preference and Base Editing Efficiency

A key limitation of current base editors, such as DdCBEs, lies in their sequence preference. The DddAtox cytosine deaminase primarily targets the TC motif within dsDNA, making it less effective on other motifs like GC, CC, or AC sequences. This restricts the range of mtDNA mutations that can be targeted for correction (Mok et al., 2020). Recent efforts to address this limitation involve protein engineering approaches such as phage-assisted continuous evolution (PACE), which has yielded evolved DddAtox variants with improved editing efficiency and broader sequence compatibility. For example, DddA11 and Q2L7-DdCBE variants have demonstrated enhanced activity on alternative sequences like HC and GC motifs, respectively (Mok et al., 2022; Sun et al., 2023).

2.2. Delivery Constraints and Tool Size

Effective delivery of mitochondrial base editors remains a major hurdle due to the double-membrane structure of mitochondria and the substantial size of editing tools like TALE-based DdCBEs. TALE arrays, which are used for sequence-specific binding, consist of repetitive amino acid sequences that contribute to their large size.

This creates challenges for viral delivery systems, such as AAVs, which have a packaging limit of approximately 4.8 kilobases (Lee et al., 2022). To overcome this, compact base editors such as ZFDs and ZFD-DdCBEs have been developed. Zinc finger arrays are significantly smaller than TALE arrays and can recognize multiple nucleotides per module, reducing the overall tool size while maintaining editing efficiency (Lim et al., 2022; Willis et al., 2022). Additionally, the development of monomeric DddAtox variants, such as GSVG, enables single-component base editors, further facilitating delivery into mitochondria using AAV vectors (Mok et al., 2022).

2.3. Off-Target Effects

Off-target editing remains a critical concern for mtDNA base editors. Deaminase activity can cause unintended C-to-T or A-to-G substitutions at non-target sites, both within mtDNA and nuclear DNA. Genome-wide analyses using methods like GOTI (Genome-wide Off-Target analysis by Two-cell Embryo Injection) and Detect-seq have revealed that DdCBEs can induce significant off-target editing, likely due to spontaneous assembly of split-DddAtox domains in unintended locations (Wei et al., 2022; Lei et al., 2022). To mitigate this issue, engineered variants such as HiFi-DdCBEs, which incorporate mutations at the dimer interface (e.g., K1389A and T1391A), reduce non-specific activity while maintaining on-target editing precision (Lee et al., 2023). Similarly, the use of nuclear export signals (NES) and DddA inhibitors, like DddIA, minimizes nuclear off-targeting by enhancing mitochondrial localization and preventing deaminase activity in the nucleus (Sun et al., 2023).

2.4. Bystander Editing and Base Editing Window

The base editing window, defined as the region within which nucleotide substitutions occur, poses a challenge for precise genetic modification. The presence of multiple editable nucleotides within the editing window increases the risk of bystander editing, where unintended base changes occur alongside the target mutation (Mok et al., 2020). Strategies to refine the editing window include the use of evolved DddAtox variants with narrower activity windows (e.g., DddAtox K1389A) and careful positioning of DNA-binding domains, such as TALE or zinc finger arrays, to modulate the editing site (Lee et al., 2023).

2.5. Strand-Selective Base Editing

Traditional mtDNA base editors target both strands of dsDNA indiscriminately, which limits their precision for therapeutic applications. Recent developments, such as mitoBEs and CyDENT systems, have introduced strand-selective editing, allowing precise modifications on a single DNA strand. By incorporating DNA nickases, such as MutH or FokI, these tools transiently expose single-stranded DNA (ssDNA), enabling selective editing by deaminases like TadA8e or APOBEC1 (Yi et al., 2023; Hu et al., 2023). These advancements offer greater control over base editing outcomes and minimize unintended modifications on the complementary strand.

Addressing the challenges of sequence specificity, delivery constraints, off-target effects, and bystander editing is critical to improving the precision and efficacy of mtDNA base editors. The development of compact, highly specific, and strand-selective tools has significantly advanced the field, offering promising therapeutic avenues for mitochondrial diseases. Continued innovation,

alongside rigorous evaluation of safety and efficiency, will be essential for translating these technologies into clinical applications.

Applications and Therapeutic Potentials

1. Genome Editing in the Treatment of Mitochondrial Diseases

Mitochondrial genome editing offers significant potential for addressing debilitating diseases caused by mtDNA mutations. Conditions such as LHON and MELAS remain without effective treatments, highlighting the need for innovative molecular therapies (Gorman et al., 2015; Taylor & Turnbull, 2005).

Recent advancements in base editing technologies have provided precise tools for correcting pathogenic mtDNA mutations. Notably, DdCBEs utilize the cytosine deaminase activity of the bacterial toxin DddA, split into halves to target double-stranded mtDNA. This enables site-specific C-to-T conversions without inducing double-strand breaks, a major limitation of traditional editing tools (Mok et al., 2020). Preclinical studies have demonstrated the efficacy of DdCBEs in disease models, including LHON and MELAS. In mouse embryos, DdCBE-based editing successfully reduced mutant mtDNA levels, restored mitochondrial function, and alleviated disease symptoms, underscoring the therapeutic potential of this approach (Guo et al., 2022).

Alongside DdCBEs, mitoTALENs have emerged as effective tools for selectively degrading mutant mtDNA. mitoTALENs utilize TALE proteins to recognize and cleave specific pathogenic mtDNA sequences, promoting the replication of wild-type mtDNA. This strategy has shown success in cellular and animal models, where the heteroplasmic balance was shifted below the pathogenic threshold, resulting in significant reductions in disease burden (Bacman et al., 2018).

These advancements highlight the feasibility of personalized mitochondrial therapies tailored to specific mutations, offering solutions to the genetic heterogeneity observed in mitochondrial disorders. However, for clinical translation, further efforts are needed to optimize delivery systems—such as AAVs—and enhance the precision and efficiency of editing technologies.

The development of these tools marks a critical step toward effective mitochondrial disease treatments, providing a foundation for addressing previously untreatable conditions and improving patient outcomes.

2. Mitochondrial Replacement Therapy (MRT)

MRT, commonly referred to as "three-parent embryo" technology, represents a groundbreaking advancement in reproductive medicine and mitochondrial genome manipulation. This technique involves replacing defective mitochondria in an oocyte or zygote with healthy mitochondria from a donor, thereby preventing the transmission of maternally inherited mitochondrial diseases. The process has demonstrated clinical success, with the first child conceived via MRT born in 2016, marking a major milestone in preventing mitochondrial disorders (Zhang et al., 2017).

Beyond preventing genetic diseases, MRT has emerged as a potential solution for improving fertility outcomes, particularly in older women experiencing oocyte aging. Mitochondrial dysfunction, characterized by reduced ATP production and increased oxidative stress, contributes to declining oocyte quality and viability with age (Babayev & Seli, 2015). By introducing healthy donor mitochondria, MRT may enhance oocyte metabolic activity, improving embryo development and increasing pregnancy success rates, although long-term efficacy and safety remain areas of active research (Amato et al., 2014).

However, the implementation of MRT raises significant ethical and legal challenges. Key concerns include the psychological implications for children born with genetic material from three individuals and societal debates over the extent of the third-party genetic contribution. The potential for germline modifications also introduces questions regarding heritability and the long-term impact on future generations. Countries like the United Kingdom have legalized MRT under stringent regulatory frameworks, ensuring oversight and safety for its application. In contrast, MRT remains banned or unregulated in countries such as the United States, reflecting ongoing societal and legal reservations (Cohen et al., 2015).

As MRT continues to evolve, its dual potential for preventing mitochondrial diseases and enhancing fertility outcomes underscores its transformative role in reproductive medicine. However, careful consideration of ethical frameworks and long-term studies will be essential to ensure its safe and equitable application globally.

3. Emerging Therapeutic Opportunities in Mitochondrial Genome Editing

Mitochondrial genome editing is emerging as a transformative approach for addressing mitochondrial disorders, fertility challenges, and a broader range of diseases influenced by mitochondrial dysfunction. Mitochondrial abnormalities, including mutations, impaired oxidative phosphorylation, and increased oxidative stress, are increasingly recognized as key contributors to age-related conditions such as cardiovascular diseases, neurodegenerative disorders, and metabolic conditions like type 2 diabetes. By directly targeting and correcting pathogenic mtDNA mutations, genome-editing tools offer the potential to restore

mitochondrial function and slow disease progression (Gammage et al., 2018; Silva-Pinheiro & Minczuk, 2022).

In addition to age-related disorders, mitochondrial genome editing is being explored as a novel strategy in cancer treatment. Alterations in mtDNA, including mutations and large-scale deletions, are implicated in tumorigenesis, metastasis, and chemotherapy resistance (Kopinski et al., 2021). Tumor cells often exhibit enhanced glycolysis (the Warburg effect) alongside mitochondrial dysfunction. Genome editing aimed at correcting these abnormalities could restore mitochondrial respiration, potentially improving the effectiveness of existing therapies. Emerging research suggests that targeting mtDNA to reverse mutations or disrupt cancer-associated pathways could reduce tumor growth and enhance responses to chemotherapeutic agents (Yu et al., 2022).

Mitochondrial genome editing also shows promise for neurodegenerative disorders like Parkinson's and Huntington's diseases, where impaired mitochondrial dynamics and mtDNA instability contribute to neuronal degeneration. Tools such as mitoARCUS and DdCBEs can be leveraged to correct mitochondrial dysfunction, mitigate oxidative damage, and protect neurons, addressing the underlying pathophysiology of these diseases (Mok et al., 2020). Similarly, metabolic disorders such as diabetes and obesity, which involve defective mitochondrial metabolism, could benefit from interventions aimed at restoring energy production (Bratic & Larsson, 2013).

Future Directions and Conclusion

Mitochondrial genome editing is advancing rapidly, with tools like DdCBEs and mitoTALENs showing significant potential

in preclinical studies. However, key challenges must be addressed to realize clinical applications.

Developing safe and efficient delivery systems is paramount. Advances in nanoparticle-based delivery and mitochondrial-targeting peptides may improve precision and in vivo efficacy. While technologies like DdCBEs and mitoTALENs exhibit high specificity, concerns over off-target effects remain. Computational modeling and high-throughput screening are needed to enhance accuracy and reduce unintended edits. Current editing methods are largely confined to base editing, limiting their use to specific mutations. Expanding capabilities to include insertions and deletions could greatly broaden therapeutic applications.

Robust preclinical studies in animal models, followed by clinical trials, are essential to establish the long-term safety and efficacy of mitochondrial genome editing. Standardized protocols and clear regulatory pathways will be critical for clinical adoption.

Addressing ethical and societal concerns is equally important. Collaboration with ethicists, policymakers, and the public will foster transparency and build consensus. International cooperation will help harmonize regulations and ensure equitable access to these transformative technologies.

Mitochondrial genome editing heralds a new era in precision genetic medicine, offering the potential to treat mitochondrial diseases at their root. Despite technical and ethical challenges, ongoing progress in molecular biology, bioengineering, and ethics will pave the way for clinical translation, unlocking transformative benefits for patients with mitochondrial disorders.

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BÖLÜM II

Click Chemistry Strategies on Natural Product Modification: An updated approach

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Aleyna Bade Karahan[2]

Introduction

In recent years, click chemistry has emerged as a powerful and versatile tool in the field of synthetic chemistry. Coined by Nobel Laureate K. Barry Sharpless in the early 2000s, click chemistry refers to a set of highly efficient, selective, and easily controllable reactions that allow for the rapid assembly of complex molecules from simple building blocks. This phenomenon has revolutionized the way scientists approach molecular synthesis, offering new solutions to challenges that have long plagued organic and medicinal chemistry. One of the most exciting applications of

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click chemistry is in the modification of natural products—complex compounds derived from natural sources that have historically served as the foundation for drug discovery (Zhang et al. 2021).

Naturally occurring products have long been the starting point for the development of many life-saving pharmaceuticals. However, modifying these compounds to optimize their biological activity, solubility, and stability has proven to be a difficult task. Traditional methods of modifying natural products often involve cumbersome synthetic steps, which can be time-consuming, inefficient, and yield only small quantities of the desired derivatives. Click chemistry, with its high efficiency, broad functional group compatibility, and mild reaction conditions, offers an updated and streamlined approach for overcoming these challenges. By enabling chemists to rapidly functionalize and modify natural products with precision, click chemistry is transforming the landscape of drug discovery and development.

Click Chemistry Mechanisms and Reactions

Click chemistry has garnered significant attention due to its simplicity, efficiency, and versatility in synthesizing complex molecular architectures. The reactions within click chemistry are characterized by their high yield, functional group tolerance, and ability to proceed under mild conditions. This makes them especially useful in the modification of natural products, where the introduction of specific functional groups is often challenging. Below, we will explore some of the most widely used click chemistry reactions, with a particular focus on their application in natural product modification.

The Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC)

One of the most widely studied and applied click reactions is the Copper-Catalyzed Azide-Alkyne Cycloaddition, a variant of the Huisgen 1,3-dipolar cycloaddition. This reaction involves the reaction between an alkyne and an azide group to form a 1,2,3-triazole ring. The process proceeds efficiently under the catalytic action of copper (I), which facilitates the formation of the triazole linkage (Tiwari et al. 2024).

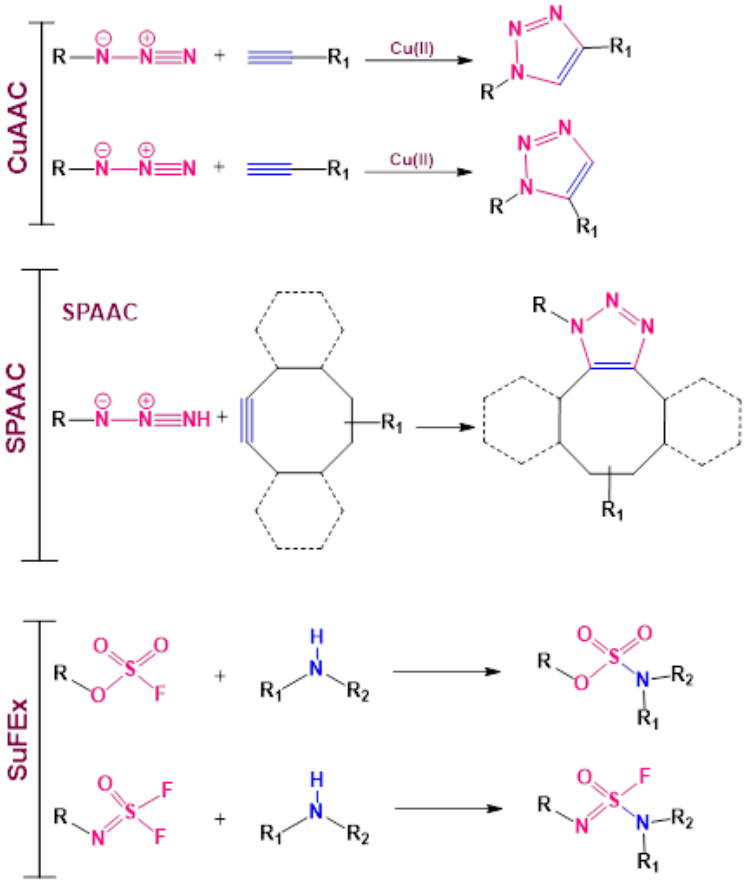

Figure 1: Representative click chemistries (Zhang et al. 2021)

The CuAAC reaction is highly selective, occurs with near-quantitative yield, and proceeds under relatively mild conditions (ambient temperature and pressure). This makes it ideal for modifying complex natural products, where delicate functional groups must be preserved while introducing new chemical functionalities. For example, the CuAAC reaction can be used to conjugate a natural product, such as a steroid or alkaloid, to a synthetic or bioactive molecule like a fluorophore, peptide, or drug delivery carrier (Ahmad et al.2013).

Aforementioned reaction is particularly useful in natural product modification because it allows the straightforward functionalization of natural compounds without the need for extensive purification of intermediates. It also enables the creation of new classes of natural product derivatives by adding a wide variety of bioactive and functional groups.

Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC)

While CuAAC is highly effective, the presence of copper can sometimes be problematic, particularly when working with biologically active natural products, where copper may induce toxicity or interfere with bioactivity. The Strain-Promoted Alkyne-Azide Cycloaddition is an alternative click reaction that occurs without the need for a metal catalyst. This reaction takes advantage of the strain in cyclooctynes (strained alkynes), which can react with azides to form the same 1,2,3-triazole linkage (Li et al. 2021).

SPAAC offers several advantages over CuAAC, including its biocompatibility and the fact that it proceeds in aqueous media, making it suitable for biological and medicinal applications. The reaction is highly efficient and selective, even in the presence of other functional groups that might otherwise interfere with other reactions. SPAAC has been particularly useful in the modification of natural products that are used in drug discovery, as it enables the

creation of bioactive derivatives that can be tested for improved pharmacological properties.

Thiol-Ene Click Chemistry

Thiol-ene chemistry is another type of click reaction where a thiol group (-SH) reacts with a carbon-carbon double bond (ene) to form a new carbon-sulfur bond. The reaction is initiated by light, heat, or a radical initiator, and it can proceed in the absence of solvents or under mild conditions. The thiol-ene reaction is highly versatile, offering functional group tolerance and high efficiency (Hoyle and Bowman 2010).

In the context of natural product modification, thiol-ene chemistry is particularly useful for the modification of compounds containing unsaturated bonds, such as terpenoids and flavonoids. For example, a thiol group can be introduced to a natural product scaffold, followed by the ene reaction to conjugate it with a variety of bioactive molecules, such as drugs, peptides, or imaging agents. This reaction is also valuable for creating more complex molecular architectures by linking natural products to polymers, nanoparticles, or other biofunctional entities (Hoyle and Bowman 2010).

Diels-Alder Reaction

The Diels-Alder reaction is a powerful pericyclic reaction in which a diene (a molecule with two conjugated double bonds) reacts with a dienophile (a molecule that contains a double or triple bond) to form a six-membered ring. While not always classified as a traditional click reaction, the Diels-Alder reaction exhibits many of the hallmark features of click chemistry, including high efficiency, regio- and stereoselectivity, and the ability to occur under mild conditions (Castro-Esteban et al. 2021).

The Diels-Alder reaction can be applied to the modification of natural products that contain diene or dienophile motifs. For

instance, certain polyphenolic natural products, such as flavonoids or stilbenes, contain conjugated double bonds that can participate in Diels-Alder reactions, forming novel scaffolds with enhanced biological activity. This reaction has also been explored for the modification of natural products in the context of drug delivery, where it can facilitate the attachment of therapeutic agents to a natural product carrier (Nicolaou et al. 2002).

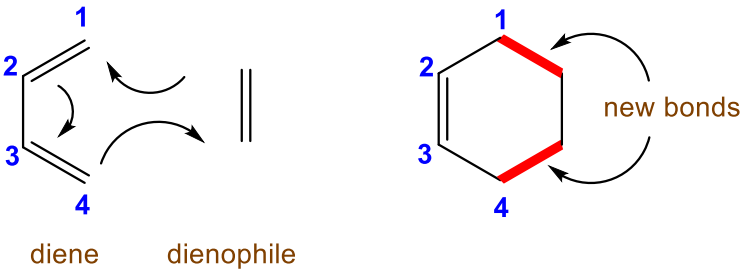

Figure 2: Representative click chemistries (Nicolaou et al. 2002)

Azide-Alkyne Ligations for Bioconjugation

In addition to the reactions described above, azide-alkyne click chemistry can also be employed in bioconjugation strategies, where a natural product is linked to biomolecules like peptides, proteins, or oligonucleotides. These reactions allow for the precise functionalization of natural products with targeting moieties or imaging agents, enabling them to be used in a variety of therapeutic and diagnostic applications (Presolski et al. 2011).

One notable example is the use of click chemistry to attach biotin to a natural product, creating a biotinylated derivative that can be used in affinity-based assays or for targeted drug delivery. Another important application is the conjugation of natural products with antibodies, enabling targeted cancer therapies or molecular imaging. Through click chemistry, these bioconjugates can be easily

synthesized with high precision, allowing for the rapid development of new natural product-based therapeutic platforms.

Click chemistry reactions, such as CuAAC, SPAAC, thiol-ene, and the Diels-Alder reaction, provide chemists with a diverse toolkit for modifying natural products. These reactions share common features: they are highly selective, often proceed with quantitative yields, and are compatible with a wide range of functional groups, making them ideal for the modification of complex natural product structures. By harnessing these click chemistry strategies, researchers can create new derivatives of natural products with enhanced biological activities, improved pharmacokinetic profiles, and novel therapeutic applications. As click chemistry continues to evolve, its applications in natural product modification will likely expand, enabling the creation of a new generation of bioactive compounds.

Examples of Click Chemistry in Natural Product Modification

The power of click chemistry in natural product modification is best demonstrated through a variety of case studies, where researchers have successfully employed click reactions to enhance the bioactivity, solubility, stability, and targeting properties of natural products. In this section, we will explore several notable examples of how click chemistry has been applied to modify natural products, including alkaloids, glycosylated compounds, and drug conjugates.

Modification of Alkaloids Using CuAAC

Alkaloids, a diverse class of naturally occurring nitrogenous compounds, are well-known for their potent biological activities, including antimicrobial, anticancer, and analgesic effects. However,

many alkaloids suffer from issues like poor solubility, toxicity, and limited bioavailability. Click chemistry offers a promising strategy for overcoming these challenges by enabling the selective functionalization of alkaloid structures.

One notable example is the use of the Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) to modify the alkaloid vinblastine, an anticancer agent derived from the periwinkle plant (*Catharanthus roseus*). Vinblastine, like many alkaloids, has limited solubility in aqueous solutions, which hampers its clinical application. To address this, researchers utilized CuAAC to conjugate vinblastine to hydrophilic polymers, such as polyethylene glycol (PEG). The resulting PEGylated vinblastine demonstrated significantly improved solubility and stability in aqueous environments, while maintaining its anticancer activity (Shah et al. 2017; Tiwari et al. 2024).

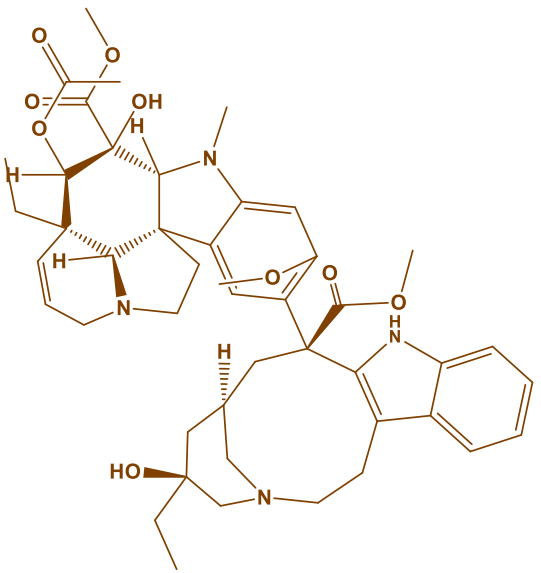

Figure 3: Chemical structure of vinblastine an anticancer drug

Moreover, the conjugation of vinblastine to PEG through click chemistry not only enhanced its pharmacokinetics but also improved its selectivity for cancer cells, as the polymer could be designed to target specific receptors overexpressed on tumor cells. This case highlights the potential of click chemistry to modify alkaloids and other natural products, enabling their optimization for therapeutic use.

Glycosylation of Natural Products via Click Chemistry

Glycosylation is a powerful strategy for improving the pharmacokinetics, solubility, and bioactivity of natural products. However, traditional glycosylation methods can be challenging, particularly when working with complex natural product scaffolds. Click chemistry, particularly the CuAAC reaction, has provided an efficient and selective approach to glycosylate natural products, enhancing their bioactivity and solubility (Zi et al. 2017).

A notable example of this application is the glycosylation of flavonoids. Flavonoids, which are polyphenolic compounds found in fruits, vegetables, and herbs, are known for their antioxidant, anti-inflammatory, and anticancer properties. However, their poor solubility in water limits their bioavailability and therapeutic potential. By using CuAAC, researchers have successfully attached sugar moieties to the hydroxyl groups of flavonoids, forming flavonoid-glycoside conjugates (Pereira et al. 2020).

In one study, researchers modified quercetin, a well-known flavonoid, by attaching glucose and other saccharides to its structure via click chemistry. The resulting glycosylated quercetin exhibited significantly enhanced solubility in aqueous solutions compared to the parent compound. Furthermore, the glycosylation improved the compound's stability in physiological environments, thereby increasing its bioavailability. This case demonstrates how click chemistry can be applied to glycosylate natural products, improving

their pharmacological properties and making them more effective in therapeutic applications.

Development of Natural Product-Drug Conjugates

One of the most exciting applications of click chemistry in natural product modification is the creation of natural product-drug conjugates. These conjugates combine the bioactivity of natural products with the targeting capabilities of other therapeutic agents, such as small molecule drugs, peptides, or antibodies. Click chemistry allows for the precise and efficient conjugation of these molecules, enabling the development of targeted therapies with reduced side effects and enhanced therapeutic efficacy.

For example, researchers have used click chemistry to attach cytotoxic drugs to naturally occurring antibodies or peptides that specifically target cancer cells. One study involved the conjugation of the natural product curcumin, an active component in turmeric with known anticancer and anti-inflammatory properties, to a cancer-targeting antibody using a CuAAC reaction. The resulting curcumin-antibody conjugate exhibited enhanced selectivity for cancer cells and improved therapeutic efficacy compared to free curcumin, which is known to have poor bioavailability and limited potency *in vivo* (Seghetti et al. 2020).

In another example, click chemistry was used to conjugate paclitaxel, a potent chemotherapeutic agent, to a natural polysaccharide derived from seaweed. The polysaccharide served as a targeting moiety, directing paclitaxel specifically to cancer cells overexpressing certain surface receptors. This strategy significantly improved the efficacy of paclitaxel, as the conjugate could deliver

the drug directly to the tumor site, reducing systemic toxicity and enhancing its antitumor effects.

These case studies demonstrate the power of click chemistry to facilitate the creation of natural product-drug conjugates, a promising approach for targeted drug delivery in cancer therapy and other diseases.

Bioconjugation of Natural Products for Imaging and Diagnostics

In addition to therapeutic applications, click chemistry has also been applied to modify natural products for imaging and diagnostic purposes. By attaching fluorescent probes, radionuclides, or other imaging agents to natural products, researchers can track the biodistribution and localization of these compounds in vivo.

A prominent example is the use of click chemistry to functionalize natural product-based scaffolds with fluorescent probes for use in imaging. In one study, the alkaloid atropine, a naturally occurring antimuscarinic agent, was conjugated with a fluorescent dye via CuAAC. The resulting conjugate allowed for the real-time tracking of atropine's distribution in living animals, providing valuable insights into its pharmacokinetic properties. This application is particularly valuable in drug discovery, as it enables researchers to monitor the behavior of natural products in vivo, optimizing their development as therapeutic agents (Ma et al. 2015).

Another notable example is the modification of natural antibiotics with radioactive isotopes for use in positron emission tomography (PET) imaging. By attaching a radioactive tracer to the antibiotic molecule via click chemistry, researchers can non-invasively monitor the accumulation of the antibiotic at infection

sites, improving the accuracy of diagnostics and the development of targeted antimicrobial therapies (Gouws et al. 2022).

Creation of Libraries of Modified Natural Products

Click chemistry is also a powerful tool for the high-throughput synthesis of libraries of modified natural products. The ability to rapidly and efficiently generate large libraries of natural product derivatives opens up new avenues for drug discovery and optimization. Researchers can screen these libraries for compounds with enhanced bioactivity, selectivity, or other desirable properties.

For instance, researchers have used click chemistry to generate a library of modified flavonoids by attaching different functional groups to the parent structure through CuAAC. These flavonoid derivatives were then screened for their anticancer activity, leading to the discovery of several compounds with improved potency and selectivity compared to the parent flavonoid. This approach demonstrates how click chemistry can be used to create diverse libraries of natural product derivatives, enabling the rapid identification of lead compounds for further development.

Conclusion

Click chemistry has undoubtedly emerged as a transformative tool in the field of natural product chemistry, offering efficient, versatile, and highly selective reactions that have significantly advanced the modification of natural compounds. As demonstrated throughout this chapter, the integration of click chemistry with natural product modification has not only streamlined traditional synthetic methods but also opened up new possibilities for enhancing the bioactivity, solubility, stability, and targeting properties of these complex molecules.

The mechanisms and reactions underlying click chemistry—such as the Copper-Catalyzed Azide-Alkyne Cycloaddition (CuAAC), Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC), thiol-ene reactions, and the Diels-Alder reaction—are powerful tools that enable the rapid and precise functionalization of natural products. These reactions have been widely applied in various modifications, from glycosylation to the creation of bioactive conjugates, facilitating the generation of new derivatives with improved pharmacological profiles.

The case studies presented illustrate the broad applicability of click chemistry, highlighting successful applications in the modification of alkaloids, flavonoids, and other natural products. By utilizing click chemistry, researchers have enhanced the solubility, bioavailability, and targeting potential of natural products, paving the way for the development of more effective therapeutics. Notably, the creation of natural product-drug conjugates and bioconjugates, as well as the generation of modified libraries for drug discovery, are prime examples of how click chemistry is revolutionizing modern drug development.

Despite its remarkable successes, challenges remain in the field, including issues with steric hindrance, selectivity, and the scalability of reactions. However, as the field continues to evolve, click chemistry holds immense promise for addressing these challenges and expanding its potential applications. Future innovations in emerging click reactions, as well as the integration of click chemistry with other synthetic and biological techniques, will likely further enhance its role in natural product modification.

In conclusion, click chemistry offers a cutting-edge approach to modifying natural products in ways that were once unimaginable. Its ability to rapidly functionalize complex molecules with high efficiency and precision positions it as a cornerstone of modern medicinal chemistry and drug discovery. As researchers continue to harness the power of click chemistry, we can expect the development of novel natural product-based therapies, personalized medicine, and bioactive molecules that will have a lasting impact on the treatment of diseases and the improvement of human health.

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BÖLÜM III

Secondary Metabolites from Nitrogen-Fixing Bacteria in Fabaceae Members

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Introduction

Nitrogen is a critical macronutrient for plant growth, and its availability in soil is often a limiting factor for agricultural productivity. In nature, certain bacteria are capable of converting atmospheric nitrogen into ammonia, a form usable by plants. Members of the Fabaceae family, commonly known as legumes, have developed symbiotic relationships with nitrogen-fixing bacteria, primarily Rhizobium and Bradyrhizobium species, to enhance soil nitrogen levels (Tan et al. 2001). Beyond their role in nitrogen fixation, these bacteria also produce an array of secondary

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metabolites that can influence the growth and health of the host plants.

Secondary metabolites are non-essential compounds that, while not directly involved in primary metabolic pathways, can have significant ecological and physiological effects. These metabolites, including alkaloids, flavonoids, and antibiotics, often play roles in plant defense, signaling, and interspecies competition. In the context of nitrogen-fixing bacteria, these compounds may influence both the symbiotic relationship with the host plant and the broader rhizosphere environment (Al-Khayri et al. 2023; Ozyigit et al. 2023).

This paper aims to review the secondary metabolites produced by nitrogen-fixing bacteria in the Fabaceae family, focusing on their biosynthesis, roles in plant-microbe interactions, and potential applications in agriculture.

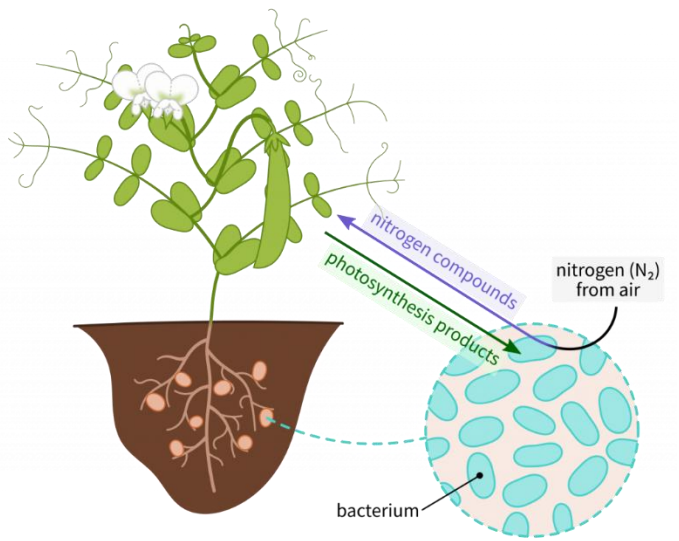

Figure 1. Rhizobium and Bradyrhizobium species fix nitrogen

Nitrogen-Fixing Symbiosis in Fabaceae

The relationship between nitrogen-fixing bacteria and leguminous plants is one of the most well-studied examples of mutualism in nature. Rhizobium, Bradyrhizobium, and other nitrogen-fixing bacteria infect the roots of leguminous plants, forming specialized structures called nodules, within which the bacteria fix nitrogen. This process benefits the plant by providing it with a readily available source of nitrogen, while the bacteria obtain carbohydrates and other organic compounds from the plant. Nitrogenase is one of the significant enzyme in the universe and catalyse the nitrogen-fixing reactions which has an iron-molybdenum active site (Figure 1) (Bhutto et al. 2019).

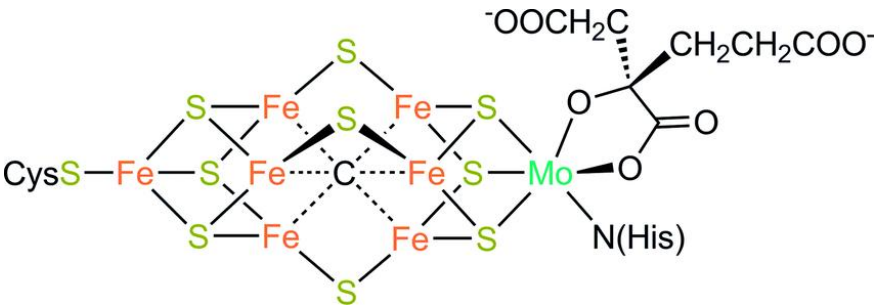

Figure 2. Active site of the iron–molybdenum nitrogenase enzyme

This symbiosis is facilitated by a series of molecular signaling events, where plant-derived compounds, such as flavonoids, induce the expression of bacterial nodulation genes. The bacteria, in turn, secrete signaling molecules, including Nod factors, that enable the plant to initiate nodule formation. However, beyond these primary interactions, secondary metabolites produced by both the host plant and the nitrogen-fixing bacteria play crucial roles in the regulation of the symbiosis, plant defense mechanisms, and the microbial community within the rhizosphere.

Secondary Metabolites Produced by Nitrogen-Fixing Bacteria

Alkaloids

Alkaloids are a group of nitrogen-containing compounds that exhibit a wide range of biological activities, including antimicrobial, antiplant, and antipathogenic properties. In nitrogen-fixing bacteria, alkaloids such as rhizobitoxine (produced by Rhizobium) have been identified as key regulators of the symbiosis.

Rhizobitoxine has been shown to inhibit the production of ethylene in plants, an important hormone that regulates nodule development. By reducing ethylene levels, rhizobitoxine promotes effective nodule formation, thereby enhancing nitrogen fixation efficiency and the chemical structure was given in Figure 3 (Sugawara et al. 2006).

Flavonoids and Isoflavonoids

Flavonoids are a diverse group of plant-derived secondary metabolites that play significant roles in plant-microbe interactions. In nitrogen-fixing symbiosis, legume plants produce specific flavonoids that trigger the expression of bacterial nod genes. Interestingly, certain nitrogen-fixing bacteria, particularly those of the genus *Bradyrhizobium*, produce their own isoflavonoids, which can influence nodule formation and plant growth.

These compounds also serve as signaling molecules that modulate plant immune responses, potentially enhancing the host plant's resistance to pathogens and environmental stressors (Subramanian et al. 2006).

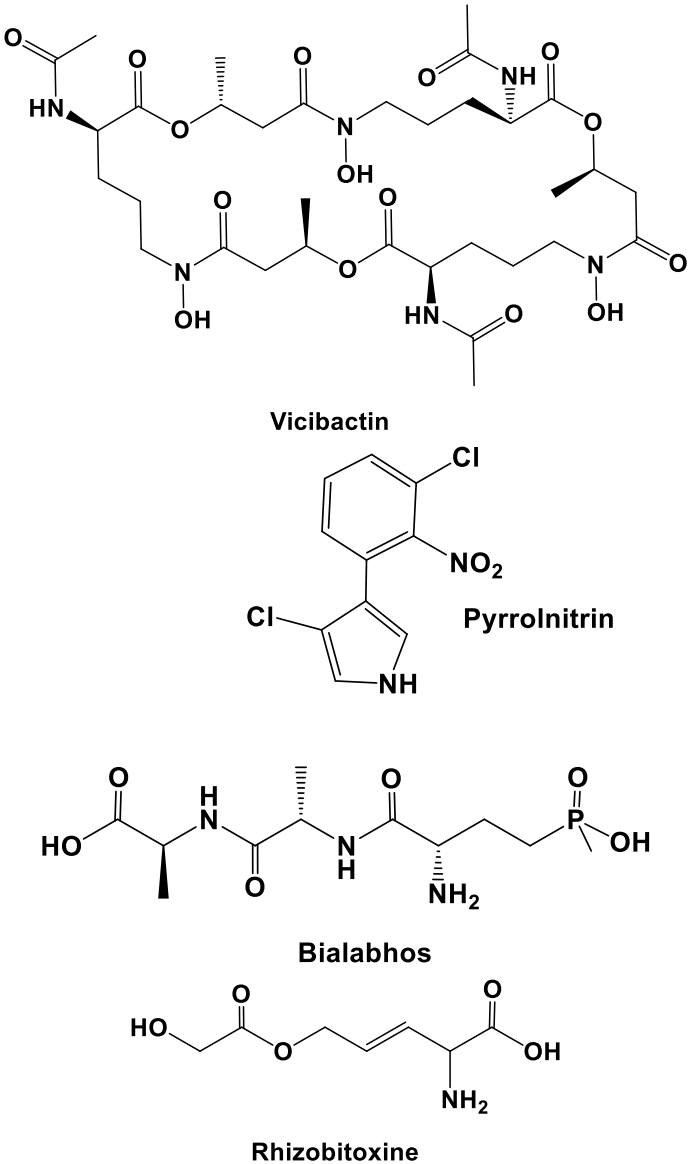

Figure 3. Phytochemicals originated from nitrogen-fixing bacteria

Antibiotics and Antifungal Compounds

Many nitrogen-fixing bacteria produce antibiotics and antifungal compounds that help them compete with other microbes in the rhizosphere. These metabolites include compounds like cyclodipeptides, pyrrolnitrin, and bialaphos, which have demonstrated antibacterial and antifungal activities (Hosoya et al. 1998). These secondary metabolites can not only protect the bacteria from pathogens but may also contribute to the plant's health by promoting a favorable microbial community in the rhizosphere (Pawar et al. 2020).

Siderophores

Siderophores are small molecules produced by microorganisms to sequester iron from the environment. This is particularly important in environments with low bioavailability of iron. Nitrogen-fixing bacteria like Rhizobium produce siderophores such as vicibactin to acquire iron from the soil or plant roots and the chemical structure was given in Figure 3. By enhancing iron availability, these metabolites promote the growth and activity of the nitrogen-fixing bacteria. In turn, plants benefit from the enhanced bacterial performance in nitrogen fixation (Datta et al. 2014).

Exopolysaccharides

Exopolysaccharides (EPS) are high-molecular-weight compounds produced by many nitrogen-fixing bacteria. These compounds are involved in biofilm formation, protection against desiccation, and modulating plant responses. EPS also plays a critical role in facilitating bacterial attachment to plant roots and nodule formation. While they are not technically secondary metabolites in the strictest sense, the production of EPS by nitrogen-fixing bacteria in the rhizosphere is a key aspect of their metabolic profile.

Ecological and Agricultural Roles of Secondary Metabolites

The secondary metabolites produced by nitrogen-fixing bacteria have profound effects on both the host plant and the surrounding environment. In addition to their roles in enhancing nitrogen fixation and promoting plant growth, these compounds can also influence plant defense responses and help plants resist stress. For example, alkaloids and antibiotics can protect the plant from harmful soil-borne pathogens, while flavonoids and isoflavonoids may enhance plant resistance to environmental stresses such as drought and extreme temperatures.

Moreover, secondary metabolites produced by nitrogen-fixing bacteria may help shape the microbial community in the rhizosphere. By producing antimicrobial compounds, these bacteria can suppress the growth of pathogenic microorganisms, fostering a healthier soil microbiome. This, in turn, can reduce the reliance on chemical fertilizers and pesticides, offering a more sustainable approach to agriculture.

Potential Applications in Biotechnology and Agriculture

The potential applications of secondary metabolites from nitrogen-fixing bacteria in agriculture are vast. Understanding the biosynthesis and ecological roles of these compounds can lead to the development of biotechnological products that enhance crop productivity and health. For instance, the application of bacterial inoculants containing specific strains of nitrogen-fixing bacteria that produce beneficial secondary metabolites could improve soil fertility, promote plant growth, and increase resilience to abiotic stress.

Furthermore, biocontrol agents derived from nitrogen-fixing bacteria, which produce antimicrobial and antifungal metabolites, can be used to protect crops from diseases, reducing the need for chemical pesticides. Additionally, the use of nitrogen-fixing bacteria in crop rotations and intercropping systems could enhance soil health

by boosting nitrogen levels and promoting beneficial microbial communities.

Conclusion

Nitrogen-fixing bacteria in the Fabaceae family are not only essential for nitrogen fixation but also produce a variety of secondary metabolites that play important roles in plant health, growth, and defense. These metabolites influence the dynamics of plant-microbe interactions and offer potential applications in sustainable agriculture and biotechnology. Further research into the biosynthesis, ecological roles, and applications of these secondary metabolites will provide valuable insights into their use as natural alternatives to chemical fertilizers and pesticides, contributing to more sustainable agricultural practices.

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