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Understanding the structure and function of *Plasmodium* aminopeptidases to facilitate drug discovery

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Abstract

Malaria continues to be the most widespread parasitic disease affecting humans globally. As parasites develop drug resistance at an alarming pace, it has become crucial to identify novel drug targets. Over the last decade, the metalloaminopeptidases have gained importance as potential targets for new antimalarials. These enzymes are responsible for removing the N-terminal amino acids from proteins and peptides, and their restricted specificities suggest that many perform unique and essential roles within the malaria parasite. This mini-review focuses on the recent progress in structure and functional data relating to the *Plasmodium* metalloaminopeptidases that have been validated or shown promise as new antimalarial drug targets.

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Metalloaminopeptidases (MAPs) are protease enzymes that play crucial roles in cell maintenance, growth, development and defence. These enzymes use metal ions in their active sites to activate a water molecule, which then acts as a hydroxide nucleophile to cleave the peptide bond and release the amino acid in the P1 position.[1] In 2013, we reviewed the known structure and function of the nine MAPs identified in *Plasmodium falciparum* (3D7) genome [1]. Since that review, we have expanded our knowledge of both their physiological role(s) in parasites and their future use as antimalarial targets. Advances in structural biology techniques have also provided new insights and hints about complexities of protein assemblies, substrates and proteolytic regulation. Here we review the new advancements in our knowledge of the structure and function of the *Plasmodium* MAPs that are validated or show potential as new antimalarial drug targets. We have not reviewed the *Plasmodium* methionine aminopeptidases in this review as recent advances have focussed on biological activity, rather than structure.

Targeting the *Plasmodium* M1 and M17 aminopeptidases for new antimalarials

The *Plasmodium* M1 and M17 MAPs, known as *Pf*A-M1 and *Pf*A-M17, function at the terminal stages of intra-erythrocytic haemoglobin digestion [2]. Both *Pf*A-M1 and *Pf*A-M17 appear to be essential for parasite survival in *P. falciparum* [3–6], making them attractive potential drug targets. Selective inhibition of *Pf*A-M1 has been shown to result in swelling of the DV and parasite death [7,8]. Whilst there were previously reports that inhibition of *Pf*A-M17 resulted in ring-stage parasite death, this now appears to be due to off-target effects, with genetic knockdown of *Pf*A-M17 resulting in parasite death at the later trophozoite stage [5]. Importantly, inhibition of *Pf*A-M1 or *Pf*A-M17 results in a build-up of undigested short peptide chains that are likely to originate from haemoglobin, confirming their *in vivo* function [5,7]. Inhibition of both enzymes has been additionally explored in the murine malaria models where dual inhibition resulted in reduction in parasitemia [9].

[1] The nomenclature of Schechter and Berger [Schechter I, Berger A: On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun* 1967, 27:157–162.] is used here. P1 and P1′ refer to substrate residues while the S1, S1′ refer to the corresponding enzyme subsites.

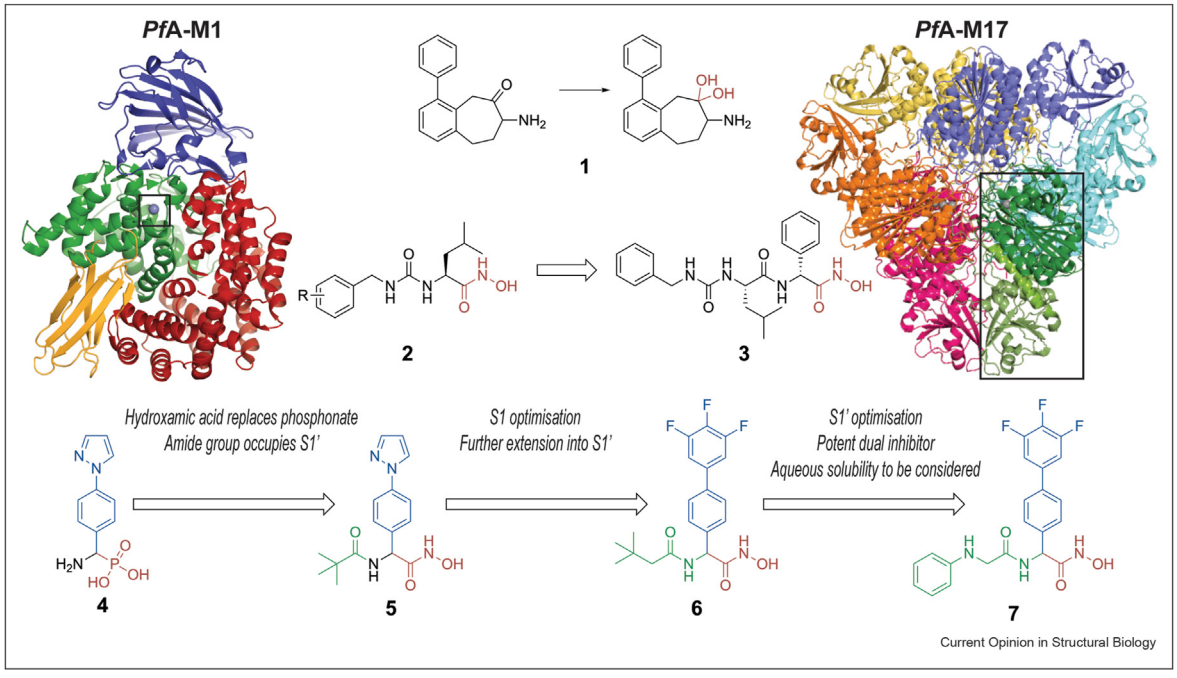
Consequently, the last decade has seen a number of drug discovery campaigns targeting the *Plasmodium* M1 and M17 aminopeptidases, either individually or as a dual target for a single inhibitor [10,11]. To date, these campaigns have focussed on metal chelation to target the active site of these enzymes. Early *Pf*A-M1 and *Pf*A-M17 inhibitors included peptidomimetics with carboxylates (bestatin), phosphinates (Co4) and hydroxamates (BDM14471 and CHR-2863) as their metal binding groups [1,10]. These compounds suffered from poor cell permeability and pharmacokinetic properties and were largely used as proof of concept studies [1,10].

Amino-benzosuberone derivatives with selective inhibition of *Pf*A-M1 ($K_i^{(app)}$ 50 nM) over *Pf*A-M17 ($K_i^{(app)} > 100$ μM) have been reported [8]. The crystal structure of lead compound **1** (Figure 1) in complex with *Pf*A-M1 showed the seven-membered ring adopting a chair conformation with the ketone being presented as the hydrated diol form (shown in red) [12]. The hydroxyls are involved in metal coordination and hydrogen bonding while the phenyl ring occupies the S1′ pocket and forms hydrophobic π-stacking [12]. Compound **1** was found to have low micromolar inhibitory activity in target enzyme assays and against *P. falciparum* parasites in culture. However, poor solubility, high clearance and low half-life are some of the drawbacks of this selective *Pf*A-M1 inhibitor [13].

Additionally, a series of leucine-derived hydroxamates (**2**, Figure 1) with potent mammalian M1 aminopeptidase inhibition [14,15] were found to also act as *Pf*A-M1 and *Pf*A-M17 inhibitors [16]. The *Pf*A-M1 bound crystal structures of these inhibitors inspired analogues forming simultaneous interactions with S1, S1′ and S2′ regions. This identified the ureido benzyl analogue **3** with significantly improved affinity against *P. falciparum* aminopeptidases ($K_i^{(app)} \leq 50$ nM) as well as selectivity over their mammalian counterpart (APN $K_i^{(app)} > 15$ μM) [16]. Although **3** retained activity in drug-resistant strains of *P. falciparum*, its low micromolar activity against the parasite [16] suggests further work could be done to improve drug-like properties.

Structure-based drug design has aided the development of another major class of hydroxamic acids as *Pf*A-M1 and *Pf*A-M17 inhibitors (**4**–**7**, Figure 1). Replacement of the original phosphonic acid (**4**, phosphonate in red) metal binder with hydroxamic acid (**5**–**7**, hydroxamates in red) resulted in stronger interactions with the zinc ion(s) and more potent inhibition [17]. It was also found that a pivalamide extension (**5**, in green) from the amine of **4**, allows S1′ pocket interactions to improve the binding to the two enzymes [17]. The subsequent study of S1 pocket substituents showed that a 3,4,5-trifluorobiphenyl unit (**6**, in blue) can take part in hydrophobic interactions and act as a hydrogen bond

Figure 1



Recent examples of inhibitors targeting either or both *Pf*A-M1 and *Pf*A-M17. Amino-benzosuberone **1** inhibits *Pf*A-M1 selectively with the ketone being presented as the hydrated diol (in red) form and involved in metal coordination [12]. Leucine-derived analogue **3** was identified as an exciting lead with significant selectivity in *P. falciparum* aminopeptidases over their mammalian counterpart [16]. **4**–**7** depict key steps in chemical evolution from a structure-based drug design campaign where the most recent hits, **6** and **7**, inhibit both enzymatic targets and the parasite with high affinity [19,20].

acceptor resulting in potent dual inhibition of both targets [18]. Compound **6** was found to have potent activity against drug-resistant and drug-sensitive parasites in the low nanomolar range. Additionally, hydroxamic acid-containing compounds are often thought to promiscuously bind to other metalloproteinases and the selectivity of **6** against a panel of matrix metalloproteinases made it a promising lead [19]. Notably, other inhibitors from this study with bulky hydrophobic S1′ substituents had a previously unobserved binding mode with *Pf*A-M17 where they displaced the catalytic zinc ion [19]. Most recently, aniline containing **7** extended further into the S1′ pocket with potent dual inhibition of both enzymes [20]. The analogues in this study were found to have lower affinity against parasites *in vitro* which may be due to solubility issues resulting in poor cell permeability.

Malcom et al. recently demonstrated the homology of M1 and M17 MAPs across different species of *Plasmodium* with conserved substrate preferences at the P1 position [21]. Evaluation of the activity of inhibitors showed that affinity trends were largely conserved between the *P. falciparum* and *Plasmodium vivax* M17 targets, but most compounds were able to inhibit *Pv*-M1 with approximately 10-fold greater affinity than *Pf*A-M1 [19]. Further studies [21] suggested that the slower transition between *Pf*A-M1's open and closed conformation might explain the slower processing of substrates resulting in higher $K_i^{(app)}$ values. Overall, the profile of the inhibitors suggested that the structure-based drug design of inhibitors using *P. falciparum* structures could be highly effective against the *P. vivax* and *P. berghei* homologs to identify cross-peptidase and cross-species inhibition across the six aminopeptidases [21]. Moreover, other computational studies [22–24] of *Pf*A-M1 and *Pf*A-M17 and *in silico* screening for inhibitors [25,26] have also been completed.

Overall, the *Pf*A-M1 and *Pf*A-M17 inhibitors identified through various campaigns are encouraging, showing that with careful design and utilisation of structural data, potent and selective inhibitors can be discovered. However, most compounds serve as promising late-stage hits that require further efforts before they can be considered preclinical leads. Moving forward, there needs to be a focus on improving their shortcomings including solubility, permeability, clearance and selectivity. This needs to be done to better align these chemical entities with the current target product profiles set by the Medicines for Malaria Venture for new antimalarials entering the clinic.

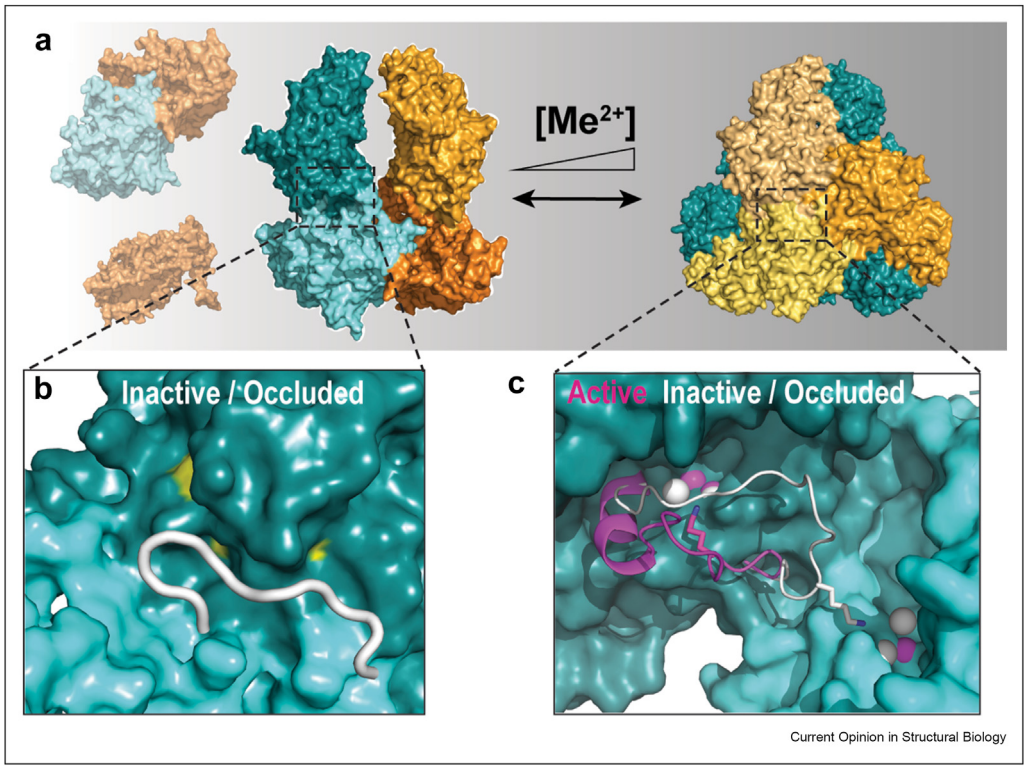
The important role of metals in the multimeric MAPs

P. falciparum encodes three multimeric MAPs that each require co-catalytic metal ions but adopt different biological assemblies. The M24 aminopeptidase P, *Pf*APP, forms a dimer [27] whereas *Pf*A-M17 assembles into a hexamer [28] and aspartyl *Pf*A-M18 into a dodecameric tetrahedral structure [29,30]. As mentioned, a number of drug discovery campaigns have targeted *Pf*A-M17, however, targeting *Pf*APP or *Pf*-M18 is still at an early stage with some *in silico* studies showing promising inhibition [31,32]. Over the past decade, crystal structures have been determined for all three MAPs and more recent advances in cryo-electron microscopy techniques have enabled the elucidation of full-length *P. vivax* M17 [33] and native *Pf*A-M18 structures [30]. These studies, along with other biochemical analyses, now provide evidence to suggest the catalytic metals in combination with a multimeric assembly underpin regulation, activity and substrate binding.

The multimeric architecture provides additional mechanisms to enhance catalytic function. Studies on M17 and M18 MAP families have highlighted the role catalytic metals play in MAP activity in addition to their role in oligomerisation. For *Pf*A-M17 and *Pv*-M17, recent studies have confirmed both must adopt a hexameric state to be proteolytically active and smaller oligomers are inactive [33]. The formation of the hexamer was metal-dependent, with the metal ion environment driving the formation of hexamer assembly from smaller inactive oligomers (Figure 2a) [33]. Without divalent cations in the environment, the active site structure was disordered and/or unstable, rendering the enzyme inactive (Figure 2b) [33]. The metal-dependent assembly of the hexamer also appeared linked to the active site arrangement, where coupled metal-protein dynamics have been discovered to control substrate access to the active site and therefore proteolysis. The position of the metal ions in the active site controls the conformation of a loop that acts to stabilise the hexamer and control access to the active site (Figure 2c) [34]. When the loop is in the inactive or occluded position, the metal ions are also not in a position required for substrate engagement and hydrolysis. When the loop is engaged with its own subunit, the metal ions return to a catalytically competent position (Figure 2c).

A similar behaviour was observed in the dodecameric M42 aminopeptidases (from the same peptidase subgroup as the *Pf*A-M18) that showed the importance of metal site 1 within the active site fold and how it directly drives the transition from dimers to the functional dodecamer [35,36]. These findings suggest that the M17 and M42/M18 MAPs exist in an oligomeric equilibrium depending on the metal cofactor availability, further suggesting a level of physiological regulation that could alter depending on life cycle stage, environment, stress or other factors. For *Plasmodium* spp, there is limited knowledge of the intracellular physiological

Figure 2



Plasmodium **M17 aminopeptidases use metals to regulate proteolytic activity.** (**a**) Metal ion concentration (indicated by arrow with [Me^{2+}]) controls the formation of functional hexamers from smaller inactive oligomeric species [33]. (**b**) Structural resolution of the inactive tetrameric form of *Pv*-M17 shows the active site is disordered and access to the site is blocked by a loop (shown in white) from a neighbouring subunit. Residues that coordinate zinc ions (zincs not observed in structure) are shown in yellow. Increasing the concentration of metal cations allows the hexamer to form and the enzyme becomes catalytically competent [33]. In *Pf*A-M17, further regulatory mechanisms exist via coupled metal-protein dynamics where the metal ions can change position, dragging a protein loop that alters access to the active site. This is shown in (**c**) where the top trimer (gold surface in A) has been removed to reveal a zoom of an internal active site within the teal trimer. The coupled metal-loop dynamics shifts the active site from an active conformation (loop in magenta cartoon, zincs in magenta spheres) to the inactive form (loop in white cartoon, zincs in white spheres) where the loop from one subunit reaches out and occludes the neighbouring active site [34].

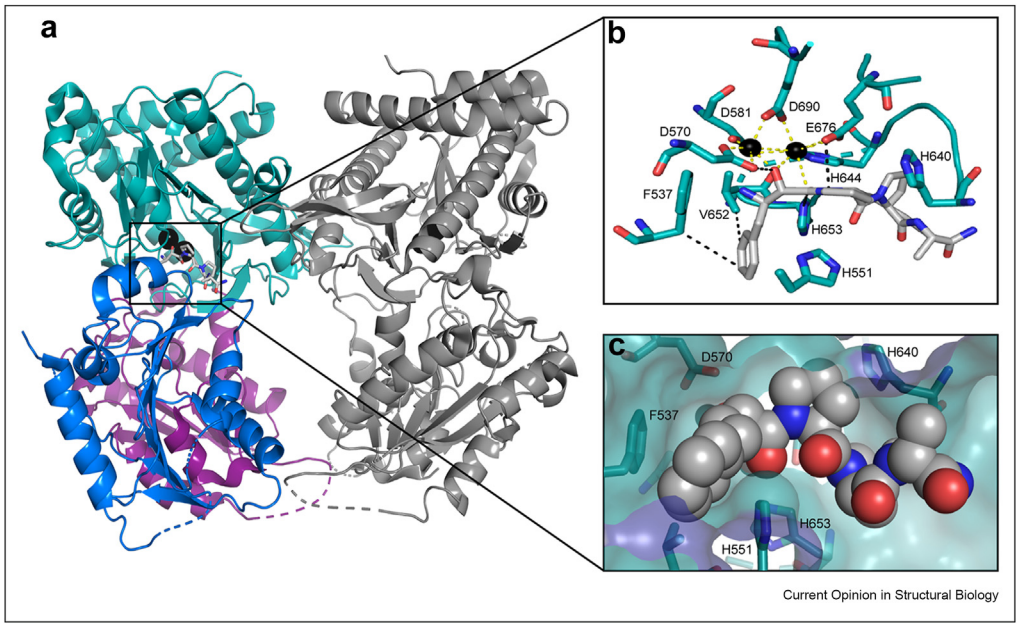
metal environment(s) but fluxes of zinc availability are essential for *P. falciparum* intraerythrocytic stage progression [37] suggesting that transition metal concentration may indeed be a relevant biological mechanism for proteolytic control.

Dissecting metal co-factor identity and availability in native *Plasmodium* MAPs, however, is challenging. Whilst generally annotated as zinc metalloenzymes, all three multimeric MAPs show improved catalytic efficiency in the presence of Mn^{2+} and Co^{2+}, compared to Zn^{2+} and other cations. However, biochemical studies on native protein are rare. Ragheb et al. (2009) showed native *Pf*APP was active in Mn^{2+} [38] but in general, recombinant proteins — produced in media-containing zinc and then purified in buffers supplemented with cation of choice — have been used to perform detailed biochemistry and structural studies. Additionally, it has been known for some time that the nature of the metal ion can change catalytic efficiency and substrate preference [39]. Recently, Malcolm et al., showed that *Pv*-M17 and *Pf*A-M17 possessed cysteinyl—glycinase activity but only in the presence of Mn^{2+} [33]. The importance of native metal identity and availability appears to be critical to our understanding of biological function as well as structure (and therefore inhibition). Additionally, targeting the assembly of multimeric MAPs may provide an alternative and more selective path of enzyme inhibition.

Proline recycling in *Plasmodium* spp

Proline recycling requires specialised protease machinery to engage and cleave the rigid conformation of proline [40]. *P. falciparum* encodes one non-essential S33 prolyl aminopeptidase (*Pf*PAP, PF3D7_1401300) [41,42] that appears to play a role in erythrocyte remodelling [4,43] and one aminopeptidase P (*Pf*APP, PF14_0517) that is implicated in late-stage haemoglobin digestion and is

Figure 3



***Pf*APP homodimer and apstatin binding pose.** (**a**) The *Pf*APP homodimer (PDB ID: 5JQK) is shown in cartoon representation that is coloured by chain and domain. Chain A is shown coloured according to domain, with domain I in blue, domain II in purple and domain III in teal. Chain B is shown in grey. (**b**) *Pf*APP active site with manganese ions shown as black circles and coordinating residues and apstatin inhibitor as teal and grey sticks respectively (PDB ID: 5JR6). Metal coordination interactions are shown as yellow dashed lines; interactions between apstatin and substrate pockets are indicated by black dashes. (**c**) Apstatin binding pose (grey space filling representation) in the *Pf*APP pocket (surface representation, domain I coloured blue, domain III coloured teal. Adapted from Drinkwater et al., 2016 [27].

essential for the erythrocytic life cycle [3,4,43]. Saturation mutagenesis revealed there were no insertion events in *Pfa-app*. [4], supporting earlier studies that were unable to generate genetic knockouts in *P. falciparum* [3]. The essential nature of *Pf*APP makes it an attractive antimalarial drug target [27] but to date, no potent inhibitors have been discovered that can validate its druggability.

*Pf*APP is a M24 MAP [44] and can catalyse the removal of any N-terminal residue with a proline at the P1′ position [27]. The X-ray crystal structure revealed a three-domain homodimer (Figure 3a), similar to the human aminopeptidase P (hAPP1, sequence identity 32%). In *Pf*APP, domains I and II are structurally similar but domain I shows higher sequence diversity from hAPP1, as well as a degree of dynamics or flexibility [27]. The active site is located at the base of domain III and like other M24 enzymes, *Pf*APP contains two catalytic manganese (II) ions coordinated by conserved residues (Figure 3b) [27]. Apstatin (Phe-Pro-Pro-Ala-NH2) is a selective aminopeptidase P inhibitor and a tool compound used to map peptide binding [27]. Apstatin shows low micromolar inhibition of *Pf*APP activity *in vitro* but no efficacy against parasite growth, presumably due to a combination of moderate potency and poor membrane permeability. Resolution of the apstatin-bound *Pf*APP X-ray crystal structure revealed a dual nature within the S1 pocket, with hydrophobic residues lining one side and polar residues on the other, and that the S1′ pocket was shaped by polar residues with a key histidine (H551) introducing an acute bend between the S1 and S1′ pockets (Figure 3c) [27,45]. Future small molecule inhibitors will need to navigate such spatial features to achieve potent binding if targeting the active site of *Pf*APP.

Conclusions

The emergence of resistance to current frontline malaria therapeutics highlights the need for identifying drugs with novel mechanisms of action. The *Plasmodium* MAPs are an exciting area of research as potential antimalarial targets. This review summarises the structural and functional advancements from the last decade on this exciting class of enzymes, including the studies on *Pf*A-M1 and *Pf*A-M17 validating their essential roles in the parasite and as antimalarial drug targets with a number of lead-like inhibitors identified. Additionally, understanding the multimeric assembly of M17 and M42/M18 MAPs provides a path for future studies and a potential new route for enzyme inhibition. Finally, the essential nature of *Pf*AAP suggests further work could validate its function as a drug target and identify potent inhibitors.

Credit author statement

Mahta Mansouri: Writing – original draft preparation, reviewing and editing. Visualisation. Kajal Daware: Writing – original draft preparation. Visualisation. Chaille Webb: Writing – original draft preparation. Visualisation. Sheena McGowan Conceptualization. Writing – reviewing and editing.

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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.

Data availability

No data was used for the research described in the article.

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