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SHORT COMMUNICATION

The crystal structure of protease Sapp1p from *Candida parapsilosis* in complex with the HIV protease inhibitor ritonavir

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Abstract

Secreted aspartic proteases (Saps) are extracellular proteolytic enzymes that enhance the virulence of *Candida* pathogens. These enzymes therefore represent possible targets for therapeutic drug design. Saps are inhibited by nanomolar concentrations of the classical inhibitor of aspartic proteases pepstatin A and also by the inhibitors of the HIV protease, but with the K_i of micromolar values or higher. To contribute to the discussion regarding whether HIV protease inhibitors can act against opportunistic mycoses by the inhibition of Saps, we determined the structure of Sapp1p from *Candida parapsilosis* in complex with ritonavir (RTV), a clinically used inhibitor of the HIV protease. The crystal structure refined at resolution 2.4 Å proved binding of RTV into the active site of Sapp1p and provided the structural information necessary to evaluate the stability and specificity of the protein-inhibitor interaction.

Keywords: Secreted aspartic protease, virulence factor, X-ray structure, candidiasis

Introduction

The treatment strategy used against HIV/AIDS relies on a combination of at least three compounds: two nucleoside reverse transcriptase inhibitors, and one non-nucleoside reverse transcriptase inhibitor or one protease inhibitor (PI). This regimen, known as Highly Active Antiretroviral Therapy (HAART) leads to a dramatic decrease in viral loads and an improvement in CD4 counts¹. The marked reduction of oropharyngeal candidiasis (OPC), which is one of the common HIV-related diseases, has been observed particularly in the patients receiving treatment with protease inhibitors. It has been hypothesized that HIV PR inhibitors are active also against aspartic proteases secreted by *Candida* species, and can thus alleviate opportunistic candidiasis².

Most of the pathogenic yeasts of the genus *Candida* possess a gene family encoding secreted aspartic proteases (Saps) that are important for the virulence of these

microorganisms. Saps belong to the same family as HIV PR, sharing the catalytic mechanism and some of the structural features. They participate in the establishment of infection by facilitating adhesion to host surfaces. Saps also degrade host defense molecules such as the Fc portion of immunoglobulin molecules and contribute to host tissue damage^{3,4}. Pepstatin A, a specific inhibitor of aspartic proteases, was found to exert a protective effect in the early stages of a *Candida* attack, and comparable results were obtained also with the HIV PR inhibitors ritonavir, amprenavir, saquinavir, nelfinavir, indinavir^{5–9}. These studies also indicate that protease inhibitors may be active at least against surface mycoses including OPC, although in deep-seated systemic infection models the efficacy of protease inhibitors has not been unequivocally proven¹⁰.

A doubt concerning the anti-fungal effect of HIV PR inhibitors arises from high K_i values that were obtained

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for these compounds and Saps from several pathogenic *Candida* species^{10,11,12}. While pepstatin A and its analogs inhibit Saps in nanomolar concentrations, inhibition constants for Saps and HIV PR inhibitors ritonavir, saquinavir, indinavir and nelfinavir are within the micromolar range or higher. It is therefore questionable, whether OPC reduction in AIDS patients should be attributed to the inhibition of Saps, or rather to the overall recovery of host anti-microbial immunity. On the other hand, micromolar concentration of HIV PR inhibitors can be regularly found in the plasma of patients under the HAART treatment¹³.

To prove the binding of HIV PR inhibitors to *Candida* secreted aspartic proteases on a molecular level, we co-crystallized Sapp1p, the secreted protease of *Candida parapsilosis*, in complex with HIV-1 PR inhibitor ritonavir (RTV).

OPC is most frequently caused by *C. albicans*, however, other *Candida* species are being increasingly isolated from oropharyngeal lesions, and *C. parapsilosis* often ranges as the second most common OPC causative agent^{14,15}. Ritonavir (RTV) was designed by Abbot laboratories and gained FDA approval in 1996 under the name Norvir¹⁶. Due to the frequent occurrence of side effects and a high cross-resistance with other PIs, its use as a HIV protease inhibitor was gradually abandoned. Unexpectedly, RTV has proven to be a potent inhibitor of cytochrome P-450 3A4, the microsomal enzyme responsible for the bulk of the metabolism of other HIV PR inhibitors. Therefore, RTV is currently used almost exclusively as a pharmacokinetic boosting agent.

The crystal structure of Sapp1p from *Candida parapsilosis* in complex with ritonavir was determined at a resolution of 2.4 Å. The interactions of RTV in the active site of Sapp1p were analyzed and compared to the available structure of HIV-1 PR in complex with RTV and the structure of Sapp1p complex with pepstatin A, which is the classical inhibitor of aspartic proteases.

Materials and methods

Protein preparation

Sapp1p was purified from its natural source. *C. parapsilosis* strain P-69 was obtained from the mycological collection of the Faculty of Medicine, Palacky University, Olomouc, Czech Republic. The yeasts were cultivated in a YCB-BSA medium (1.2% yeast carbon base, 0.2% BSA, 15 mM sodium citrate, pH 4.0). Sapp1p was purified from the cell-free supernatants obtained from *C. parapsilosis* cultivation for 72 h at 30°C in a rotation shaker. The cells were harvested by centrifugation (5000g for 15 min). The supernatant was filtered using Stericup (Millipore, Billerica, MA, USA). The cell-free supernatant containing secreted proteases was applied to an SP Sepharose fast flow (Sigma-Aldrich, St. Louis, MO, USA) equilibrated with 15 mM of sodium citrate pH 4.0. The fractions containing Sapp1p/Sapp2p were eluted with gradient 0–300 mM NaCl, collected and concentrated using

an Amicon Ultra – 30k (Millipore) to a volume of 5 ml. The concentrate was loaded on the HiLoad Superdex™ 75 26/60 (GE Healthcare, Piscataway, NJ, USA) column equilibrated in 25 mM Bis-Tris HCl, pH 6.3. After gel chromatography, the active fractions containing the mixture of Sapp1p and Sapp2p were collected. Sapp1p and Sapp2p were separated by chromatofocusing on Mono P 5/200 GL (GE Healthcare) column equilibrated with 25 mM Bis-Tris, pH 6.3. Elution of Sapp1p and Sapp2p was performed using a gradient of 10% polybuffer 74, pH 4.0 (GE Healthcare). The efficiency of purification steps was analyzed using SDS-PAGE, Western blotting, and activity assays. Protein analyses and proteolytic activity assays were previously described¹⁷.

Protein crystallization

The Sapp1p-ritonavir (RTV) complex was prepared by mixing the enzyme with a 100-fold molar excess of RTV (dissolved in dimethyl sulfoxide) and concentrated by ultrafiltration to a concentration of 25 mg·ml⁻¹ using Amicon Ultra– 0.5 ml 30k (Millipore). Initial crystallization trials were performed with the help of the Gryphon crystallization workstation (Art Robbins, Sunnyvale, CA, USA) by the vapor diffusion method in sitting drop mode at 19°C in 96-well plates; 0.2 µl protein solution was mixed with a 0.2 µl well solution and the mixture was equilibrated over a 50 µl reservoir solution. The PEGs Suite and JSCG Core I Suite (QIAGEN, Germantown, MD, USA) were used for the initial crystallization condition screen. Initial microcrystals appeared after several days in various conditions containing 100 mM MES pH 6.5 and PEG 200–400 as the precipitant. Further optimization was done manually, and involved changing to the hanging drop mode in the 24-well crystallization plates (EasyXtal DG-Tool, QIAGEN). Crystals were obtained by mixing 3 µl of the protein-RTV complex solution with 1 µl of reservoir solution composed of 100 mM MES pH 6.5, and 30% PEG 400. Crystals appeared after one day in the form of crystal clusters (Supplementary Figure S1) and reached a full size of 200 × 150 × 100 µm within 2 days. These crystals were unstable and during several days started to disintegrate. For data collection, one crystal was removed from the cluster and cryocooled in liquid nitrogen after 2 days of growth.

Data collection and structure determination

Diffraction data for the 2.4 Å resolution set were collected at 100 K using the MX14.2 beamline at BESSY, Berlin, Germany. Data were integrated and reduced using MOSFLM¹⁸ and scaled using SCALA¹⁹ from the CCP4 suite²⁰. Crystal parameters and data collection statistics are given in Table 1.

Structure was determined by molecular replacement using the program Molrep²¹. The search model was derived from the structure Sapp1p-pepstatin A inhibitor complex structure (PDB code 3FV3¹⁷). Model refinement was carried out using the program REFMAC 5.2²² from the CCP4 package²⁰. Manual building was performed using Coot²³. Medium NCS (non-crystallographic symmetry)

Table 1. Crystal data and diffraction data collection and refinement statistics.

Data collection statistics	
Wavelength (Å)	0.917
Space group	$P2_12_12_1$
Cell parameters (Å)	61.6 61.8 158.0
Resolution range (Å)	43.6–2.4 (2.46–2.40)
Number of unique reflections	22587 (1807)
Multiplicity	4.9 (4.9)
Completeness (%)	92.5 (99.9)
R_{merge}^a	0.093 (0.388)
Average $I/\sigma(I)$	8.9 (3.0)
Wilson B (Å ²)	47.2
Refinement statistics	
Resolution range (Å)	157.77–2.40 (2.46–2.40)
No. of reflections in working set	20570 (1652)
No. of reflections in test set	1131 (89)
R value (%) ^b	24.9 (33.4)
R_{free} value (%) ^c	31.2 (37.8)
RMSD bond length (Å)	0.02
RMSD angle (°)	1.9
Number of atoms in AU	10124
Number of water molecules in AU	4
Mean B value protein / solvent (Å ²)	54.1/36.5
Ramachandran plot statistics ^d	
Residues in favored regions (%)	94.5
Residues in allowed regions (%)	4.6

Values in parentheses refer to the highest-resolution shell.

^a $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is an individual intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity of reflection hkl with summation over all data.

^b $R = \|F_o\| - \|F_c\| / \|F_o\|$, where F_o and F_c are the observed and calculated structure factors, respectively.

^c R_{free} is equivalent to R value but is calculated for 5% of the reflections chosen at random and omitted from the refinement process³¹.

^dAs determined by MolProbity²⁵.

restraints were applied during the refinement. The final refinement steps included TLS (translation/libration/screw) refinement²⁴. The quality of the final model was validated with Molprobity²⁵. The shape complementary value²⁶ was calculated by the SC program implemented in CCP4²⁰. The reported value represents an average from the individual protein-inhibitor complexes present in the asymmetric unit. Refinement statistics are given in Table 1. Figures showing structural representations were prepared with the program PyMOL²⁷. Atomic coordinates and experimental structure factors have been deposited with the Protein Data Bank with the code 3TNE.

Results and discussion

Crystal structure

The crystal structure of the *Candida parapsilosis* Sapp1p, produced from its natural source, in complex with ritonavir (RTV) was determined by molecular replacement and refined using data to 2.4 Å resolution (Figure 1A).

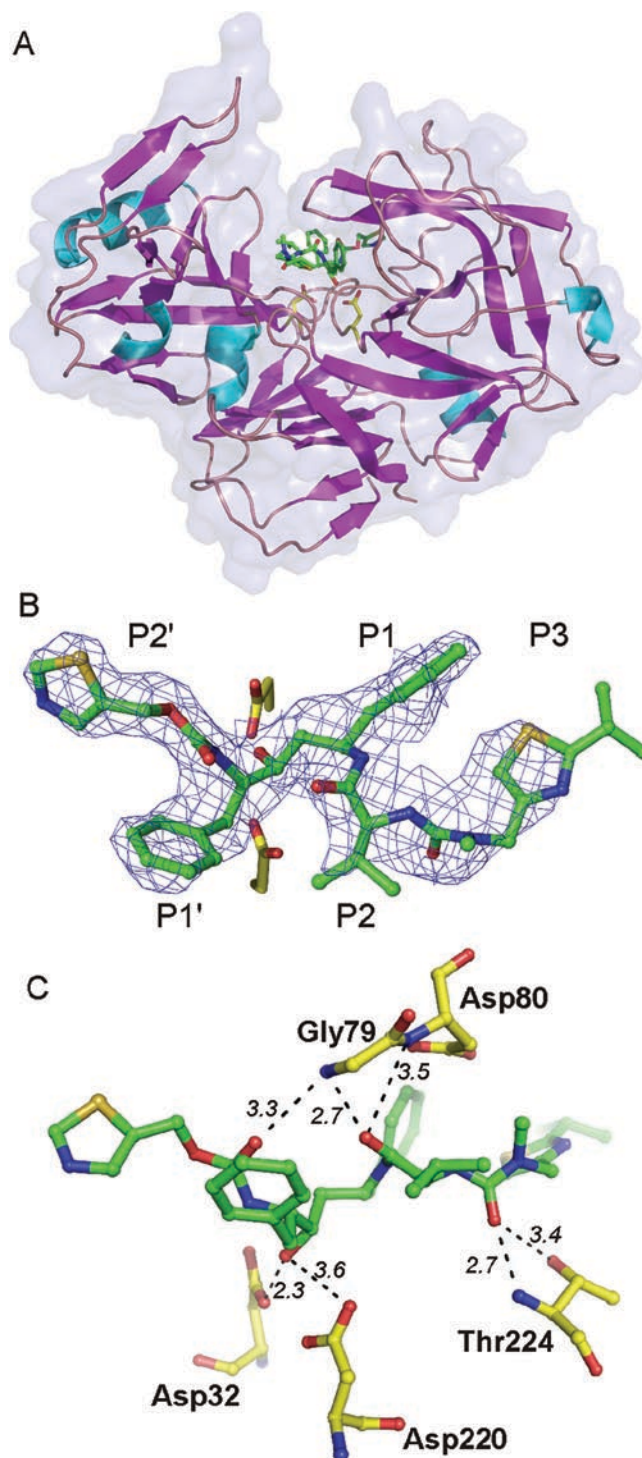


Figure 1. (A) Overall three dimensional structure and secondary structure elements of Sapp1p complexed to RTV. The protein in the ribbon representation is colored by secondary structure, the RTV molecule is represented by sticks. The transparent protein solvent accessible surface area is also shown. (B) The $2F_o - F_c$ electron density map for RTV is contoured at 0.8σ . The carbon atoms of RTV are shown in green, oxygen, nitrogen and sulfur atoms are colored red, blue and golden, respectively. Two catalytic aspartates are depicted with carbon atoms colored yellow. (C) The detail of the RTV interaction with residues in the Sapp1p active site. Hydrogen bonds are shown as dashed lines; the number represents distance between hydrogen bond donor and acceptor in Å.

Obtaining a well-shaped single crystal for the diffraction analysis of the Sapp1p-RTV complex represented a major obstacle. Due to the low affinity of RTV toward Sapp1p, a mixture of different protein species (Sapp1p in free form and complexed with RTV) was present in the crystallization drops. This heterogeneity resulted in a suboptimal crystal nucleation and growth, and resulted in the formation of crystal clusters (Supplementary Figure S1).

As revealed by diffraction analysis, the individual parts of the clusters belonged to two different crystal forms with diverse compositions. Crystal form I belonged to the $P2_12_12_1$ space group with cell dimensions $a=87.45$ Å, $b=87.69$ Å, and $c=160.5$ Å. Crystal form I contained four protein subunits in the unit cell with no inhibitor present in active site. Crystal form II belonged to space group $P2_12_12_1$ with cell dimensions $a=61.6$ Å, $b=61.8$ Å, and $c=158.0$ Å. The crystal form II contained two protein subunits in the unit cell and the continuous map for RTV was found in the active sites of both protein molecules. The optimization of crystallization conditions to obtain single crystals of crystal form II failed even at an extremely high excess of inhibitor over protein and thus a part of the crystal cluster was used to collect a complete dataset. The diffraction pattern consisted of diffraction from several crystal lattices (Supplementary Figure S1); only the major lattice was selected during the data processing and we ascertained that the error models for data integration and scaling were properly chosen. Data collection statistics are shown in Table 1.

The orthorhombic crystal form contains two protein-inhibitor complexes in the asymmetric unit (AU) with a solvent content of 36.7%. The final crystallographic model consists of two molecules of the Sapp1p-RTV complexes, each molecule containing 339 protein residues and one RTV molecule.

The two protein chains in the AU are quite similar: the root mean square deviation (RMSD) for superposition of 339 C α atoms is 0.281 Å; a value within the range observed for different crystal structures of identical proteins²⁸. The refined Sapp1p-RTV structure shows no significant conformational changes in the protein chain compared to the structure of the same protein in complex with pepstatin A (PDB code 3FV3¹⁷). The RMSD for superposition of 339 C α atoms of different protein chains was 0.445–0.502 Å.

All atoms of RTV were modeled into an electron density map with a well-defined central region and rather disordered parts for substituents in positions P2 and P3 (Figure 1B). Due to a suboptimal crystal and consequently the diffraction data quality, the quality of the resulting crystal structure is also suboptimal. Nevertheless, all crystallographic statistical indicators of structure quality are acceptable (see refinement statistics in Table 1) and the crystallographic model can evidently be used to analyze the structural features of RTV binding to Sapp1p.

Inhibitor binding

Sapp1p structure is composed of two domains forming a central binding cleft that accommodates a substrate (Figure 1A). Each domain provides one catalytic aspartate positioned at the bottom of the active site. The active site is covered by an anti-parallel β -sheet flap (residues 71–89), a common feature of aspartic proteases.

RTV binds to the Sapp1p active site in an extended conformation and occupies the S2-S3' enzyme substrate binding subsites. The hydroxyl group of the central RTV moiety forms two hydrogen bonds with the side-chains of the two catalytic aspartates Asp32 and Asp220 (Figure 1C). Additional polar interactions involve hydrogen bonds with main-chain atoms of Ser35 (N), Gly79 (N), and Thr224 (N), and also a side-chain of Thr224 (O γ 1) (Figure 1C). Upon the complex formation, RTV buries 313 Å² of solvent accessible surface area, which represents 92.3% of its total solvent accessible surface area.

Superposition of our structure with pepstatin A (PepA), a typical inhibitor of aspartic proteases, bound to the active site of Sapp1p is shown in Figure 2A. The K_i values for inhibition of Sapp1p by PepA and RTV are 0.3 nM¹¹ and 1.9 μ M¹¹, respectively, a difference of four orders of magnitude. A comparison of inhibitor interactions in the Sapp1p active site showed suboptimal interactions of RTV over PepA. The central hydroxyl group of RTV and PepA interacts with catalytic aspartates in a similar way, however the interactions with individual substrate binding pockets differ substantially. The only exception is the P2 substituent, which is structurally similar in RTV and PepA. Bulky aromatic substituents in positions P1 and P1' of RTV form very few interactions (P1' phenyl) or even some unfavorable interactions (e.g. P1 phenyl with Ile30) with their corresponding Sapp1p substrate binding pockets. Thiazolyl substituents in P3 and P2' positions do not fit into corresponding S3 and S2' sites. Suboptimal filling of the enzyme subsite binding pockets by RTV can be documented by a comparison of the shape complementarity of the Sapp1p active site with RTV and PepA, respectively. The shape complementarity (SC²⁶) value for PepA is 0.80 ± 0.02 , while the SC value for RTV is 0.68 ± 0.02 . Such a low value points to a limited stability and specificity of the protein-ligand interaction.

RTV was designed and selected to fit the active site of HIV-1 PR and the reported K_i value for inhibition of HIV-1 PR with RTV is 0.12–0.17 nM^{29,30}. The superposition of the Sapp1p structure with the structure of HIV-1 protease is shown in Figure 2B and the superposition of the binding modes of RTV in the Sapp1p and HIV-1 PR binding sites, respectively, is depicted in Figure 2C. The central part of RTV binds similarly to HIV-1 PR and Sapp1p as a result of the sequence and structural conservation of the catalytic triads in the aspartic proteases. Position of P1 and P1' substituents are also quite similar, although the conformation of the aromatic rings differs. The substituent in positions distal from the central hydroxyl of RTV differ in structure, especially the position of thiazolyl groups in P2 and P3', which were specifically designed to form

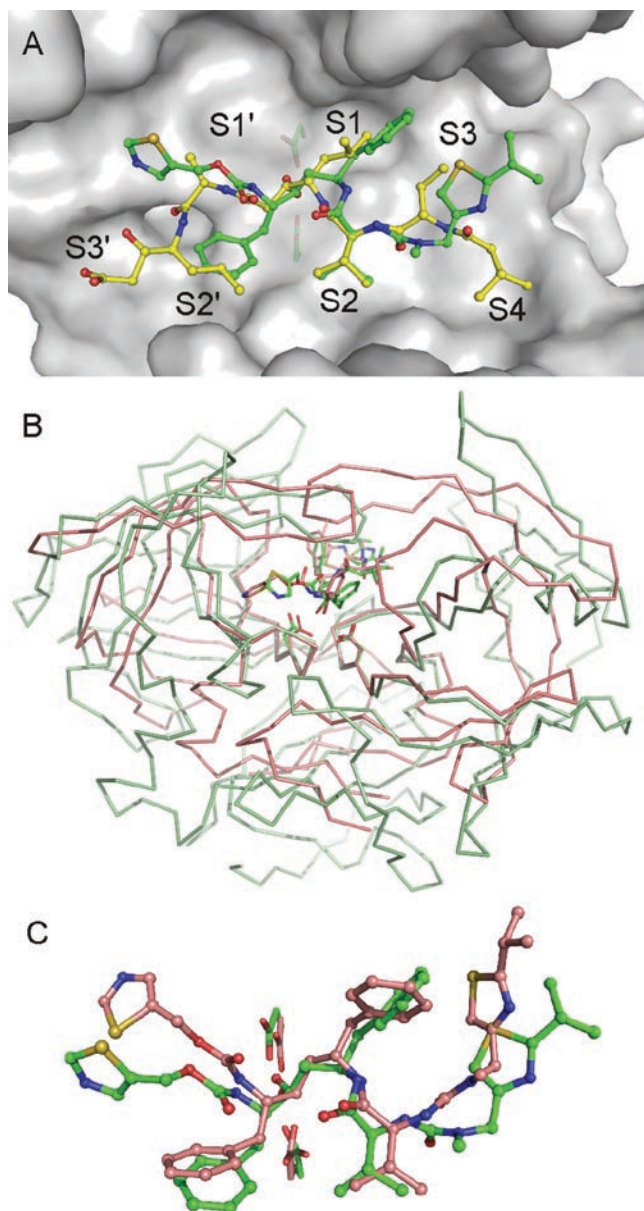


Figure 2. (A) Comparison of RTV and PepA binding modes in the Sapp1p active site. Carbon atoms of RTV and PepA are shown in green and yellow, respectively. Oxygen, nitrogen and sulfur atoms are colored red, blue and golden, respectively. Protein is shown by a solvent accessible surface area with underlying catalytic aspartates shown as sticks. Substrate binding sites S4-S3' are labeled. Flap region (residues 71–89) is omitted from the figure for clarity. (B) Superposition of Sapp1p and HIV-1 PR complexes with RTV. Structure of HIV-1 PR in complex with RTV (PDB code 1HXW¹⁶) is colored pink; the structure of Sapp1p in complex with RTV is colored green. Superposition was done by matching the catalytic triads (catalytic aspartates are highlighted as sticks at the bottom of the active sites). (C) Comparison of RTV binding modes in the active sites of Sapp1p (carbon atoms colored green) and HIV-1 PR (carbon atoms colored pink).

an optimal interaction with hydrophobic residues Pro81 and Val82 of HIV-1 PR in the S3 subsite, which is not obviously present in Sapp1p.

In conclusion, the structural analysis showed that RTV can specifically bind to the Sapp1p active site and forms

specific polar interactions with catalytic aspartates and additional hydrogen bonds with the residues in S1 and S1' subsites. The interaction of the central part of RTV with the enzyme catalytic site is similar to the interaction of PepA with Sapp1p as well as to interaction of RTV with HIV-1 PR. Non-polar interactions of RTV with Sapp1p are quite limited. The low shape complementarity of RTV with individual substrate binding subsites results in a low inhibition efficiency of RTV toward Sapp1p in the micromolar range. Although the structure of the Sapp1p-RTV complex by itself does not explain the reported anti-mycotic activity of the HIV protease inhibitors, it sheds light on the concept of a multi-target effect of ritonavir.

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Declaration of interest

The authors declare no conflicts of interest.

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