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Hydrophobic tagging-induced degradation of NAMPT in leukemia cells

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ABSTRACT

Nicotinamide phosphoribosyl transferase (NAMPT) is considered as a promising target for cancer therapy to its crucial role in cancer metabolism. Despite the therapeutic potential of NAMPT enzymatic inhibitors, their effectiveness is limited by dose-related toxicity and the inability to suppress nonenzymatic functions of extracellular NAMPT (eNAMPT). Herein, we designed and synthesized the first hydrophobic tagging NAMPT degraders. Among them, compound **NH-11** selectively degraded NAMPT in leukemia cells through the ubiquitin-proteasome system. Compound **NH-11** effectively induced apoptosis and showed low toxicity to normal cells, representing a promising anti-leukemia lead compound.

Nicotinamide phosphoribosyl transferase (NAMPT) is expressed in nearly all cells and involves in the biosynthesis nicotinamide adenine dinucleotide (NAD), a vital coenzyme for NAD-dependent enzyme (Fig. 1A) [1–5]. By regulating the intracellular NAD concentration, NAMPT orchestrates the critical biofunctions, such as adaptive responses to inflammatory, mitochondrial biogenesis and cellular metabolism [6–8]. However, NAMPT also implicates in the development of aging, obesity, nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM) [9–12]. Beyond these functions, NAMPT plays a pivotal role in the metabolism of many cancers including leukemia, colorectal, ovarian, breast, gastric, and so on [13]. In addition to its enzymatic function, extracellular NAMPT (eNAMPT) could also be secreted out of the cells and has cytokine-like activity [14,15]. Abnormal levels of eNAMPT engaged in tumor progression by modulating angiogenesis, remodeling tumor immune microenvironment, and promoting the proliferation, migration, and epithelial–mesenchymal transition (EMT) of tumor cells [3,16,17].

It has been demonstrated that the enzyme activity of NAMPT can be inhibited by small-molecule inhibitors. A few NAMPT specific inhibitors, such as OT-82, CHS828 and FK866, have entered clinical trials for cancer therapy [18,19]. Despite encouraging preclinical evidence of the potential utility of these inhibitors in cancer models, none of them is able to progress to the later stages of clinical trials owing to the limited anti-tumor efficacy [13,20]. One possible reason is that the inhibition of intracellular NAMPT enzymatic activity may not be sufficient to fully impair the oncogenic function of NAMPT because the roles of eNAMPT are enzymatically-independent [14]. Therefore, the adaptation of additional tactics to boost the efficacy of NAMPT blockage is highly desirable.

Targeted protein degradation (TPD), represented by proteolysis-targeting chimera (PROTAC), is an emerging therapeutic strategy in drug development [21–24]. Although PROTACs have numerous advantages such as catalytic mechanism, avoiding drug resistance and tackling undruggable targets, clinical development of PROTACs is still limited by high molecular weight, poor cellular permeability, unfavorable pharmacokinetics, and low bioavailability [25]. Hydrophobic tagging (HyT) technology presents an alternative strategy for TPD, which relatively has lower molecular weight and reduced the elimination of potential teratogenic side effects associated with E3 ligase ligands (*e.g.* thalidomide-derived cereblon ligands) [26,27].

A typical HyT molecule consists of an identifying peptide or small-molecule ligand that specifically binds to the protein of interest (POI), and a hydrophobic tag that mimics a misfolded protein state, thus leading to the degradation of protein by recruiting chaperones or proteasomes [28,29]. A small number of hydrophobic tags (*e.g.* adamantane and fluorene) have been discovered and successfully employed in the design of HyT degraders [27]. However, the target scope, pharmacological activity and mechanism of

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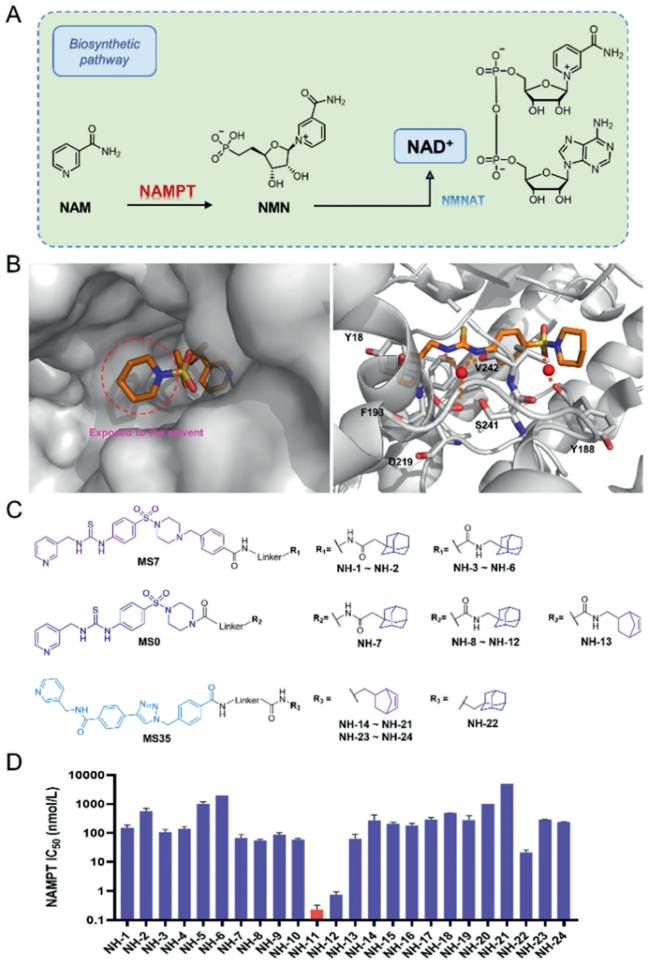

Fig. 1. Rational design and screening of hydrophobic tagging NAMPT degraders. (A) The biosynthetic pathway of NAMPT. (B) Predicted binding model of **MS0** with NAMPT (PDB: **4JR5**). (C) The chemical structures of NAMPT inhibitors **MS7**, **MS0**, **MS35** and designed degraders **NH-1**–**NH-24**. (D) NAMPT inhibitory activity of compounds **NH-1**–**NH-24**.

action remain largely unexplored. Previously, we confirmed that targeted protein degradation was an effective strategy to interfere with the function of NAMPT and designed the effective NAMPT degraders using the PROTAC approach [30,31]. Nevertheless, it is still unknown whether NAMPT could be effectively degraded by the HyT strategy. Herein, we designed the first HyT-mediated NAMPT degraders, which showed potent NAMPT degrading potency and selective anti-leukemia ability.

Previously, we identified potent NAMPT inhibitors (**MS7** and **MS0**) through high-throughput screening and structure-based drug design [32]. These compounds shown excellent *in vitro* inhibitory activity against NAMPT, offering favorable ligands for the design of NAMPT degraders. Binding mode analysis of **MS7** with NAMPT revealed that the benzyl piperazine was exposed to the solvent (Fig. 1B). Therefore, various linkers and HyT tags were introduced on the benzyl or piperazine group, affording compounds **NH-1**–**NH-13** (Fig. 1C). To further explore the effects of NAMPT ligands on the degrading activity, our previously identified inhibitor **MS35** [33] was also used to design HyT-based degraders **NH-16**–**NH-24** (Fig. 1C).

Chemical synthesis of the target compounds **NH-1**–**NH-13** was depicted in Schemes S1 and S2 (Supporting information). 4-Nitrobenzenesulfonyl chloride underwent substitution reaction with *tert*-butyl piperazine-1-carboxylate under basic conditions to obtain compound **3**. The nitro group of compound **3** was then reduced to the amino group in the presence of Pd/C and H_2, and the resulting product (compound **4**) was further reacted with sodium azide and 3-pyridylamine separately to obtain urea compound **6**. Compound **6** was deprotected by trifluoroacetic acid (TFA) to remove the Boc protecting group, resulting in key intermediate **7** (**MS0**). Compound **7** underwent substitution with methyl-4-(bromomethyl)benzoate, followed by the removal of the ester protecting group, to afford key intermediate **9** (**MS7**). Subsequently, different lengths of fatty chains were connected to adamantane to obtain linker containing hydrophobic tags **12a**-**b**, **15a**-**d**, **17**, and **19a**-**e**. These products were then coupled with key intermediate **7** or **9** to form target compounds **NH-1**–**NH-12**. Key intermediate **7** was also connected to the linker tags and then coupled with norbornene to obtain the target compound **NH-13**. The synthetic routes for target compounds **NH-14**–**NH-24** were shown in Schemes S3 and S4 (Supporting information). 4-Ethynylbenzoic acid and 3-(aminomethyl)pyridine were coupled under the *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and Et_3N conditions to obtain intermediate **24**. Intermediate **24** underwent click reaction with intermediate **25** to obtain key intermediate **26**. After hydrolysis, key intermediate **26** was connected to different linker tags, and then coupled with norbornene or adamantane group to obtain target compounds **NH-14**–**NH-24**.

Initially, the NAMPT inhibition activity of the designed HyT degraders were assayed to confirm whether they could bind with NAMPT binding. The results indicated that most compounds effectively inhibited NAMPT with half-maximal inhibitory concentration (IC_{50}) values in the nanomolar range except for compounds **NH-6**, **NH-20** and **NH-21** (Fig. 1D). Then, the NAMPT degradation ability of compounds **NH-1**–**NH-12** was detected by the Western blot analysis in A2780 cells, an ovarian cancer cell line that was widely used in the NAMPT degradation assay [34]. However, there was no obvious degradation effect on this cell line after exposure with 1 or 10 μmol/L compounds for 24 h (Fig. 2A). Thus, we further investigated the NAMPT degradation activity on various cancer cell lines including colon cancer (HCT-116), cervical cancer (HeLa), and leukemia cells (K562, Jurkat, HL60). Interestingly, significant degradation was only observed in leukemia cells, particularly for K562 and HL60 cells (Figs. 2B and C). Generally, **MS0**-based HyT degraders (**NH-7**–**NH-12**) were more potent than **MS7**-based HyT degraders (**NH-1**–**NH-6**), which also have relatively lower molecular weight. Among these compounds, **NH-11** showed the best NAMPT inhibitory and degrading activity. When the NAMPT ligand was replaced by **MS35**, the resulting compound (**NH-22**) had less activity to induce NAMPT degradation than compound **NH-11**. Recently, norbornene was discovered to be an effective HyT tag and was successfully employed in the design of HyT degraders targeting anaplastic lymphoma kinase and intractable enhancer of zeste homolog 2 [27]. Inspired by that, we replaced the HyT part of **NH-11** with norbornene and obtained **NH-13**. However, the NAMPT degrading activity was significantly decreased. We further synthesized norbornene tagged degraders **NH-16**–**NH-22** and **NH-23**–**NH-24** on the basis of NAMPT inhibitor **MS35** (Fig. 2D).

NH-11 dose-dependently induced NAMPT degradation with DC_{50} values of 3.18 μmol/L (K562), 6.05 μmol/L (HL60) and 7.32 μmol/L (Jurkat), respectively (Figs. 3A and B). The DC_{50} values of **NH-13** on K562, Jurkat and HL60 cells were 10.34, 10.47 and 10.80 μmol/L, respectively (Fig. 3A and Fig. S1A). Compound **NH-16** had DC_{50} values of 10.21, 11.71 and 12.11 μmol/L against K562, Jurkat and HL60 cells, respectively. **NH-22** had DC_{50} values of 9.75 μmol/L on K562, 9.10 μmol/L on Jurkat, and 11.21 μmol/L on HL60, respectively (Fig. 3C, Figs. S1B and C). Additionally, the NAMPT degradation induced by compound **NH-11** was also dependent on the duration of exposure. Following incubation with 8 μmol/L **NH-11** for 48 h, a maximum degradation of approximately

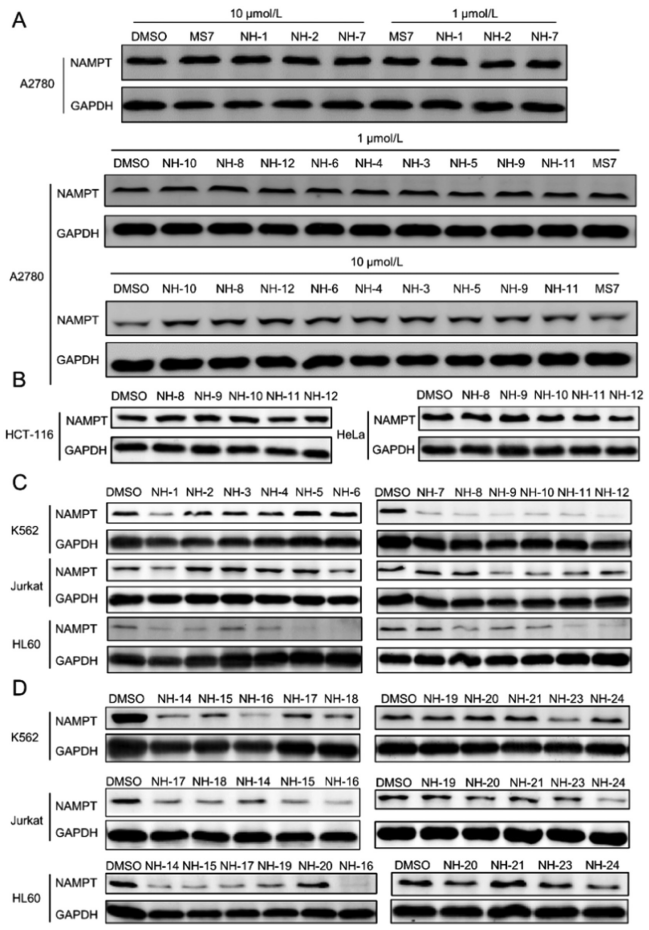

Fig. 2. Evaluation of NAMPT degradation activity of the designed compounds. (A) Compounds **NH-1**–**NH-12** induced the degradation of NAMPT at 10 μmol/L and 1 μmol/L in A2780 cells. (B) Evaluation of NAMPT degradation activity treated by **NH-8**–**NH-12** in HCT-116 and HeLa cells. (C) Evaluation of NAMPT degradation activity treated by **NH-1**–**NH-12** in different leukemia cells. (D) The NAMPT degradation activity in different leukemia cells treated with **NH-14**–**NH-24** (15 μmol/L) for 48 h. GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

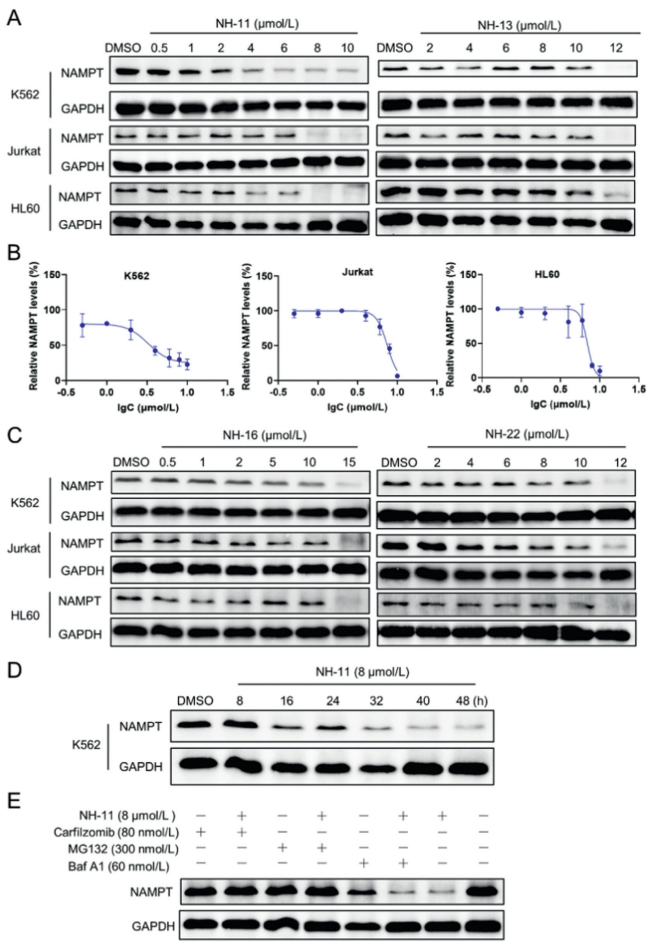

Fig. 3. Evaluation of NAMPT degradation activity in leukemia cells. (A) The NAMPT degradation activity in K562, Jurkat, and HL60 cells treated with **NH-11** or **NH-13** at various concentrations. (B) The relative NAMPT levels induced by **NH-11** in K562, Jurkat, and HL60 cells. (C) The NAMPT degradation activity in different leukemia cells treated with **NH-16** or **NH-22** at various concentrations. (D) The NAMPT degradation in K562 cells treated with 8 μmol/L **NH-11** for 8–48 h. (E) K562 cells were treated with carfilzomib (80 nmol/L), MG132 (300 nmol/L), Baf A1 (60 nmol/L) or combination treatment with the degrader **NH-11** (8 μmol/L) for 48 h and the NAMPT levels were detected.

95% of NAMPT protein was observed (Fig. 3D). These results indicated that **NH-11** might be the lead compound for NAMPT degradation in leukemia cells.

Compound **NH-11** possessed the highest capacity for NAMPT degradation and was selected for further mechanism and antitumor activity evaluations. In eukaryotic cells, damaged proteins can be degraded by proteasomes or lysosomes [35,36]. To investigate the potential degradation mechanism of **NH-11**, we performed rescue assays. The K562 cells was co-treated with **NH-11** and lysosomal inhibitor bafilomycin A1 (Baf A1) [37], or ubiquitin (Ub)-proteasome inhibitors MG132 [38], carfilzomib [39]. It was found that the MG132 and carfilzomib largely abolished **NH-11**-induced NAMPT degradation (Fig. 3E), indicating that **NH-11** mediated the degradation of NAMPT *via* the Ub-proteasome system (UPS). Given the potent NAMPT inhibition activity and degradation efficiency of **NH-11**, its antiproliferative activity against leukemia cell lines were also investigated by using a cell counting kit-8 (CCK8) assay. The results revealed that **NH-11** showed potent antiproliferative activity against K562, Jurkat and HL60 cells with IC_{50} value of 0.73 ± 0.31, 0.03 ± 0.01, and 0.95 ± 0.38 μmol/L, respectively (Fig. S2 and Table S2 in Supporting information).

We next investigated the mechanism of **NH-11** in inhibiting proliferation of leukemia cells. The Annexin V-FITC and propidium iodide (PI) staining assay indicated that **NH-11** significantly induced apoptosis of K562 cells after 48 h of treatment and the apoptosis rate was enhanced in a dose-dependent manner (Figs. 4A and C). In addition, flow cytometric analysis was conducted to investigate the effects of compound **NH-11** on the cell cycle of K562 cells. After treatment with 1 or 5 μmol/L of degrader **NH-11** for 48 h, 37.42% and 26.71% of K562 cell remained in the G1 stage, which was significantly lower than that in the DMSO treatment group. Notably, after treatment with 5 μmol/L of degrader **NH-11** for 48 h, 52.05% of K562 cells showed a prominent cell cycle arrest in the S phase (Figs. 4B and D). Moreover, consistent with the flow cytometric assay, **NH-11** could sustain the downregulation of Bcl-2, a key anti-apoptotic protein, whereas it could increase the levels of Bax, a protein promotes cell death (Figs. 4E and F). Finally, NAD^+/NAMPT activity was also detected. Compared with inhibitor **MS7**, degrader **NH-11** could more effectively inhibit the synthesis of NAD by NAMPT ($IC_{50} = 35.71 \pm 2.09$ nmol/L, Fig. 4G). In addition, the cytotoxicity of **NH-11** on normal cell lines L02 and MCF-10A was also evaluated. The results indicated that there was no obvious cytotoxic effect on normal cells (Fig. 4H). Taken together, these results suggested that **NH-11** effectively and selectively degraded NAMPT in K562 cells and then induced the cell apoptosis.

In summary, a series of novel HyT NAMPT degraders were designed and synthesized by connecting hydrophobic tags adamantane and norbornene to several NAMPT inhibitors. HyT degrader

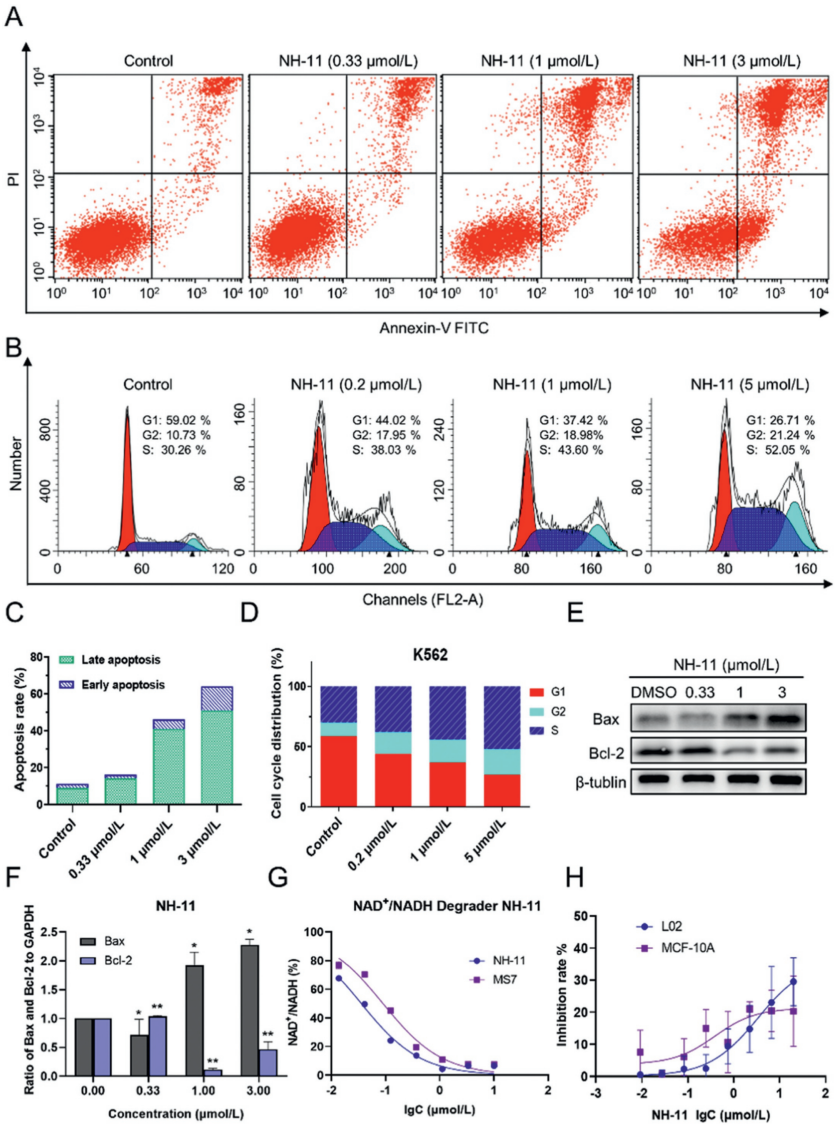

Fig. 4. Antitumor activity of NAMPT degraders. (A) **NH-11** induced apoptosis of K562 cells after 48 h of treatment. (B) K562 cells were treated with **NH-11** for 48 h and the cell cycle was evaluated *via* flow cytometry. (C) The bar graph shows the early and late apoptosis. (D) The bar graph shows the percentages of cells in G1, S, and G2 phases. (E) Bax, Bcl-2 protein levels in K562 cells treated by **NH-11** for 48 h. (F) The bar graph shows the relation of Bax and Bcl-2 to GAPDH. (G) The percentage of NAD^+/NADH treated by **NH-11**. (H) L02 and MCF-10A cells were treated with indicated concentration of **NH-11** and inhibition rate was determined.

NH-11 selectively degraded NAMPT in leukemia cells, blocking the secretion of intracellular NAMPT. Thus, we hypothesized that the level of eNAMPT could be reduced. Compound **NH-11** showed potent antiproliferative activities and effectively induced cell apoptosis and cell cycle arrest in leukemia cells. Comparing with reported NAMPT PROTAC degraders, **NH-11** possesses relatively lower molecular weight and good selectivity for leukemia cells. Thus, the hydrophobic tag may be an alternative NAMPT degradation strategy and further optimizations are currently in progress.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.109392.

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