Pak. J. Chem. 13(3-4): 57-66, 2023 Review article
ISSN (Print): 2220-2625
ISSN (Online): 2222-307X DOI: 10.15228/2023.v13.i03-4.p01

Chemical Modulation of RNA Epigenetic Modifications

L. Cheng
Beijing National Laboratory for Molecular Sciences (BNLMS), CAS Key Laboratory of Molecular Recognition and Function, CAS Research/Education Center for Excellence in Molecular Sciences, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China, and University of Chinese Academy of Sciences, Beijing 100049, China
Corresponding Author Email.chengl@iccas.ac.cn

Abstract
Given the increasing evidence demonstrating the notable functions of RNA modifications, the precise regulation of a particular epigenetic mark is high desirable. Current genetic techniques like over-expression or knockout of corresponding genes/proteins, as well as the development of molecular inhibitors/antagonists of these enzymes facilitated a prompt epigenetic associated phenotype observation. However, these approaches heavily relied on the characterization of RNA-modifying enzymes. *In vitro* or *in vivo* alteration of RNA epigenetics, especially for those modifications whose pathways have not been identified, through direct chemical interventions with small organic molecules would fulfill this gap and is thus highly desirable. Considering the fact that those enzymatic modifications and de-modifications are chemical reactions occurred in the homeostasis constraints of a living system, we believe it would be possible for developing the corresponding their biomimetic counterparts. In this *Review*, we will present our recent advances regarding the direct chemical interventions towards several biologically important RNA modifications and their applications in live cells or growing plants. We have demonstrated that the demethylation process of N^6-methyladenosine m^6A in live cells could be accomplished through Flavin mononucleotide prompted biocompatible photo-oxidation. This is the first example of small organic molecules modulating *in vitro* RNA epigenetic modifications. We also presented a straightforward transformation for the generation of 5-formylcytidine f^5C from 5-methylcytidine m^5C using anthraquinone-2-sulfonate without oxidizing other biomacromolecules or redundancy production of 5-hydroxymethylcytidine hm^5C. Most recently, we reported that oxoammonium cations might be applied as artificial deprenylases for the highly sensitive and selective demodification of N^6-prenyladenosine i^6A in oligonucleotides, cells and even live plants. One of the most striking features of these "*artificial enzymes*" is their low cytotoxic activity on cells and extraordinary capability in regulating designated modifications without over-expression or inhibiting corresponding enzymes or disturbing other epigenetics, nucleic acids, or proteins.
The objective of this *Review* is to present the research findings regarding the design, synthesis and evaluation of highly efficient chemical reagents and biocompatible transformations toward selected RNA epigenetics that have enabled chemists' ability to not just controlling *in vitro/in vivo* RNA modifications, but also introducing artificial variants onto natural RNAs, without disturbing any related enzymes. Such double direction regulating may considerably help in the function interpretation/installation/modulation of versatile modifications pertaining to RNAs. The ability of those chemical motifs will undoubtedly illuminate undisclosed functions and regulatory mechanisms affiliated to the vast majority of discovered modifications and has the potential to enable treatments for related human diseases.
Keywords: RNA epigenetic modification; Direct modulation; Small organic molecules; Biocompatible transformations; Chemical tools

1. INTRODUCTION

The first RNA modification, pseudourindine, was discovered over 60 years ago [1]. Since then, the genome-wide discovery and mapping of new modifications have expressively boosted with the assistance of advancements in high throughout detection and sequencing methods. Till now, over 170 post-transcriptional RNA epigenetic modifications have been identified on mRNAs, tRNAs, rRNAs and other non-coding RNAs [2]. Similar to that highly diverse distribution, the functions of those modifications varied from genetic information translation to biomacromolecule regulation processes, representing a relatively new angle for the illumination of control mechanisms in biologic pathways [3]. To fully elucidate the detailed function of each modification in the development of cellular events or human disease, a precise regulation of a particular RNA variant is highly desirable. Nonetheless, the manipulation of a specific RNA modification, even in large quantity, is a laborious and time-consuming task. The use of genetic approaches and the identification of small molecular inhibitors/antagonists towards RNA-modifying proteins (RMPs) is considered as a promising strategy for the modulation of the corresponding RNA modifications. It has shown great potency for drug discovery in association with cancer-related epigenetic alteration [3]. However, this indirect intervention strategy heavily depended on the characterization of RMPs [4]. For those epigenetic change whose modifying enzymes have not been identified, this pathway becomes extremely difficult. Another constraint with RMP-related approach is that one enzyme may catalyze the modulation reactions with multiple substrates. For example, the perturbation of a methyltransferase may cause a simultaneous variation to several epigenetics, making it challenging to link the phenotypic changes to a specific modification.

One alternative for the abovementioned approaches is to explore direct intervention of designated RNAs with precise and bioorthogonal transformations [5]. All modified residues characterized in cellular RNA molecules are unexceptionally produced by ingenious chemical reactions (such as alkylation, carbonylation, oxidation and hydrolysis) with the assistance of dedicated RMPs (Figure 1a). For example, the simple methylation is actually an enzymatic-catalyzed base-promoted S_N2 alkylation with SAM (Figure 1b) [6], while the coordination chemistry and the non-heme iron^{IV} species provide the essential mechanistic machinery of the oxidative demethylation (Figure 1c) [7]. Consequently, it is reasonable that sophisticated biocompatible chemical transformations may be used for biomimetic alternation of a given RNA substrate. However, current concepts of bioorthogonal chemistry predominately relied on pre-installed bioorthogonal handles (like alkynes, azides) within biomacromolecules [8]. It remains a huge challenge to establish new bioorthogonal reactions (or more precisely, biocompatible transformations) that are inert to naturally occurring functionalities and yet able to recognize their RNA substrates with high specificity. Such precise epigenetic reorganization strategies, if possible, will significantly empower chemical biologists with the ability in the recognition of detailed functions of epigenetic modifications in the native cellular environment.

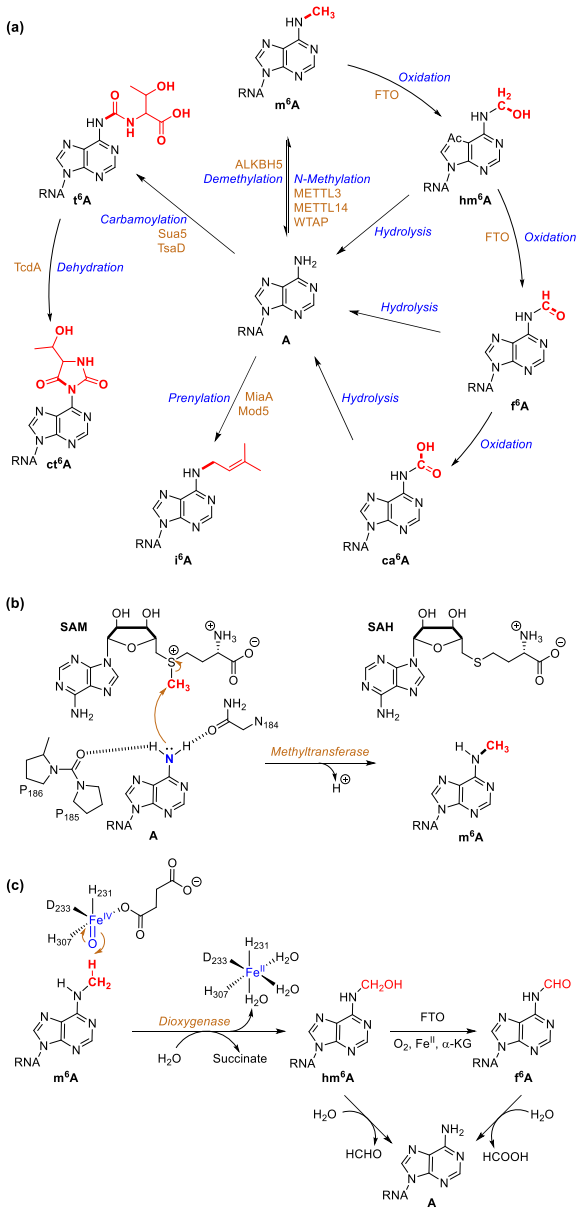

Figure 1. (a) Biochemical pathways for selected epigenetic modifications within adenosine; (b) Methyltransferase METTL16-catalyzed N^6-methylation of adenosine to produce m^6A; (c) Demethylase FTO-catalyzed demethylation of m^6A affording adenosine *via* two oxidative intermediates hm^6A and f^6A.

On this basis, we will present our recent advances regarding the direct chemical interventions towards several biologically important RNA modifications and their applications in live cells or growing plants (Figure 2). The design, synthesis and evaluation of highly efficient chemical reagents and biocompatible transformations has enabled us to not only control *in vitro*/*in vivo* RNA modifications, but also introduce artificial variants into natural RNAs, without disturbing any related enzymes. Such double direction regulating may considerably help in the function interpretation of a given RNA modification. Another application of the approach is that it may provide context and guidance to researchers seeking novel techniques in their own domains, such as high-resolution sequencing.

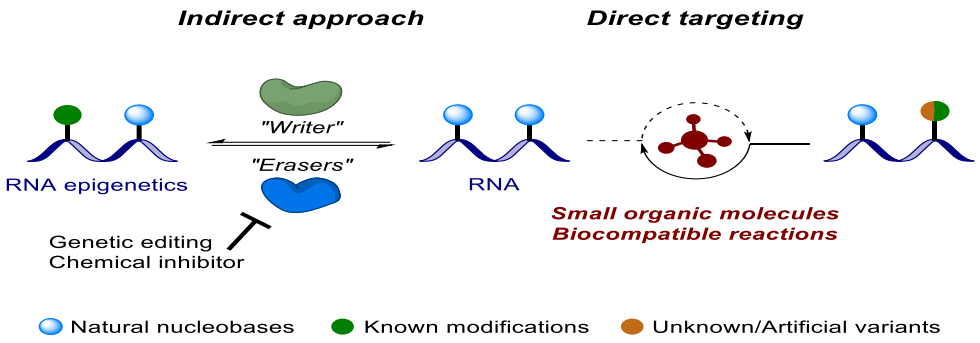

Figure 2. Indirect (left) and direct (right) interventions to RNA modifications.

2. OXIDATIVE DEMETHYLATION OF m^{6}A

N^6-Methyladensoine m^{6}A is one the most abundant internal modifications in RNAs, presenting in 1~4‰ of all adenosines in global cellular RNAs and accounting for more than 80% of all RNA base methylations [9]. It mainly distributes in mRNAs and occurs in non-coding RNAs, such as rRNAs and tRNAs. Substantial studies show that abnormal m^{6}A methylation levels may affect RNA metabolism processes and hence lead to RNA dysfunction and diseases. For example, decreased levels of m^{6}A caused by increased FTO activity contribute to the onset of obesity and other diseases [10], while increased levels of m^{6}A methylation can be associated with the progression of human abdominal aortic aneurysm and human brain developing [11-12]. The chemical process of m^{6}A creation and decomposition in RNA (Figures 1b-c), *i.e.*, the installation of methylation onto *N*^{6}-poisition as well as its removal, has been fully investigated. The dynamic combination of RMPs like methyltransferases (for example, METTL3) or demethylases (FTO or ALKBH5) ensures a balanced equilibrium of m^{6}A modification in the transcriptome. Therefore, considerable efforts have been devoted to the exploration of small organic molecules as useful inhibitors/antagonists of designated RMPs for potential therapeutics. Strictly speaking, such activation or inhibition can only partially intervene m^{6}A, given the fact that those RMPs may target multiple modifications [13]. In this regard, direct alternations enabling selective methylation (if possible) or demethylation of adenosine residues are much more promising.

In recent years, numerous oxidizing combinations have emerged for the selective functionalization of methyl groups attached to nitrogen atoms [14]. These site-selective functionalization of C-H bonds afforded abundant tools to synthetic chemists in the editing of existing molecules such as complex natural products. Nonetheless, distinguishing the simple methyl substituents in RNA epigenetics remains an extraordinary challenge due to the small gap between the bond dissociation energies within the nucleosides and the fragility of the phosphate linkages within the nucleotides. Prof. Zhou pioneered the use of ammonium bicarbonate-activated hydrogen peroxide solution (NH_4HCO_3-H_2O_2), which has long been utilized in the oxidation of tertiary amines or pyridine analogs, towards the oxidation of m^{6}A nucleosides (Figure 3a) [15]. Similar to the enzymatic demethylation process, this new chemical method generated two known intermediates, hm^{6}A and f^{6}A, as well as an unprecedented oxm^{6}A. In addition, a decrease of 10% in m^{6}A/A ratio was observed when HeLa cells were incubated with the chemical's combination, indicating that the generated hydroxyl radical (HO•) indeed attacked the methyl groups and thus demethylated m^{6}A *in vitro*.

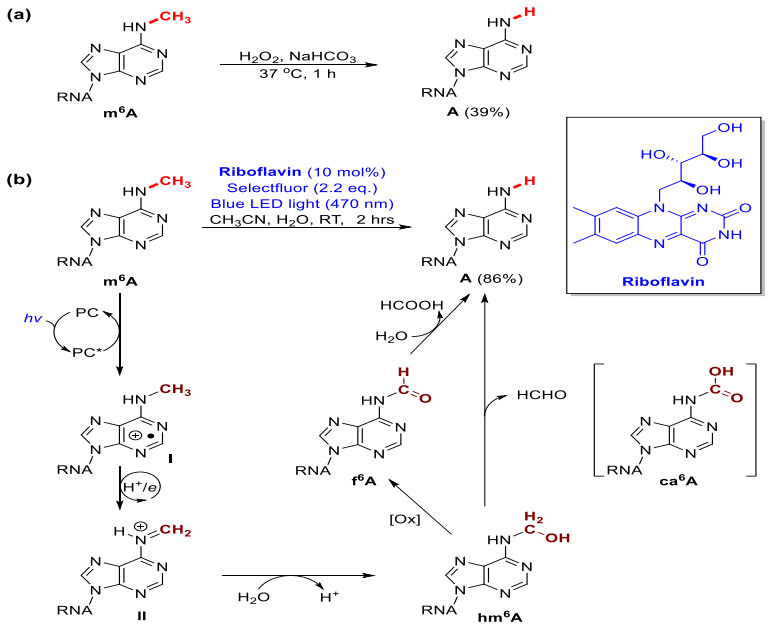

Figure 3. (a) Oxidative demethylation of m^{6}A with ammonium bicarbonate-activated hydrogen peroxide solution; (b) Riboflavin (PC) promoted oxidative demethylation of m^{6}A under the irradiation of blue light and the proposed mechanism for the selective *N*^{6}-methyl activation.

Inspired by above-mentioned work and others [16-18], we were determined to develop an alternative strategy to activate the sp^3 C-H bonds without generating toxic ROS. In 2017, we reported a simple protocol for the oxidative demethylation of m^{6}A and 6mA modifications with Riboflavin (vitamin B_2), and a common redox cofactor in natural systems, under the irradiation of blue LED light ($\lambda = 470$ nm) with the assistance of external oxidant - Selectfluor (Figure 3b) [19]. This photo-induced oxidative demethylation exhibited excellent efficiency with m^{6}A (100% conversion within 2 hours, and isolated yield of adenosine as 86%), without effecting other epigenetic modifications, including m^{5}C, A_m and the very similar m^{1}A. We demonstrated that the artificial demethylation of m^{6}A was initiated by the novel light-driven system, from which the adenosyl radical cation **I**, an opened-shell cationic species was generated upon one electron removal with excited Riboflavin (PC*). The charge was presumably delocalized over the whole nucleoside. However, **I** in principle existed instantaneously in a localized structure, with the *N*^{6} bearing most of the charge, and maintaining the aromaticity in the adenosine. The unpaired electron made the "*inert*" *N*-methyl group chemically reactive prone to hydrogen abstraction, and yield the iminium cation **II**. The latter was an excellent electrophile amenable to interception by water to produce the key oxidation intermediate - hm^{6}A. Interestingly, we found a new intermediate ca^{6}A over the course of further oxidation of hm^{6}A to f^{6}A. It was proposed that ca^{6}A might be involved in the FTO/ALKBH5 mediated process to modulate RNA-protein interactions [7]. Nevertheless, it has not been captured until the introduction of non-enzymatic transformation to demethylation. All of those metastable intermediates are hydrolytically decomposed in the water, concluding the demethylation process and producing the natural adenine.

One limitation of the above study was that Selectfluor in aqueous solution caused significant decomposition of RNA. Therefore, we further studied the potential of using the cellular oxidizing agents - oxygen (O_2) for the artificial demethylation (Figure 4) [20]. Riboflavin was capable of promoting the oxidative demethylation with atmospheric oxygen, but in an extremely low efficiency (37% of m^{6}A demethylation after 48 hours). Considering that the isoalloxazine ring in riboflavin is responsible for its photophysical and photochemical properties, we envisioned that changes at the isoalloxazine would prudently adjust its redox capacity. Thus, we synthesized and screened a bunch of riboflavin derivatives 1-10 with diverse substituents at C-7 and 8 positions of the isoalloxazine ring (Figure 4a). It turned out that the introduction of either strongly electron-withdrawing or donating substituent groups exerts a profound influence upon both the physical (solubility, for example) and photochemical properties (Figure 4b). Most of these derivatives resulted in a decreased oxidizing ability except Lumichrome **1**, which was later suspended from further optimization due to its poor solubility in water. Interestingly, *N*-10 substituents on the isoalloxazine ring (**11-12**, FAD and FMN) that may influence the protonation status under physiological conditions greatly improved the catalytic efficiency. FMN, the 5'-phosphate of riboflavin as well as the prosthetic group of various oxidoreductases, was an excellent biocompatible blue-light photocatalyst for the demethylation of m^{6}A (79% of isolated adenosine after 6 hours). Consequently, it was chosen as the optimal regulator, and did function

as a true demethylase mechanistically similar to FTO or ALKBH5, converting all m^6A into hm^6A and f^6A rapidly (Figure 4c), and yet with surprisingly high substrate specificity (Figure 4d). FMN exhibited remarkable recognition towards designated substrates - N^6-methylated adenosine derivatives (m^6A, $m^{6,6}A$, and 6mA), from other structurally similar modifications. Regio-isomers of m^6A like m^1A or A_m, were all untouched under the standard conditions. Additionally, other common RNA modifications like 5-methyl uracil m^5U or m^5C were also unaffected. Investigations are still ongoing and the reason for the high discrimination of FMN remains unclear. One possibility was that FMN recognizes the adenosines *via* Hoogsteen base interaction, similar to A-U base pairing with its third ring resembling uracil [21]. We further confirmed that FMN would induce bioorthogonal demethylation in live cells (Figure 4e). Control experiments of incubating HeLa cells with FMN demonstrated trivial change of m^6A, indicating that FMN did not interfere the methylation process by activating or inhibiting the methyltransferases or demethylases, or disturbing other intracellular biomacromolecules. In addition, it was capable of demethylating *ca.* 53% of m^6A in genomic RNAs without any noticeable apoptosis. To the best of our knowledge, this is one of the most significant methods useful in the reduction of m^6A levels using chemical or enzymatic means. Besides, we found that FMN was also able to downregulate excess m^6A in methyltransferase overexpressed cell lines (Figure 4f). The combination of FMN and blue light irradiation considerably promoted the decline of m^6A in METTL3 over-expressed 293T cells (*ca.* 34%). Together, these findings revealed that FMN is the first cell-active and artificial demethylase of m^6A RNAs.

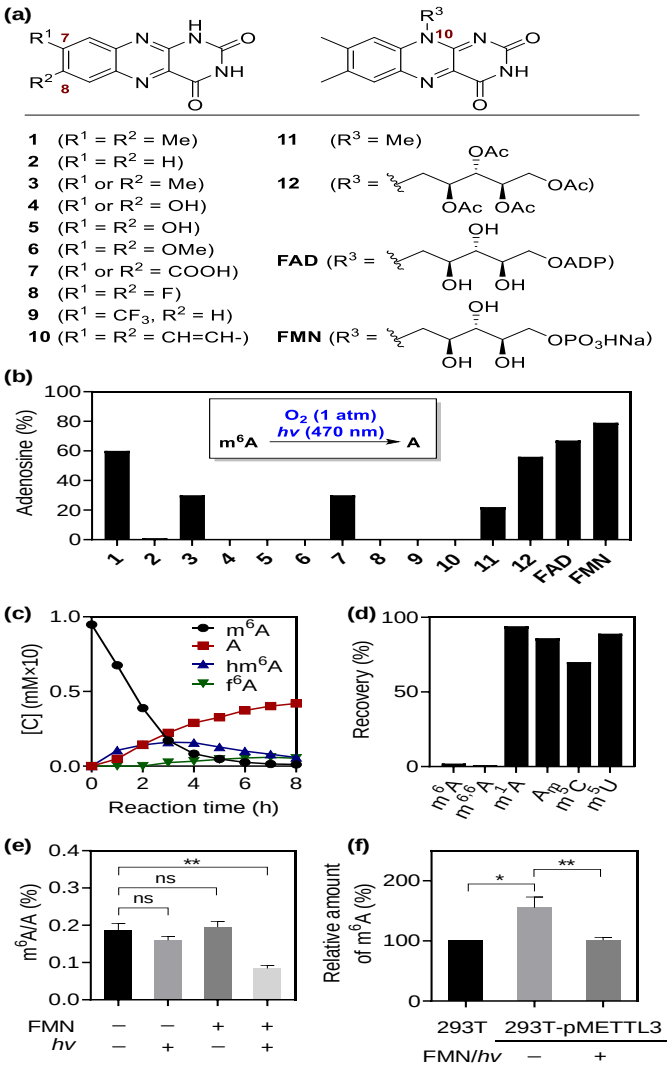

Figure 4. (a) Structures of natural and synthetic isoalloxazine derivatives; (b) Demethylation evaluation with m^6A and oxygen under blue LED light irradiation. Y axial referred to isolated adenosine; (c) Kinetic profiles of the oxidative demethylation promoted by FMN; (d) Epigenetic modification selectivity of FMN under the standard conditions. Y axial referred to isolated modifications; (e) Determination of m^6A/A in genomic RNAs of HeLa cells with or without

FMN/irradiation; (f) Determination of relative m^6A/A in genomic RNAs of wide type and METTLE-overexpressed 293T cells with or without FMN/irradiation. Figures (c)-(f) reproduced with permission from ref. 21. Copyright 2019 John Wiley and Sons.

3. OXIDATIVE DEPRENYLATION OF i^6A

Prenylation (or isopentenylation) is one of the ubiquitous post-translational modifications in numerous proteins, where the extremely large and hydrophobic substituent helps to facilitate protein-protein interactions (PPIs) [22]. Interestingly, the same motif has only been discovered at the N^6-position of A_{37} (i^6A) in a few tRNAs from bacteria and eukaryotes. As one of the hypermodified epigenetics, i^6A is believed to be the precursor of ms^2i^6A and ms^2io^6A, which together with other bulky modifications like t^6A or ms^2t^6A, acted in divergent regulatory manners on physiological circuits within the cell [23]. However, the mechanism of action pertaining to i^6A remains unclear, which is largely because there has been little success in the identification of biocompatible probes to specifically bind to and alter the prenyl substituent.

Inspired by the structural characteristics of i^6A possessing both *N*-alkyl and dimethyl allylic functionalities, we proposed a new strategy for a chemical deprenylation of this motif (Figure 5a) [24]. We designed and synthesized a variety of electron-deficient reagents that may undergo reversible nucleophilic addition with i^6A to afford the adduct **III**. With the assistance of adjacent allylic group, the charge-separated intermediate undertook intramolecular and irreversible hydrogen abstraction to generate the α,β-unsaturated imine **IV**, which was quickly hydrolyzed to remove the substituent while releasing 3-methyl-2-butenal as the side product. Other common *N*-modification types such as *N*-acetyl or *N*-methyl, which could not initiate either the addition step or the H-elimination, should be untouched in this circumstance. Indeed, we discovered that oxoammonium cation **13** derived from TEMPO met all the criteria and exhibited the highest efficiency to deliver the deprenylated adenosine quantitatively. As our expectation, it showed excellent discrimination between i^6A and m^6A (Figure 5b). Canonical and non-canonical nucleic acids, such as A_m, m^5C, f^5C and the saturated analogue iPH^2A were all unaffected. hm^5C was slightly converted to f^5C, but in an extremely slow pace. It is worth to mention that most tested amino acid residues, even those likely to be oxidized by mild oxidants (Ser, Thr and Met), remained unchanged. Interestingly, natural products (9-Prenyladenine and Imperatorin) possessing identical isopentenyl substituent were inert substrates under the same conditions, which proved the advantage of this artificial deprenylation in high sensitivity and selectivity for i^6A, and low response for other functionalities in biomacromolecules. Moreover, the i^6A oligo abstracted from $tRNA^{Met}$ fragment treated with **13** (40 equiv.) showed a significant decrease after 2 hours and the appearance of the generated new peak was comparable with authentic RNA, confirming the very limited impact of the compound on phosphate linkage in nucleic acids (Figure 5c).

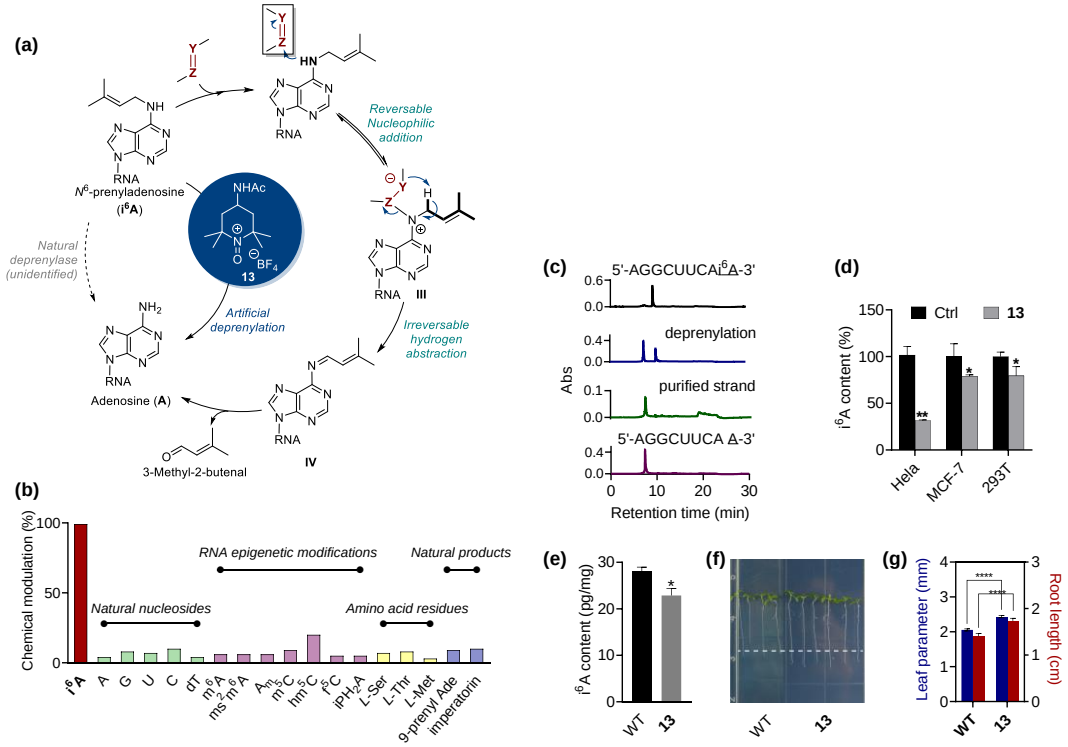

Figure 5. (a) Design and proposed pathway of chemical deprenylation of i^6A, and the optimized structure of oxoammonium cation 13; (b) Substrate selectivity of **13** towards nucleic acids, amino acid residues and natural products; (c) HPLC traces of i^6A RNA and treatment with **13**; (d) i^6A contents in different cell lines when treated with **13**; (e) i^6A contents in wide type (WT) and **13** treated *Arabidopsis thaliana* seeds; (f) Phenotypic changes of WT and **13** treated *Arabidopsis thaliana* seeds; (f) Leaf widths and root lengths of WT and **13** treated *Arabidopsis thaliana* seeds. Figures (b) and (f) reproduced with permission from ref. 24. Copyright 2020 John Wiley and Sons.

The free nucleoside i^6A exists as a naturally occurring cytokinin, regulating cell division, gene expression, and differentiation in plants [25]. After satisfactory implementation of the oxoammonium cation **13** as selective chemical deprenylation reagent, pilot screening was carried out by testing the compound for *in vitro*/*in vivo* efficiency in the 24-well plate culture system.[4] Cells treated with up to 100 μM of **13** were normally shaped after 72 hours, implying an extremely low toxicity. However, a marked decrease (up to 60%) in the number of free i^6A was observed (Figure 5d). With this strong downscaling, consideration was given to testing the effectiveness of this compound in reducing the i^6A of live plants. Conventional *in vivo* techniques (such as overexpression, knockout, and knock-in models) towards prenyltransferases have produced a demonstrable impact on the phenotype. However, it is time-consuming and expensive compared with the chemical probe approach. In addition, the i^6A-related RMPs are a group of closely related gene members with functional redundancy, and the duration and specificity of one gene knockout towards i^6A is limited, which may result in a complete lack of connection. Our compound **13**, on the other hand, proved its capability of modulating endogenous i^6A (Figure 5e) in live plants for the first time, with a significant impact on the morphological traits (leaf and root growth) (Figures 5f-g). Considering the limited impact of the compounds towards biomacromolecules and the robust preparation of this molecule, we believe it is the first time in plant sciences that an artificial epigenetic alternation system could modulate *Arabidopsis thaliana* seedling growth under real conditions and even on economically important crops. The major advantages of this approach include its straightforward operation, inexpensive cost, as well as ability of achieving high levels of i^6A reduction and avoiding genetic alternation.

4. OXIDATIVE POST-MODIFICATION OF m^5C

5-Formylcytidine f^5C was a natural nucleoside that was discovered in mitochondrial tRNAs from a large number of species [26]. The electron-withdrawing formyl group enabled an exceptional shift in the equilibrium of cytidine from common amino-oxo to less stable imino-oxo tautomer, which interacted with adenosine (A^3) and thus decoded the non-universal AUA codon [27]. Inspired by this essential role of 5-formyl modification as a structural signature in the translation process, numerous f^5C-sensitive probes have been developed during the past few years for *in vivo* visualization and detection [28]. Considering the low overall f^5C occurrence in RNAs (0.0001-0.002% of all Cs), the enrichment of f^5C through an artificial m^5C post-modification would significantly benefit the analysis accuracy. Besides, the temporally alteration of f^5C would be valuable both for basic understanding of its biological function and the role of its amount changes in disease states. Current technique heavily depended on the manipulation of ALKBH1, the α-KG and Fe^{II}-dependent dioxygenase, which is responsible for oxidizing m^5C in total RNA of mammalian cells. However, enzymatic post-oxidation of the methyl group in m^5C produces another important epigenetic mark hm^5C, which promotes translation of specific mRNAs in basic cellular processes and embryogenesis [29]. Modulation of the dioxygenase *via* mutation is a powerful means of modulating its cellular function and enzymatic activity. However, a selective f^5C dioxygenase was still not realized.

Instead of that sluggish process, we envisioned that small organic molecules might serve as selective promotors for this purpose [30-34]. We established a sensitive labelling and detection assay for f^5C based on a blue-light facilitated cyclization with 2-aminothiophenol [35], and then conducted a high throughput screening to identify potential artificial post-oxidases. A significant challenge encountered in this process was that several small molecule probes nonspecifically activated the chemically inert methyl substituent in m^5C, as well as other methyl variants, such as m^5U and m^6A. An ideal candidate should also possess the ability of generating the desired formyl product, without pre-mature intermediate hm^5C or hyper-oxidized byproduct ca^5C. One of the anthraquinone derivatives, sodium 2-anthraquinonesulfonate, or SAS, turned out to be the most potent artificial post-oxidases of m^5C under the irradiation of ultra-violet light (Figure 6a). Of hundreds of molecules that we screened, SAS had an unprecedented ability to discriminate m^5C/5mC from other epigenetic methyl modifications (Figure 6b). This compound's post-modifying selectivity over other epigenetic variants especially m^5U, and its preferential generation of f^5C (isolated yield 67%) with extremely few hm^5C (less than 0.5%, Figure 6c), are both excellent properties that might contribute to minimizing unwanted side effects when, and if, it is used as a starting point for the next step in the *in vivo* processes. Interestingly, the amount of hm^5C followed a parabola curve (Figure 6c), but it can not be converted to f^5C under the standard conditions, indicating that it might not be the precursor towards f^5C and the two were generated in distinct pathways. The methyl discrimination ability of SAS is still in process. However, we proposed

that the oxidation stated from the photo-induced excitation of the molecule, which can oxidize m^5C forming a radical **V** (or initially a radical-cation followed by rapidly deprotonation) (Figure 6d). The primary cytidine methyl radical is a target for oxygen and undergoes rapid reaction to give peroxyl radical **VI**. This was believed to be the key intermediate in the process. Subsequent decay of the peroxyl radical *via* the Russell mechanism [36] would yield the hm^5C, which was inert under the circumstances to further oxidation. Otherwise, the peroxyl radical predominately abstracted one electron and one hydrogen to generate hydroperoxide oxm^5C, which conceded the formyl product f^5C upon decomposition.

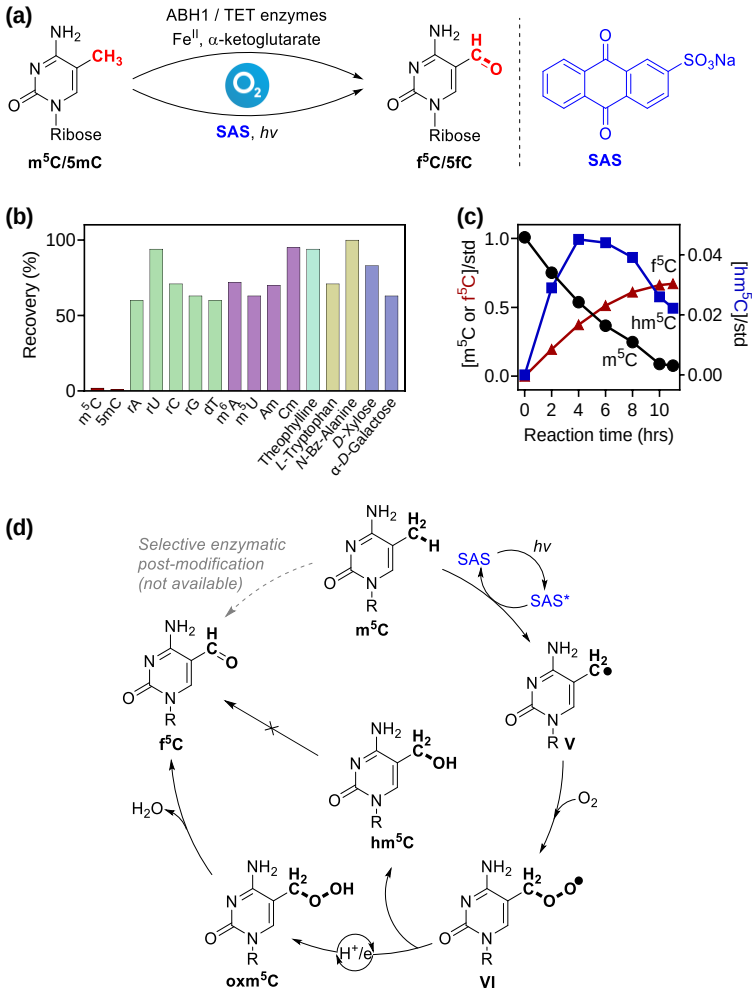

Figure 6. (a) Enzymatic post-oxidation of m^5C with ALKBH1 and the chemical manipulation with small organic molecule SAS to generate f^5C; (b) Substrate discrimination of SAS with m^5C/5mC under the standard conditions; (c) Kinetic profiles of the decomposition of m^5C, production of f^5C and hm^5C; (d) Proposed mechanism for the artificial oxidation of m^5C. Figures (b) and (c) reproduced with permission from ref. 3. (b) Copyright 2019 John Wiley and Sons.

5. CONCLUSION

Modifications to RNA molecules are critical for the regulation of diverse biological processes. The capacity of precisely manipulating an epigenetic mark of interest in living cells could help clarify its thorough functions and regulatory mechanisms. It also has the potential for the treatments of human diseases related to genetic alternations. At present, genome editing has enabled a precise manipulation of nucleic acid sequences and brought about desired genetic changes at will *in vitro* and *in vivo*. With the assistance of powerful CRISPR/Cas9 toolbox, scientists have achieved the artificial altering of m^6A without underlying RNA sequences. [37-39]. Nonetheless, in order to genetically edit the designated variant, an engineered dCas-RMP fusion should be equipped, which considerably limited its *in vivo* application if no RMP has been identified or multiple RMPs were involved.

Small organic molecules and related biocompatible transformation have proven to be invaluable chemical tools for the investigation of biological processes of interest. However, their application in epigenetic research is still in its infancy due to the target specificity. RMPs antagonists/inhibitors are powerful instruments for pinpointing specific enzyme

activities in natural circumstance. Nonetheless, only a few specific molecules have been disclosed (e.g., inhibitors for dioxygenases and antagonists for methyltransferases). As a result, their use are rather limited. Furthermore, chemical molecules and reactions mimicking RMPs have gained plentiful interest as potentially feasible alternatives to current approaches. In addition with modifying activities and efficiencies comparable to their enzymatic counterparts (if they exists), these artificial enzymes possess preferred benefits such as low cost, outstanding tolerance in physiological environments, and most importantly, tunable structures with easy optimization and preparation [40]. Although in the early days of development, impressive advances have already been made. Those artificial m^6A demethylase, i^6A deprenylase and m^5C oxidases have presented immense potential in the removal and post-modification of specific RNA epigenetics. Undoubtedly, much bigger challenges lie ahead. For instance, oxidoreductases is the only class of RMPs that current molecules can mimic. Group transfer modifications (methylation, prenylation), isomerization (uridine to pseudouridine), ligase reactions (RNA-peptide conjugation), and numerous other RNA epigenetic modifications are far beyond our ability. On this basis, high-throughput screening are needed. Another limitation of current molecules refers to their lack of discrimination of a given chemical modification in a specific location. This can be challenging when using those structures either as therapeutic agents or as biological probes. A global alternation without location narrowness might cause unexpected toxicity and misinterpretation of their effects, thus preclude the development of promising candidate moving forward to clinic trails or even into a drug. To overcome that, it would be preferable to identify and utilize binding motif (small organic binders or complementary sequences) to enhance the functional delivery and shuttle the functional motif to designated locations. Therefore, we may have a better understanding of the multifaceted roles of each epigenetic modification that are vital for life and death. The challenge is huge, but that day will come eventually.

ACKNOWLEDGMENT

I sincerely thank all past and present members of my research group for their hard work and dedication over the past decade. This work was supported by the National Key R&D Program of China (2020YFA0707901), National Natural Science Foundation of China (22022704, 21977097, and 22271291) and Chinese Academy of Sciences.

References

1. F. F. Davis, F. W. Allen, *J. Biol. Chem.*, 227, 907-915. **(1957)**
2. Modomics database: https://iimcb.genesilico.pl/modomics/modifications.
3. T. O. Tollefsbol, *Handbook of Epigenetics*; 2nd Ed. Elsevier/Academic Press: London, **(2017).**
4. R. Esteve-Puiga, A. Bueno-Costa, M. Esteller. *Cancer Lett.*, 474, 127-137. **(2020)**
5. M. Roovers, J. Wouters, J.M. Bujnicki, C. Tricot, V. Stalon, H. Grosjean, L. Droogmans. *Nucleic Acids Res. 32*, 465–476. **(2004)**
6. E.M. Sletten, C.R. Bertozzi. *Acc. Chem. Res.* 2011, 44, 666-676. **(2011)**
7. A. Ruszkowska, M. Ruszkowski, Z. Dauter, J.A. Brown. *Sci. Rep.*, 8, 5311-5324. **(2018)**
8. Y. Fu, G.F. Jia, X.Q. Pang, R.N. Wang, X. Wang, C.J. Li, S. Smemo, Q. Dai, K.A. Bailey, M. A. Nobrega, K.L. Han, Q. Cui, C. He. *Nat. Commun.*, 4, 1798-1806. **(2013)**
9. B.R. Stockwell. *Nature, 432*, 846-854. **(2004)**
10. C.M.Wei, A. Gershowitz, B. Moss. *Cell*, *4*, 379-386. **(1975)**
11. B.J. Zhang, H. Jiang, Z. Dong, A.J. Sun, J.B. Ge. *Genes Dis.* **(2020)**
12. K. J. Yoon, F. R. Ringeling, C. Vissers, F. Jacob, M. Pokrass, D. Jimenez-Cyrus, Y.J. Su, N.S. Kim, Y.H. Zhu, L. Zheng, S. Kim, X.Y. Wang, L.C. Doré, P. Jin, S. Regot, X.X. Zhuang, S. Canzar, C. He, G.L. Ming, H.J. Song. *Cell*, *171*, 877-889. **(2017)**
13. Y. He, J. Xing, S. Wang, S. Xin, Y. Han, J. Zhang. *Ann. Transl. Med.*, *7*, 797. **(2019)**
14. L.J. Xie, L. Liu, L. Cheng. *Biochemistry*, *59*, 230-239. **(2020)**
15. L. Shi, W.J. Xia. *Chem. Soc. Rev.*, *41*, 7687-7697. **(2012)**
16. J.J. Wu, H. Xiao, T.L. Wang, T.T. Hong, B. Fu, D.S. Bai, Z.Y. He, S. Peng, X.L. Xing, J.L. Hu, P. Guo, X. Zhou. *Chem. Sci.*, *6*, 3013-3017. **(2015)**
17. T. Endo, J. Zemlicka. *J. Org. Chem.*, *44*, 3652-3656. **(1979)**
18. T. Kato, S. Ogawa, I. Ito. *Tetrahedron Lett.*, *22*, 3205-3208. **(1981)**
19. M. Sako, T. Makino, Y. Kitade, K. Hirota, Y. Maki. *J. Chem. Soc., Perkin Trans. 1*, 1801-1805. **(1992)**
20. L.J. Xie, R.L. Wang, D. Wang, L. Liu, L. Cheng. *Chem. Commun.*, *53*, 10734-10737. **(2017)**

21. L.J. Xie, X.T. Yang, R.L. Wang, H.P. Cheng, Z.Y. Li, L. Liu, L.Q. Mao, M. Wang, L. Cheng. *Angew. Chem. Int. Ed.*, *58*, 5028-5032. **(2019)**
22. X.Y. Jin, R.L. Wang, L.J. Xie, D.L. Kong, L. Liu, L. Cheng. *Adv. Synth. Catal*., *361*, 4685-4690. **(2019)**
23. H.P. Cheng, X.H. Yang, L. Lan, L.J. Xie, C. Chen, C. Liu, J. Chu, Z.Y. Li, T.Q. Zhang, D.Q. Luo, L. Cheng. *Angew. Chem. Int. Ed.*, *59*, 10645-10650. **(2020)**
24. M. Raszka, N.O. Kaplan. *Proc. Natl. Acad. Sci. USA.*, *71*, 4546-4550. **(1974)**
25. F.L. Zhang, P.J. Casey. *Annu. Rev. Biochem.*, *65*, 241-269. **(1996)**
26. U. Schweizer S. Bohleber N. Fradejas-Villar. *RNA Biol.*, *14*, 1197-1208. **(2017)**
27. D.W.S. Mok, M.C. Mok. *Cytokinins: Chemistry, Activity, and Function*; CRC Press: Boca Raton, **(1994)**.
28. J. Moriya, T. Yokogawa, K. Wakita, T. Ueda, K. Nishikawa, P.F. Crain, T. Hashizume, S.C. Pomerantz, J.A. McCloskey, G. Kawai, N. Hayashi, S. Yokoyama, K. Watanabe *Biochemistry*, *33*, 2234-2239. **(1994)**
29. H. Lusic, E.M. Gustilo, F.A. Vendeix, R. Kaiser, M.O. Delaney, W.D. Graham, V.A. Moye, W.A. Cantara, P.F. Agris, A. Deiters. *Nucleic Acids Res.*, *36*, 6548-6557. **(2008)**
30. H.P. Jiang, T. Liu, N. Guo, L. Yu, B.F. Yuan, Y.Q. Feng. *Anal. Chim. Acta*, *981*, 1-10. **(2017)**
31. B. Delatte, F. Wang, L.V. Njoc, E. Collignon, E. Bonvin, R. Deplus, E. Calonne, B. Hassabi, P. Putmans, S. Awe, J. Wetzel, R. Kreher, C. Soin, P.A. Creppe, C. Limbach, V. Gueydan, A. Kruys, S. Brehm, C. Minakhina, M. Defrance, R. Steward, F. Fuks. *Science*, *351*, 282-285. **(2016)**
32. C. Bienvenu, J.R. Wagner, J. Cadet. *J. Am. Chem. Soc*., *118*, 11406-11411. **(1996)**
33. H. Yamada, K. Tanabe, S. Nishimoto. *Bioorg. Med. Chem. Lett*., *15*, 665-668. **(2005)**
34. H. Yamada, K. Tanabe, T. Ito, S. Nishimoto. *Chem.- Eur. J*., *14*, 10453-10461. **(2008)**
35. R.L. Wang, X.Y. Jin, D.L. Kong, Z.G. Chen, J. Liu, L. Liu, L. Cheng. *Adv. Synth. Catal*., *361*, 5406-5411. **(2019)**
36. S.L. Khursan. In *PATAI'S chemistry of functional groups*; Marek, I., Ed.; John Wiley & Sons, Ltd, **(2014)**.
37. X.Z. Liu, J. Zhou, Y. Mao, Q. Ji, S.B. Qian. *Nat. Chem. Biol*., *15*, 865-871. **(2019)**
38. J. Li, Z. Chen, F. Chen, G. Xie, Y. Ling, Y. Peng, Y. Lin, N. Luo, C.M. Chiang, H. Wang *Nucleic Acids Res*., *48*, 5684-5694. **(2020)**
39. Wilson, C.; Chen, P. J.; Miao, Z.; Liu, D. R. *Nat. Biotechnol*., *38*, 1431-1440. **(2020)**
40. Breslow, R. *Artificial Enzymes*; WILEY-VCH Verlag GmbH & Co. KGaA: Weinheim, **(2005).**

Received: 4th April 2023 **Accepted: 1th June 2023**