Insights into the molecular inactivation mechanism of human activated thrombin-activatable fibrinolysis inhibitor

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Summary. Background: Thrombin-activatable fibrinolysis inhibitor (TAFI) is a validated target for thrombotic diseases. TAFI is converted in vivo to activated TAFI (TAFIa) by removal of its pro-domain. Whereas TAFI is stable and persists in the circulation, possibly in complex with plasminogen, TAFIa is unstable and poorly soluble, with a half-life of minutes. Objectives: In order to study the molecular determinants of this instability, we studied the influence of protein inhibitors on human TAFIa. Results: We found that protein inhibitors significantly reduced the instability and insolubility of TAFIa. In addition, we solved the 2.5-A resolution crystal structure of human TAFIa in complex with a potent protein inhibitor, tick-derived carboxypeptidase inhibitor, which gives rise to a stable and soluble TAFIa species. The structure revealed a significant reduction in the flexibility of dynamic segments when compared with the structures of bovine and human TAFI. We also identified two latent hotspots, loop Lβ2β3 and segment α5–Lzβ7–β7, where conformational destabilization may begin. These hotspots are also present in TAFI, but the pro-domain may provide sufficient stabilization and solubility to guarantee protein persistence in vivo. When the pro-domain is removed, the free TAFIa moiety becomes unstable, its activity is suppressed, and the molecule becomes insoluble. Conclusions: The present study corroborates the function of protein inhibitors in stabilizing human TAFIa and it provides a rigid and high-resolution mold for the design of small molecule inhibitors of this enzyme, thus paving the way for novel therapy for thrombotic disorders.

Keywords: blood coagulation, metallopeptidase, TAFI, zymogen activation.

Introduction

Thrombin-activatable fibrinolysis inhibitor (TAFI) is a plasma zymogen that, when converted to activated TAFI (TAFIa), inhibits fibrinolysis, thus contributing to hemostasis [1–5]. It removes C-terminal lysines from the fibrin clot following the endoproteolytic action of plasmin at late stages of coagulation, thus preventing fibrinolysis from entering the propagation phase. It, therefore, contributes not only to the fine-tuning of physiological clot lysis, but also to thromboses and thrombotic diseases, and animal studies have shown that TAFI inhibition reduces such disorders. TAFI is secreted by the liver and circulates as a 58-kDa glycoprotein at high concentrations (3–15 µg mL⁻¹ [3]), possibly in complex with plasminogen [6]. It is stable in circulation and shows exoproteolytic activity despite the presence of a 20-kDa pro-domain upstream of its catalytic domain, which is characteristic for A/B-type members of the funnellin tribe of metallocarboxypeptidases [7–11]. This occurs because the active site is preformed in the zymogen and accessible to substrates [12,13]. TAFI is converted to the 36-kDa mature form, TAFIa, by cleavage at bond Arg98A–Ala4 (for the structure-based numbering convention used, see Materials and methods) at the end of the pro-domain. This proteolysis may be induced by thrombin (TH)–thrombomodulin (TM) or plasmin at the end of blood coagulation in vivo and by several serine proteinases, including trypsin, in vitro [14]. TAFIa has stronger proteolytic activity than TAFI, but no specific inhibitors of either TAFI or TAFIa have been found in the bloodstream. However, TAFIa spontaneously inactivates, showing a half-life of 5–10 min at normal body
temperature [6,15–18]. This feature is critical, as it correlates with diminished functional availability. The differences in incidence in the plasma of TAFI and TAFIa may be attributed to the pro-domain, which contains four glycosylation sites. In general, sugar moieties attached to glycoproteins increase solubility and reduce aggregation [17,19]. The glycosylation of TAFI aids in solubility and stability, as non-glycosylated TAFI and TAFIa, which has no sugars, are only poorly soluble [20]. Moreover, the pro-domain may stabilize TAFI as in other funnels, for which the pro-domains act as chaperones during folding [7,12,21]. Furthermore, pro-domain removal raises the isoelectric point from approximately 5 to 8, which gives a more basic, less soluble protein [17]. Exogenous addition of the pro-domain does not alter TAFIa stability or activity [12,21]. In contrast, long exposure to TH–TM in vitro produces a further cleavage in human TAFI after Arg210, producing fragments of 25 and 11 kDa. Other identified targets for proteolytic cleavage are approximately 1.5–2 kDa. Other identified targets for proteolytic cleavage include Lys235 and Arg237 [15,18,21–23].

Protein inhibitors control proteolytic activity, but they may also enhance protein rigidity and solubility by interacting with flexible parts of a structure [24]. Such inhibitors have proven potential as therapeutic adjuvants for the TAFI–TAFIa axis, and some are now undergoing clinical trials in various cardiovascular conditions [9]. To examine the stability of human TAFIa, we analyzed the detergent-free TH–TM-mediated maturation of TAFI in the absence and presence of inhibitors. In addition, we solved the X-ray crystal structure of human TAFIa in complex with one such protein inhibitor from a blood-sucking parasite.

Materials and methods

Proteins and reagents

Human TAFI was obtained from human blood plasma as previously described [10]. Human pancreatic procarboxypeptidase B1 (PCPB1) was overexpressed in Pichia pastoris and purified as previously described [25]. Recombinant leech-derived carboxypeptidase inhibitor (LCI) and tick-derived carboxypeptidase inhibitor (TCI) were produced in Escherichia coli and purified as reported elsewhere [26]. Human TH was purchased from Sigma (St Louis, MO, USA), rabbit lung TM from American Diagnostica (Stamford, CT, USA), and sequencing-grade porcine trypsin from Promega (Madison, WI, USA). The carboxypeptidase inhibitor 2-guanidinoethylmercaptosuccinic acid (GEMSA) and the serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc) were obtained from Calbiochem (Darmstadt, MI, USA) at the joint IBMB-CSIC/IRB/Barcelona Science Park High-Throughput Crystallography Platform. Suitable crystals appeared after 1 week in a Bruker steady-temperature crystal farm at 20 °C, with 0.2 M KBr, 0.1 M cacodylate and 15% PEG 4000 (pH 6.5) as reservoir solution. These conditions were efficiently scaled up to the microliter range with Cryschem crystallization dishes (Hampton Research; Aliso Viejo, CA, USA). Crystals were cryoprotected with 0.2 M KBr,
0.1 M cacodylate, 30% PEG 4000, and 15% glycerol (pH 6.5). A complete diffraction dataset was collected at 100 K from a single N2 flash-cryocooled crystal on a Q315R ADSC CCD detector at beamline ID23-1 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) within the Block Allocation Group ‘BAG Barcelona’. Crystals were trigonal, and harbored one complex per asymmetric unit. Diffraction data were integrated, scaled, merged and reduced with programs XDS [28] and SCALA [29] (Table 1).

Structure solution and refinement

The X-ray crystal structure was solved by Patterson search methods with program AMoRe [30], using all diffraction data between 15-Å and 4-Å resolution. A two-body search was performed by using the coordinates of the mature enzyme part of unbound human TAFI [Protein Data Bank (PDB) 3D66 [12] and those of TCI (PDB 3D4U [27]). A single solution was found for each molecule at, respectively, 14.7, 66.8, 96.5 and 8.8, 82.0, 21.2 (α, β, γ in Eulerian angles) and 0.40, 0.86, 0.00 and 0.53, 0.30, 0.13 (x, y, z, in fractional unit cell coordinates) after rigid body refinement. This solution gave a combined resolution range (Å) (outermost shell) of 46.2–2.50 (2.64–2.50).

Table 1 Crystallographic data

<table>
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<td>Resolution range (Å) (outermost shell)*</td>
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*Values in parentheses refer to the outermost resolution shell. †Rmerge = Σhkln|Fobs| - |Fcalc| / Σhkln|Fobs|; Rf.i.m., Rp.i.m. = Σhkln|Fobs| - Σhkln|Fcalc| / Σhkln|Fcalc|; and Rp.i.m. = Σhkln|Fobs| - Σhkln|Fcalc| / Σhkln|Fobs|; free Rfactor, same for a test set of reflections (> 700) not used during refinement. ¾According to program MOLPROTB.
species started to aggregate and precipitate after 30 min, and after 7 h almost all of the protein was in the insoluble fraction. The fraction of TAFI that was not converted also tended to aggregate, and was in the insoluble fraction after 24 h. However, a control experiment consisting of mixing TAFI and TAFIa in the absence of TH–TM revealed that the latter observation was due to a clustering effect of precipitating TAFIa (data not shown).

In a second experiment, the maturation reaction was only quenched just after the aliquots were removed (Fig. 1B). In this case, all TAFI had been converted to TAFIa after approximately 1 h. In accordance with previous reports [20], TAFIa began to aggregate after 15 min and, after approximately 1 h, all protein was insoluble. In order to provide a positive control for solubility/stability, human PCPB1 was activated by trypsin (it is not activated by TH–TM), as shown in Fig. 1B (see Fig. 1C). In this case, PCPB1 was completely converted to carboxypeptidase B1 after approximately 15 min, and the latter remained soluble. A second cleavage, after Arg130 (for PCPB1 numbering, see Fig. 1 in [27]), was observed after 7 h. However, in contrast to TAFIa, carboxypeptidase B1 remained soluble for 96 h (data not shown).

Stability studies during TAFI maturation in the presence of inhibitors

These experiments were motivated by the finding that the pro-domain had a function in TAFI stabilization but not in TAFIa activity or stability [21]. Pro-domains contribute to folding during biosynthesis of A/B-type funnelin zymogens, and they inhibit in trans the mature enzyme moiety [7,11]. Accordingly, we repeated the experiments shown in Fig. 1B in the presence...
of the small molecule B-type funnelin inhibitor GEMSA (Fig. 2A) as well as protein inhibitors derived from the medical leech, *Hirudo medicinalis* (LCI; 67 residues [37]), and the ixodid tick, *Rhipicephalus bursa* (TCI; 75 residues [38]). These are potent inhibitors of human TAFIa and exhibit micromolar (GEMSA) and nanomolar (LCI and TCI) inhibition constants [12,37,38]. In addition, GEMSA and the funnelin inhibitor, *potato carboxypeptidase inhibitor* (PCI), were reported to increase the thermal stability of TAFIa [39]. Furthermore, GEMSA had a positive effect on the stability and rigidity of the human TAFI structure [12]. In the present study, GEMSA prolonged TAFIa solubility, although it did not maintain it (Figs 1B and 2A). In contrast, both LCI and TCI led to complexes with TAFIa, which remained soluble after 24 h.

**Fig. 2.** Time course of human thrombin-activatable fibrinolysis inhibitor (TAFI) maturation in the presence of inhibitors. Processing of TAFI to activated TAFI (TAFIa) with thrombin–thrombomodulin at 37 °C was studied in the presence of 2-guanidinoethyl-mercaptosuccinic acid (GEMSA) (A), leech-derived carboxypeptidase inhibitor (LCI) (B), or tick-derived carboxypeptidase inhibitor (TCI) (C) (for details, see Materials and methods). Aliquots were removed at the indicated time points, blocked immediately with Pefabloc, and centrifuged, and the soluble (left panel) and insoluble (right panel) fractions were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis. All panels show representative gels from repeated experiments.

**Fig. 3.** Crystal structure of human activated thrombin-activatable fibrinolysis inhibitor (TAFIa) in complex with tick-derived carboxypeptidase inhibitor (TCI). (A) Richardson plot of human TAFIa (red/orange/yellow) in complex with TCI (blue/cyan) shown in the funnelin standard orientation [11] (left) and after a vertical 90° rotation (right). With respect to bovine TAFIa (see Figs 1 and 2A in [27]), the human enzyme displays an additional surface helix, α2¢, after α2, and loop Lb2b3 is fully ordered for its main chain atoms. The catalytic zinc ion is shown as a magenta sphere. The termini of each protein chain, the domains of TCI and the repetitive secondary structure elements of human TAFIa are labeled. (B) Cα structure representation in stereo of the superimposition of the TCI complexes of bovine (purple/white traces) and human (yellow/blue traces) TAFIa. Orientation as in (A). The surrounded areas indicate the regions of major structural differences [see (C) and (D)]. The catalytic zinc ion of human TAFIa is displayed as a magenta sphere for reference. (C) Detail in stereo of the superimposed structures of human (yellow) and bovine (purple) TAFIa, displayed for residues 53–61 (Lb2b3), together with the final σA-weighted (2mFobs − DFcalc)-type electron density map of the human enzyme contoured at 1σ. The chain of the bovine enzyme is undefined at residues 55–58 (see also Fig. 1 in [27]). Selected residues are numbered for reference. (D) Same as (C), but for segment 90–105 (La2b4 in bovine TAFIa; Lα2α2¢ + α2¢ in human TAFIa). The bovine enzyme is undefined at residues 93–97. CTD, C-terminal domain; NTD, N-terminal domain.

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The stabilizing effect was stronger for TCI, perhaps because the surface of the interaction is larger than that of LCI (see below and [27,40]). In addition, TAFI was not completely activated in the presence of TCI, in contrast to LCI. This finding can be attributed to the higher inhibitory potency of the tick inhibitor, which binds to its target at two sites (see below), and to its capacity to also inhibit TAFI [18]. It is worth mentioning that both inhibitors, isolated from hematophagous animals, prevent host blood clotting during feeding by inhibition of the TAFI-to-TAFIa conversion [9]. In particular, TCI displays the highest affinity for human TAFIa (K_i = 1.2 nM) and strongly accelerates fibrinolysis [9,38]. Accordingly, these two inhibitors seem to mimic and even enhance the role of the pro-domain, and provide TAFIa with high stability and rigidity, and hence solubility.

Crystal structure of human TAFIa in complex with TCI

To obtain structural insights into the stabilizing effect of TCI on human TAFIa and to provide a high-resolution scaffold for the future design of TAFIa inhibitors, we derived the 2.5-Å crystal structure of the protein complex (Fig. 3). In contrast to the structures of human and bovine TAFI (PDB 3D66, 3D67, 3D68, and 3DGV [12,13]), which were solved to significantly lower resolution and/or with several segments of the structure in weak electron density, both TAFIa and TCI were unambiguously defined for their complete polypeptide chains.

Similarly to its bovine ortholog, human TAFIa has a compact globular shape and shows the classic α/β-hydrolase fold of A/B-type and N/E-type funnelins [7,9]. It has a central eight-stranded, highly twisted β-sheet (strands β1–β8; secondary structure element nomenclature according to bovine TAFIa; see [27]), flanked by helices α4 and α6 on its concave face and by α1–α3, α5 and α7 and the surface N-terminus and C-terminus of the molecule on its convex side (Fig. 3A). The access to the active site cleft is reminiscent of a funnel, whose rim is formed by surface loops: the loop connecting strand β8 with helix α7 (Lβ8α7), Lβ3α2, Lβ5β6, Lβ6α5, and the long 53-residue segment, Lα3α4. The latter delimits the front and the bottom of the active site and the specificity pocket. The catalytic zinc ion is coordinated by His69 Nο1, Glu72 Oε1, Glu72 Oε2, and His196 Nο1 at the bottom of the cleft. An isolated glycine, which may have been trapped during purification, is found next to the zinc. It partially occupies the specificity pocket and is bound to the side chains of Arg127, Asn144, Arg145, and Tyr248. These residues contribute to substrate binding and catalysis in TAFIa and in other A/B-type funnelins, together with Arg71, Ser197–Ser199, Val203, Gly243, Leu247, Ala250, Asp255, Thr268, and Glu270. In particular, Asp255 accounts for the B-type specificity of TAFIa for basic C-terminal residues [11,27].

Inhibition through TCI is exerted by its two constituting β-defensin-like domains, an N-terminal domain (NTD; Asn11–Leu37I) and a C-terminal domain (CTD; Cys401–Leu74I), which are linked by a two-residue spacer and do not interact (Fig. 3A and [27,41]). The main interaction with the peptidase is exerted by CTD, which lies on top of the funnel-like rim, thus blocking access of substrates to the active site cleft. It contacts protease segments Lβ3α2, Lα3α4, Lβ5β6, Lβ7α6, and Lβ8α7, and residues from the active site. Therefore, CTD contributes not only to activity inhibition but also to the overall stability and rigidity of the TAFIa moiety. The C-terminus of CTD penetrates the enzyme moiety, and its last residue, His75I, is cleaved off, so that the ϵ-carboxylate group of Leu74I binds the catalytic zinc ion. In contrast, NTD contacts only TAFIa region Trp120–Met125 at the beginning of the long Lα3α4 segment, which can be considered as an exosite, that is, a binding site that is distinct from the catalytic site. This second site may contribute to TAFIa stability, and could be targeted in future inhibitor design studies. Overall, the TAFIa–TCI interaction spans 1068 Å² of TAFIa and 947 Å² of TCI, and includes 17 hydrogen bonds and six salt bridges. These numbers are significantly higher than those corresponding to the complex of LCI with a standard funnelin, human carboxypeptidase A2 (PDB 1DTD [40]; 770 Å², 656 Å², 10 hydrogen bonds, no salt bridges), which indicates that TCI forms tighter complexes than LCI (see above and Fig. 2).

Comparison with reported TAFI–TAFIa structures

As inferred from high sequence identity (78%), both human and bovine (PDB 3D4U [27]) TAFIa–TCI complexes fit well to each other after optimal superposition, with a root mean square deviation of 0.50 Å for all Ca atoms of each complex (Fig. 3B). See Sanglas et al. [27] for further structural details of TAFIa–TCI interactions. Only two regions show major differences: the segment that connects helix α2 with strand β4, and loop Lβ2β3 (Fig. 3C). Both segments were disordered in the bovine structure, which was solved to high resolution, and they were hypothesized to be potential ‘fibrinolysis switches’, responsible for the destabilization of bovine TAFIa [27]. In the present complex, the first segment is fully ordered and defined, even for its side chains, and it adopts the chain trace usually found in funnelins such as human carboxypeptidase B1, with an extra α-helix, here termed α2’ (Fig. 3). This segment is partially stabilized by a crystal contact with the C-terminal helix of a neighboring protease molecule in human TAFIa, and is hereafter referred to as Lα2α2’ + α2’ for the human structure and as Lα2β4 for the bovine counterpart (Fig. 3D). Loop Lβ2β3, in turn, is likewise defined in the human structure, although there is some difficulty in resolving the conformation of the side chains (Fig. 3C).

The plots depicting the average TDPs per residue of human and bovine TAFIa and human carboxypeptidase B1 show that, whereas the pancreatic enzyme has no significantly flexible regions, two hotspots are found in human TAFIa, centered on Lβ2β3 and α5–Lα5β7–β7 (Fig. 4). The former segment is characterized by a unique one-residue insertion after position 56, thus giving a surface loop that is larger than in standard funnelins [11], and a unique superficial lysine at position 55 in bovine, human, rat and mouse TAFIa but not in pancreatic funnelins (see Figs 1 in [25] and 3C in [27]).
basic residue could be targeted by bloodstream serine proteases, and its mutation to asparagine was found in human TAFIa variants with 2.6-fold (a double point mutant) and 22-fold (a four-fold mutant) longer half-lives than the wild-type enzyme [42]. Furthermore, mouse, rat and bovine TAFIa undergo cleavage within Lβ2β3 during inactivation.

In turn, $\alpha_5$–Lx5β7–β7 (residues 214–243; this study and [27]) was included in an ‘instability region’ (residues 213–242) in bovine TAFI [13] and a ‘dynamic flap’ (residues 204–257) in human TAFI [12]. This segment is a potential binding region for heparin [12,13,27], which was shown to stabilize TAFIa against spontaneous inactivation [14], thus implicating this region in the regulation of TAFIa half-life [27]. It contains cleavage sites for TH (at Arg210 [43]) and trypsin (at Arg237 [44]), and several point mutations that improve stability are found within this region. Overall, reported examples of TAFI with enhanced durability in solution comprise single to quintuple mutants affecting residues in Lβ2β3, Lα2α4, β6–Lβ6x5–$\alpha_5$–Lx5β7, and $\alpha_6$ [13,43]. These variants have between 1.5-fold and 181-fold longer half-lives than the wild type. Bovine TAFIa naturally has two of these mutations, Thr233Ile and Thr236Ile, and therefore it was expected to be more stable than human TAFIa [13]. Interestingly, $\alpha_5$–Lx5β7–β7 is disordered and flexible in the unbound human TAFI structure, and somewhat better defined in its complex with GEMSA (see Fig. 2 in [12]). In contrast, in the corresponding region of human TAFIa, locally high TDPs are only found within segment 231–238, peaking at Asn2356 (Fig. 4). This reduction in the flexible segment span in complexed TAFIa, if compared with TAFI, can be attributed to stabilization by TCI, which interacts with this ‘dynamic flap’ through a hydrogen bond between Arg210 and Gln53I of TCI.

Finally, in addition to Lβ2β3 and $\alpha_5$–Lx5β7–β7, bovine TAFIa shows a peak of flexibility at Lα2α4 (Figs 3C and 4 [27]). This segment is undefined from Glu93 to Thr97. In contrast, the equivalent region in the human enzyme, Lα2α2' + α2', is well defined by electron density (see above). The potential importance of this region for bovine TAFIa inactivation (as well as for rat and mouse; see Fig. 1 in [27]) is underpinned by the presence of Lys92, which should be freely exposed to solvent and targetable by trypsin/plasmin. In contrast, human TAFIa harbors an isoleucine at this position.

**Proposed mechanism of human TAFIa inactivation**

The data we report in this article add to previous biochemical and structural work [12,13,21,27], and confirm that human (and also bovine and rodent) TAFIa is structurally unstable and poorly soluble, and thus prone to aggregation [2,22,43]. These properties, which reduce the availability of active enzyme in vivo, constitute the main mechanism of TAFIa regulation, as no specific endogenous inhibitors have been described. This mechanism is supported by studies on point mutants with enhanced stability and more persistent activity than the wild-type protein [43]. It differs strongly from the widespread regulation of activity through protein inhibitors or proteolytic inactivation through other proteases, as found, for example, in...
other proteases of the blood coagulation cascade [45]. The instability of TAFIα contrasts with the behavior in vivo of structurally related pancreatic counterparts such as human carboxypeptidase B1, which are robust, soluble, and stable. This difference is consistent with the distinct biological function of these enzymes, and accounts for the differences in their half-lives in vivo. The action of TAFIα during blood coagulation/fibrinolysis is transient, unlike that of pancreatic funnelins in the digestive tract.

Accumulating structural evidence pinpoints two and three hotspots for instability, respectively, in human (L8β[3] and α5-L8β[7]–β7) and bovine (the preceding plus L8β[4]) TAFIα, thus highlighting slight differences in the inactivation mechanism among species. These hotspots, together with reduced solubility, may serve as initiators for enhanced mobility of structurally dynamic segments, which eventually reduce temperature-dependent stability and disrupt the enzyme moiety. The intrinsic instability and poor solubility of TAFIα are already latent in TAFI and in complexes with small molecule and protein inhibitors, as indicated by the presence of regions with high dynamic segment mobility. However, the interaction of the enzyme moiety with the pro-domain or protein inhibitor bodies reduces instability, so that these complexes can be isolated and crystallized. In the case of the zymogen, the presence of four glycosylation sites within the pro-domain further contributes to solubility.

Enzyme destabilization leads to structurally triggered activity decay and aggregation in vitro. In vivo, destabilization may lead to proteolysis, first at selected bonds (e.g. Arg210, Lys235 and Arg237 in bovine and human TAFIα, and Arg57 only in bovine TAFIα) and subsequently, possibly, at newly exposed sites. Flexible regions on the molecular surface of a protein structure can be directly correlated with proteolytic susceptibility [46], especially in a protease-rich medium such as blood. Eventually, cleaved TAFIα might be cleared from the bloodstream, being putatively ‘shuttled’ by α2-macroglobulin and pregnancy zone protein [47].

Given the role of TAFIα in the downregulation of plasmin activation and fibrinolysis, which may lead to blood coagulation disorders, there is a need for very tight regulation. Inhibition of TAFIα has already been identified as an emerging biomedical target for thrombolysis and thrombolytic therapies [3,48]. It is conceivable that TAFIα has evolved to become suited for self-destruction through instability and reduced solubility, which guarantee spontaneous inactivation and rapid clearance at the site of action.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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