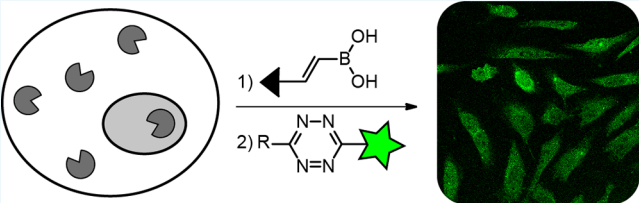# Vinylboronic Acids as Efficient Bioorthogonal Reactants for Tetrazine Labeling in Living Cells

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S *Supporting Information*

ABSTRACT: Bioorthogonal chemistry can be used for the selective modification of biomolecules without interfering with any other functionality present in the cell. The tetrazine ligation is very suitable as a bioorthogonal reaction because of its selectivity and high reaction rates with several alkenes and alkynes. Recently, we described vinylboronic acids (VBAs) as novel hydrophilic bioorthogonal moieties that react efficiently with dipyridyl-*s*-tetrazines and used them for protein modification in cell lysate. It is not clear, however, whether VBAs are suitable for labeling experiments in living cells because of the possible coordination with, for example, vicinal carbohydrate diols. Here, we evaluated VBAs as bioorthogonal reactants for labeling of proteins in living cells using an irreversible inhibitor of the proteasome and compared the reactivity to that of an inhibitor containing norbornene, a widely used reactant for the tetrazine ligation. No large differences were observed between the VBA and norbornene probes in a two-step labeling approach with a cell-penetrable fluorescent tetrazine, indicating that the VBA gives little or no side reactions with diols and can be used efficiently for protein labeling in living cells.

In the last years, the development of reactions that are unaffected by any of the molecular functionalities in a biological system has emerged as a major field of research in chemical biology.[1−4] These bioorthogonal reactions are used for tagging biomolecules with a high reaction rate by a two-step approach *in vitro* and *in vivo*, without the need to attach these tags directly onto the biomolecule. The reactants should be nontoxic to the cellular environment and should not influence the function of the labeled biomolecule of interest; therefore, a small hydrophilic reactant is often preferred. One of the most popular bioorthogonal reactions is the inverse electron-demand Diels−Alder (iEDDA) reaction between electron-poor tetrazines[5−8] and electron-rich or strained alkenes or alkynes, e.g., *trans*-cyclooctene,[9,10] norbornene,[11] or cyclopropene.[12,13] Recently, we reported a new nonstrained bioorthogonal reactant, vinylboronic acid (VBA), which reacts, depending on its substituents, with second-order rate constants (k_2) up to 27 $M^{-1} s^{-1}$ with 3,6-dipyridyl-*s*-tetrazines. These VBAs are 1 order of magnitude faster than the commonly used tetrazine reactant norbornene (Figure 1A).[14] We further showed that the VBA moiety is biocompatible with cellular components, stable in cell lysate, and applicable for protein modification.

Over the past years, boronic acids have been used in living cells for various applications.[15] The concept that phenylboronic acid moieties can form reversible complexes with 1,2- and 1,3-*cis*-diols in aqueous environments has been exploited in the development of chemosensors for carbohydrates (Figure 1C).[16] The rate of this condensation reaction is dependent on the pK_a of the phenylboronic acid, where electron-withdrawing substituents on the phenyl ring lower the pK_a and thereby favor binding to the diols.[17,18] Despite the potential

condensation reaction with vicinal diols present in and on the cell, many boronic acid-containing compounds have been successfully used inside living cells such as protease inhibitors,[19] sensors for reactive oxygen species,[20] and reactants in bioconjugation reactions.[21−23] It is not clear, however, if or to what extent the carbohydrate-rich cellular environment interferes with the cellular uptake and distribution of these boronic-acid containing compounds or with molecules containing our bioorthogonal VBA moiety.

To investigate the uptake and bioorthogonality of the VBA moiety in living cells, we chose a two-step labeling strategy of the proteasome using a VBA-modified irreversible inhibitor, followed by visualization using a dipyridyl-*s*-tetrazine functionalized with a fluorophore (Figure 1B). The proteasome is a large multisubunit proteolytic complex that is mainly responsible for the degradation of proteins into small peptides by the ubiquitin-proteasome pathway.[24−26] The proteolytic core contains seven distinct α and β subunits, whereas the β1, β2, and β5 subunit are responsible for the proteolytic activities of the proteasome. These proteolytic subunits act as N-terminal threonine proteases, where the nucleophilic hydroxyl attacks the peptide carbonyl causing cleavage of the peptide bond. Recent progression in the development of inhibitors of the proteasome or of only one of the proteolytic subunits is extensively reviewed.[27−29] The two-step labeling strategy of the proteolytic proteasome subunits using irreversible inhibitors
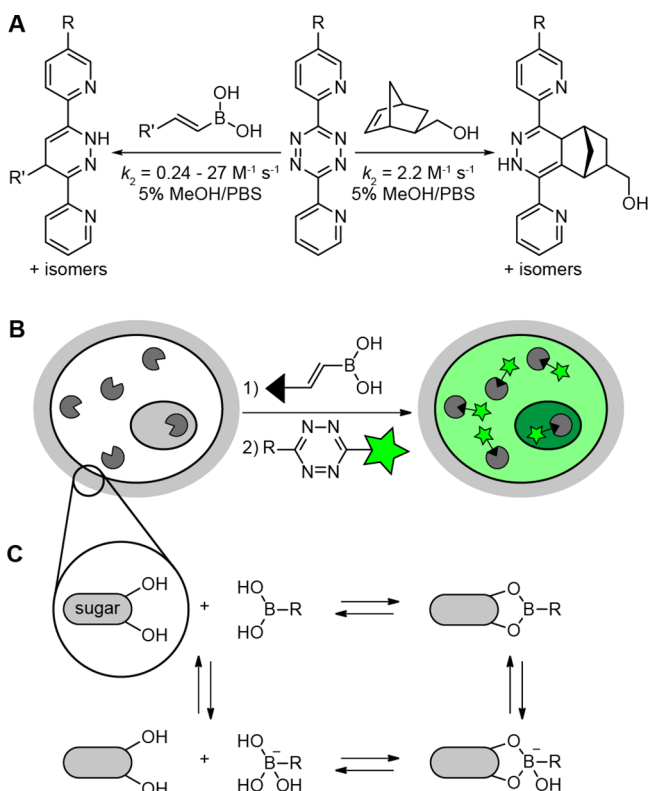
Figure 1. (A) Reported tetrazine ligation with vinylboronic acids and norbornene;[14] k_2 values were determined in 5% MeOH in PBS. (B) Two step protein labeling protocol. Addition of an irreversible protein inhibitor containing a VBA is followed by cycloaddition with a dipyridyl-s-tetrazine containing a fluorophore. (C) Equilibria of carbohydrate diols with a boronic acid.

followed by a bioorthogonal reaction with a fluorescent molecule has been previously performed using the copper-catalyzed alkyne−azide cycloaddition,[30,31] strain-promoted alkyne−azide cycloaddition,[32] the Staudinger ligation,[30,33−36] and the tetrazine ligation[30] and thereby serves as a well-established model system for our studies.

The (6-aminohexanoyl)₃-(leucinyl)₃-vinyl-(methyl)-sulfone (Ahx₃L₃VS) moiety is found to give strong inhibition of the proteasome, without being selective for any of the three catalytic subunits.[37] The three leucines are recognized by the proteasome and the vinylsulfone moiety reacts covalently and irreversibly with the γ-hydroxyl of the N-terminal threonine of the catalytic proteasome subunits. The long linker of the scaffold is built up by aminohexanoic acids that are beneficial for efficient binding to the proteasome[37] and were thought to be an advantage for the two-step labeling of the proteasome as the bioorthogonal moiety could be placed far away from the active site. Here, we describe the synthesis and application of proteasome inhibitor VBA-Ahx₃L₃VS 7, containing a protected vinylboronic acid moiety that hydrolyzes quickly in aqueous solution to the free boronic acid.[14] We compared this compound to Nor-Ahx₃L₃VS 8, which contains the commonly used bioorthogonal norbornene moiety.

The proteasome inhibitors were synthesized using modified literature procedures as depicted in Scheme 1 (SI − experimental section). In short, Boc-Leu-OH 1 was first reduced to its corresponding aldehyde 3 via Weinreb amide 2. A stabilized Wittig reaction gave trans-VS 4 containing a small amount of cis-VS that was removed using column chromatography. Next, a double round of Boc-deprotection and coupling of Boc-Leu-OH yielded Boc-L₃VS 5. Finally, Boc-

Scheme 1. Synthesis of Proteasome Inhibitors 7−9[a]
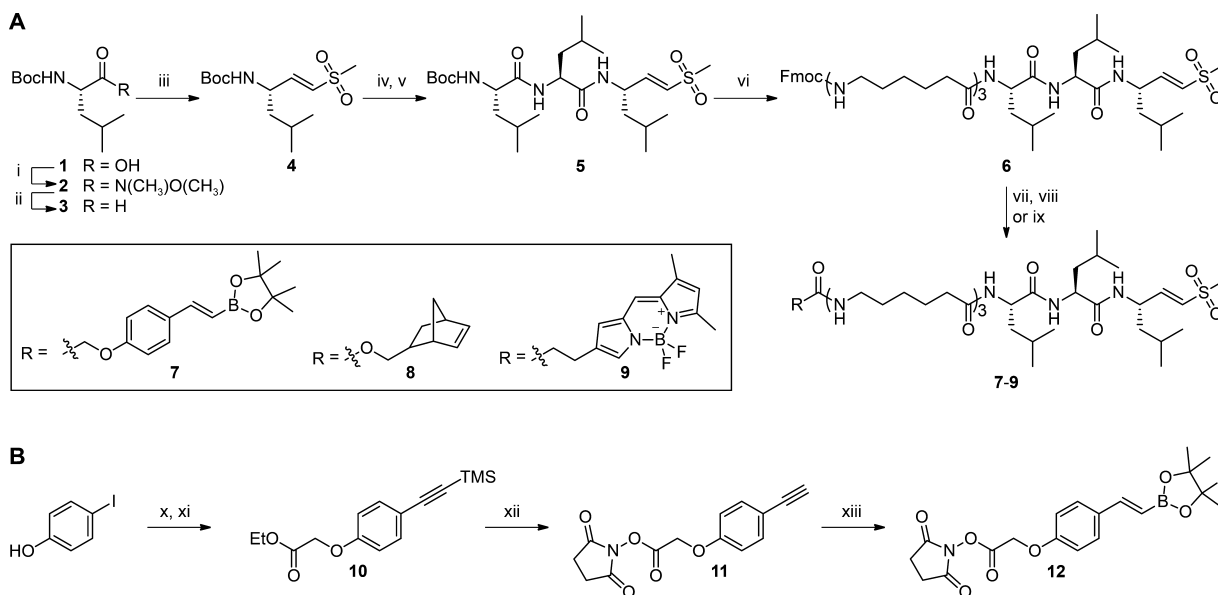
[a](i) N,O-dimethyl hydroxylamine, NMM, EDC, CH₂Cl₂, 5 h, 95%. (ii) LiAlH₄, Et₂O, 0 °C, 15 min, 81%. (iii) Diethyl(methylsulfonylmethyl)-phosphonate, NaH, THF, 0 °C, 1 h, 35%. (iv) (a) 4 M HCl in dioxane, CH₂Cl₂, 2 h, (b) Boc-Leu-OH, EDC, HOBt, Et₃N, CH₂Cl₂, o/n, 94%. (v) Same as iv, yielding 5 in 98%. (vi) (a) 4 M HCl in dioxane, CH₂Cl₂, 2 h, (b) Fmoc-Ahx₃-OH, EDC, HOBt, Et₃N, CH₂Cl₂, o/n, 54%. (vii) (a) piperidine, DMF, 15 min. (b) VBA-NHS 12, DIPEA, DMF, 1 h, yielding 7 in 73%. (viii) same as vii, only (b) with norbornene-NHS, yielding endo/exo-8 in 94%. (ix) (a) DBU, DMF, 7 min, (b) BODIPY-FL NHS ester, HOBt, DIPEA, DMF, 1.5 h, yielding 9 in 44%. (x) Ethyl chloroacetate, K₂CO₃, DMF, 16 h, 98%. (xi) Trimethylsilylacetylene, CuI, PdCl₂(PPh₃)₃, DIPEA, toluene, 30 °C, 24 h, 99%. (xii) (a) LiOH, THF/H₂O 1:1, 2 h, (b) N-hydroxysuccinimide, EDC, DMF, 16 h, 88%. (xiii) Pinacolborane, Ru(CO)ClH(PPh₃)₃, toluene, 50 °C, 16 h, 74%.

deprotection and then coupling of Fmoc-Ahx$_3$-OH, yielded Fmoc-protected Ahx$_3$L$_3$VS **6**.

Next, we prepared the NHS ester of VBA and norbornene to ensure efficient coupling to scaffold **6**, after deprotection of the Fmoc. For VBA-NHS **12**, we used the alkoxy-substituted phenylvinylboronic acid, as we previously found that this electron rich VBA gave the highest reaction rate with dipyridyl-*s*-tetrazine.[14] First, etherification of 4-iodophenol using ethyl chloroacetate followed by a Sonagashira coupling yielded TMS-protected alkyne **10** in excellent yield. Deprotection of the TMS group and coupling of the acid with *N*-hydroxysuccini-mide yielded alkyne-NHS **11**. Final hydroboration of the alkyne with pinacolborane yielded NHS-boronic ester **12**. Norbor-nene-NHS was prepared in one step from its corresponding alcohol in good yield. The VBA-NHS **12** and the norbornene-NHS were finally coupled to the free amine of Fmoc-deprotected **6**, yielding VBA-Ahx$_3$L$_3$VS **7** and Nor-Ahx$_3$L$_3$VS **8** in good yields. In addition, the fluorescent BODIPY-Ahx$_3$L$_3$VS **9** was prepared by direct coupling of the commercially available BODIPY-FL NHS ester to deprotected **6**.

Having VBA-Ahx$_3$L$_3$VS **7** and Nor-Ahx$_3$L$_3$VS **8** in hand, we initially evaluated the selectivity and potency of the inhibitors in cell lysate (Figure 2A,B). First, we established that a concentration of 0.3 μM of BODIPY-Ahx$_3$L$_3$VS **9** was essential for visualization of all proteasome subunits in cell lysate (Figure S1). Then, compounds **7** or **8** were incubated for 1 h at various concentrations in the protein lysate, after which BODIPY **9** was added to label all residual unbound proteasomal subunits (Figure 2B). Using this setup, we observed that low micromolar concentrations of inhibitor **7** and **8** were essential for full inhibition of all subunits. In addition, we observed a slight selectivity of VBA **7** for β5 compared to the other subunits, whereas norbornene **8** was more selective for β2 as well as β5.

We continued to evaluate the inhibition of the proteasome with the Ahx$_3$L$_3$VS probes **7** and **8** in living cells. Here, performing a similar competition experiment as above, a 100$\times$ higher concentration of both **7** and **8** was essential to inhibit the proteasome subunits completely. Despite the moderate cell permeability of the probes this indicates that, similarly to the norbornene handle, the VBA moiety did not hamper membrane permeability of the inhibitor (Figure 2C). Elevated concentrations for complete proteasome inhibition and the observed reduced labeling of the β1 subunit in living cells was also observed with BODIPY **9** (Figure S2) and reported previously using a different Ahx3L3VS probe.[37] Aside, significant cell death was visible when norbornene **8** was used at concentrations >100 μM, while VBA **7** did not show any toxicity up to 1 mM concentration.

Next, we investigated whether we could visualize the proteasome subunits with a two-step labeling strategy using VBA **7** or norbornene **8** followed by the tetrazine ligation with dipyridyl-*s*-tetrazine **13**, containing a BODIPY-FL fluorophore, in cell lysate as well as in living cells (Figure 3A). After inhibition of the subunits using VBA **7** and norbornene **8** in cell lysate at concentrations that showed full subunit inhibition as established above (3 μM), about 3 μM of tetrazine **13** was necessary to give a complete reaction (Figure S3). In living cells, after inhibition of the proteasome with 300 μM VBA **7** and 100 μM norbornene **8**, a comparable concentration of 10 μM tetrazine **13** was necessary to visualize the subunits (Figure 3B and C). To our delight, similar labeling intensities of the proteasome subunits were observed using the probes VBA **7**
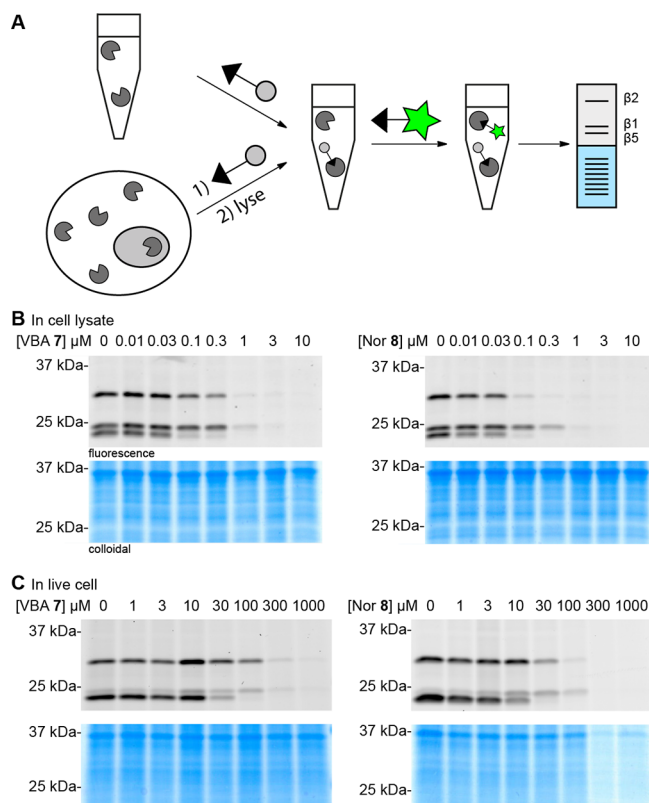

Figure 2. (A) Schematic figure of the competition assay of VBA **7** or norbornene **8** using BODIPY **9** in cell lysate and living HeLa cells. (B) SDS-PAGE analysis of the competition assay in cell lysate (10 μL of 1 mg/mL), which was first incubated with **7** or **8** (indicated concentration) for 1 h at 37 °C and next with **9** (0.3 μM) for 1 h at 37 °C. (C) Competition assay in living HeLa cells, which were first incubated with **7** or **8** (indicated concentration) for 3 h at 37 °C, whereupon the cells were lysed and the lysate (10 μL of 1 mg/mL) was incubated with **9** (0.3 μM) for 1 h at 37 °C. In-gel fluorescence (top) was measured at 488 nm followed by colloidal staining (bottom) as a loading control of the protein lysates.

and norbornene **8** in lysate and in living cells, as evidenced by SDS-PAGE gel.

In addition, we evaluated the stability of the VBA and norbornene handles in living cells and the efficiency of the tetrazine ligation by monitoring the protein labeling over the course of time. After inhibition of the proteasome with VBA **7** and norbornene **8** (300 μM and 100 μM, respectively), the cells were lysed and the subsequent tetrazine ligation was performed with tetrazine **13** for various amounts of time. Complete labeling of the subunits was observed within 2−5 min, indicating that both functionalities were intact and the reaction was very efficient (Figure S4).

Finally, we used the same two-step labeling protocol to visualize the proteasome in living HeLa cells by confocal microscopy (Figure 3B and D). Again, similar intensities were observed using VBA **7** or norbornene **8** followed by addition of 3 μM of tetrazine-BODIPY **13**, indicating successful labeling of the proteasome. For comparison, the labeling pattern of BODIPY **9** was measured, which was similar to that observed for **7** and **8**, indicating that the VBA, norbornene, and BODIPY labels did not significantly influence the cellular distribution of the inhibitors (Figure S5). Performing the ligation with a higher concentration of tetrazine **13** using the same microscopy settings resulted in higher labeling intensities; however, in this
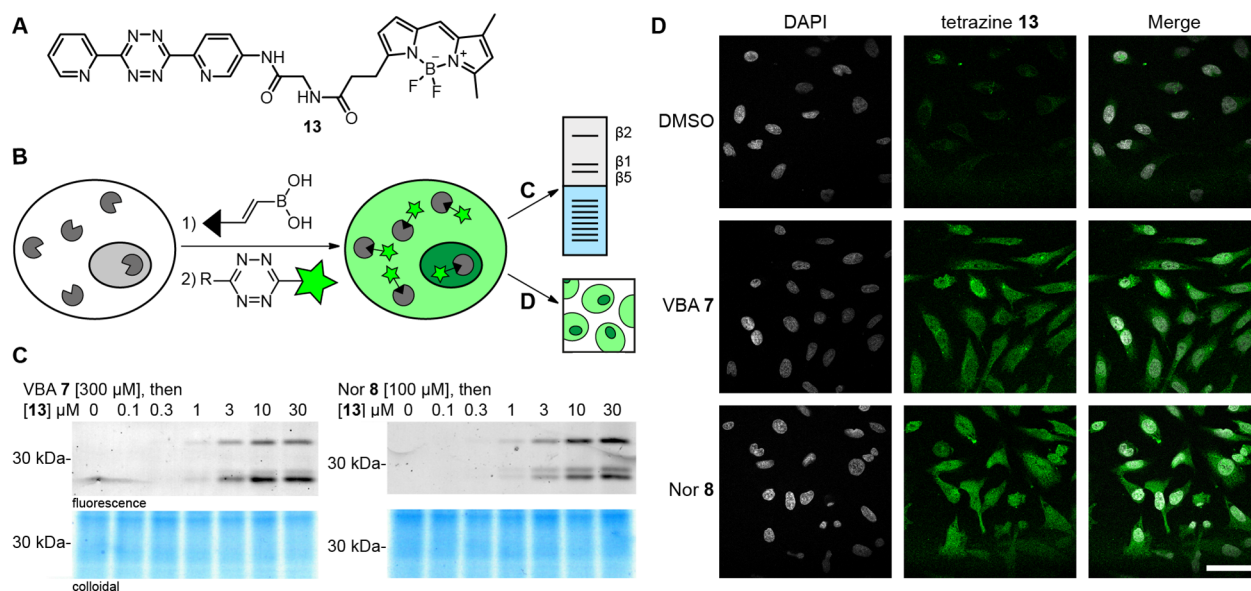
Figure 3. (A) Structure of the cell-permeable tetrazine-BODIPY **13**. (B) Schematic figure of the two-step labeling of the proteasome. (C) SDS-PAGE fluorescence analysis of a concentration range of tetrazine **13** in the two-step labeling of the proteasome subunits with VBA **7** (300 μM) or norbornene **8** (100 μM) in living cells. (D) Confocal microscopy of the two-step labeling of the proteasome. HeLa cells were first incubated with DMSO, VBA **7** (300 μM), or norbornene **8** (100 μM) for 3 h at 37 °C, after which tetrazine **13** (3 μM) was applied. Scale bar = 50 μm.

case also an increased background signal was observed (Figure S6).

In summary, we evaluated the use of the vinylboronic acid functionality for bioorthogonal labeling with tetrazines in living cells. The synthesized proteasome inhibitor containing a vinylboronic acid was successfully used for the two-step labeling of the proteasome in cell lysate and in living cells without showing diminished labeling efficiency with a dipyridyl-s-tetrazine compared to the proteasome inhibitor functionalized with a norbornene moiety. We have to emphasize that the potential condensation of the VBAs to vicinal diols would not be visible on SDS-PAGE gel or on confocal microscopy, as the boronic acid is released after the tetrazine ligation. However, if significant condensation would occur, a higher concentration of VBA **7** would be essential to fully inhibit the proteasome subunits. Our results show that three times more VBA **7** compared to norbornene **8** was needed for complete inhibition of all subunits *in vitro* as well as in living cells, indicating that the vinylboronic acid handle does not significantly hamper cellular uptake of the probes into the cell.

The number of bioorthogonal reactions that are suitable for labeling inside living cells is limited, because of the requirement of toxic reagents (e.g., copper(I)-catalyzed alkyne−azide cycloaddition) or possible side reactions of the reactants (e.g., the thiol−ene or thiol−yne side reactions of free intracellular cysteines with strained alkenes or alkynes). We have shown that the tetrazine ligation with vinylboronic acid is little or not affected by possible side reactions that can occur in the cell with biomolecules such as carbohydrates, making the reaction a valuable addition to the bioorthogonal toolbox.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconj-chem.7b00796.

Experimental details for the synthesis and proteasome inhibition in cell lysate and in living cells, full spectroscopic data for all new compounds, and additional figures (PDF)

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Notes

The authors declare no competing financial interest.

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