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Antifungal Activity of Resveratrol Derivatives against *Candida* Species

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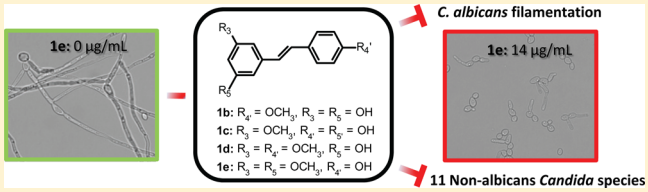
Supporting Information

ABSTRACT: *trans*-Resveratrol (**1a**) is a phytoalexin produced by plants in response to infections by pathogens. Its potential activity against clinically relevant opportunistic fungal pathogens has previously been poorly investigated. Evaluated herein are the candidacidal activities of oligomers (**2a**, **3**−**5**) of **1a** purified from *Vitis vinifera* grape canes and several analogues (**1b**−**1j**) of **1a** obtained through semisynthesis using methylation and acetylation. Moreover, *trans*-ε-viniferin (**2a**), a dimer of **1a**, was also subjected to methylation (**2b**) and acetylation (**2c**) under nonselective conditions. Neither the natural oligomers of **1a** (**2a**, **3**−**5**) nor the derivatives of **2a** were active against *Candida albicans* SC5314. However, the dimethoxy resveratrol derivatives **1d** and **1e** exhibited antifungal activity against *C. albicans* with minimum inhibitory concentration (MIC) values of 29−37 μg/mL and against 11 other *Candida* species. Compound **1e** inhibited the yeast-to-hyphae morphogenetic transition of *C. albicans* at 14 μg/mL.



trans-Resveratrol (**1a**) is a natural stilbenoid produced by several plants in response to pathogenic infections. Although present in a restricted number of plant families, **1a** forms the backbone of a wide range of stilbene biosynthetic products, leading to a spectrum of monomeric and oligomeric compounds with diverse biological activities.[1,2] Stilbenes were first described for their fungicidal action against plant fungal diseases by Langcake and Pryce.[3] Later, antimicrobial activities of stilbenes were reported against various phytopathogens, with **1a** occurring as a major phytoalexin of the family Vitaceae.[4,5] Resveratrol fully inhibits conidial germination of the gray mold agent *Botrytis cinerea*[6] and reduces roughly 75% sporangial germination of the causal agent of grapevine downy mildew, *Plasmopara viticola*.[7] Pterostilbene, the 3,5-dimethoxyresveratrol (**1e**), and *trans*-ε-viniferin (**2a**) have been found to be 5-fold more active than **1a**, indicating the potential of resveratrol derivatives as a source of effective antifungal agents.[8,9]

In the field of human health, several investigations have been conducted on **1a** due to its multiple pharmacological activities including cardioprotective, antiaging, anticarcinogenic, anti-inflammatory, estrogenic/antiestrogenic, and antioxidant properties.[10,11] However, little information is available concerning the potential antifungal activity of **1a** against common agents of human mycoses such as *Candida* yeasts.

Although the candidacidal activity of **1a** has been published against *Candida albicans* TIMM 1768[12] and against five other *Candida* species,[13] these data remain somewhat controversial.[14] Interestingly, it has been reported that semisynthetic modifications of natural stilbenes can be carried out to increase their pharmacological potential and their range of activity against new biological targets.[15−17] Considering the potential of stilbenes as fungicidal agents against phytopathogens and taking into account the large diversity of chemical structures exhibited by natural and semisynthetic stilbene derivatives, the potential of resveratrol derivatives including both natural and semisynthetic analogues as candidacidal agents was investigated.

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RESULTS AND DISCUSSION

Five methyl derivatives of *trans*-resveratrol (**1b**–**1f**; Figure 1) were synthesized from commercial **1a** in one reaction using

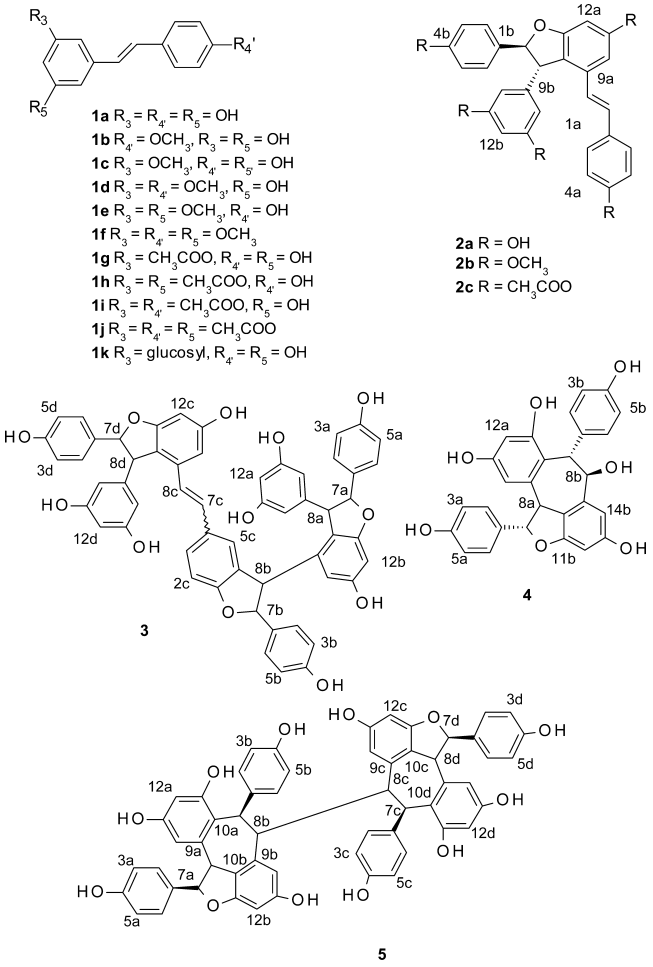


Figure 1. Structures of the resveratrol analogues investigated.

methyl iodide in the presence of sodium hydride in *N*,*N*-dimethylformamide, as described in the Experimental Section. Separation of 4′-methoxyresveratrol (**1b**), 3-methoxyresveratrol (**1c**), 3,4′-dimethoxyresveratrol (**1d**), 3,5-dimethoxyresveratrol (**1e**), and 3,5,4′-trimethoxyresveratrol (**1f**) was performed by column chromatography on silica gel with petroleum ether–ethyl acetate as the eluent. Acetyl derivatives of **1a** (**1g**–**1j**) were synthesized in one step by using acetyl chloride in a mixture of Et_3N and THF. 3-*O*-Acetylresveratrol (**1g**), 3,5-di-*O*-acetylresveratrol (**1h**), 3,4′-di-*O*-acetylresveratrol (**1i**), and 3,5,4′-tri-*O*-acetylresveratrol (**1j**) were purified on silica gel with light petroleum–ethyl acetate used for elution. The natural oligomers of **1a**, i.e., *trans*-ε-viniferin (**2a**), *cis*/*trans*-vitisin B (**3**), ampelopsin A (**4**), and hopeaphenol (**5**), were purified from grape cane extracts by centrifugal partition chromatography (CPC) and semipreparative HPLC using adapted conditions.[18,19] *trans*-ε-Viniferin pentamethyl ether (**2b**) and *trans*-ε-viniferin pentaacetate (**2c**) were obtained by treatment of **2a** with methyl iodide and acetyl chloride, respectively (Scheme 1).

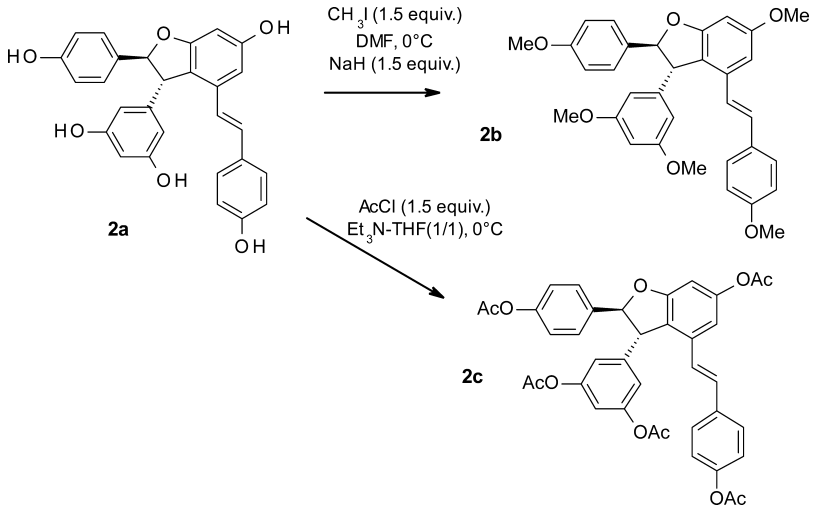
In a first series of experiments, compounds **1a**–**1k**, **2a**–**2c**, and **3**–**5** were evaluated for their in vitro antifungal activities against *C. albicans*, the most common agent in all clinical forms of candidiasis (Table 1). At the highest test concentration (300

Table 1. Inhibition of *C. albicans* SC5314 by Natural and Semisynthetic Stilbenoid Compounds[a]

compound	MIC_{95}[b] (μg/mL)
	C. albicans SC5314
1a	na[c]
1b	150
1c	300
1d	37
1e	29
1f–**1k**	na[c]
2a–**2c**	na[c]
3	300
4, **5**	na[c]
5-FC[d]	2

[a]Data are mean values of two independent experiments, each with three replicates. [b]Minimum inhibitory concentration (the lowest compound concentration causing 95% growth inhibition). [c]No activity at the highest test concentration, 300 μg/mL. [d]5-Fluorocytosine.

Scheme 1. Substitutions of *trans*-ε-Viniferin



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μg/mL) used, compounds **1a**, **1f**−**1k**, **2a**−**2c**, **4**, and **5** did not show any activity against the *C. albicans* reference strain SC5314. In contrast, five compounds displayed moderate (**1b**, **1c**, and **3**) to high (**1d** and **1e**) antifungal activities. Of note, the absence of biological activity of **1a** observed in the present work confirmed previous results.[14] Interestingly, the methyl derivatives of **1a** showed varying degrees of antifungal activity depending on the number and the position of the methyl groups present. The highest candidacidal activities were observed for the dimethoxy-resveratrol derivatives (**1d** and **1e**), with minimum inhibitory concentration (MIC) values of 29−37 μg/mL, whereas two monomethoxy-resveratrol derivatives (**1b** and **1c**) showed moderate activities, with MICs of 150−300 μg/mL. In contrast, the trimethoxy-resveratrol derivative **1f** exhibited no discernible activity. In addition, given the importance of the yeast-to-hyphae transition in the virulence of *C. albicans*,[20] the effect of **1e** was tested on the SC5314 strain morphogenetic transition (Figure 2). The results

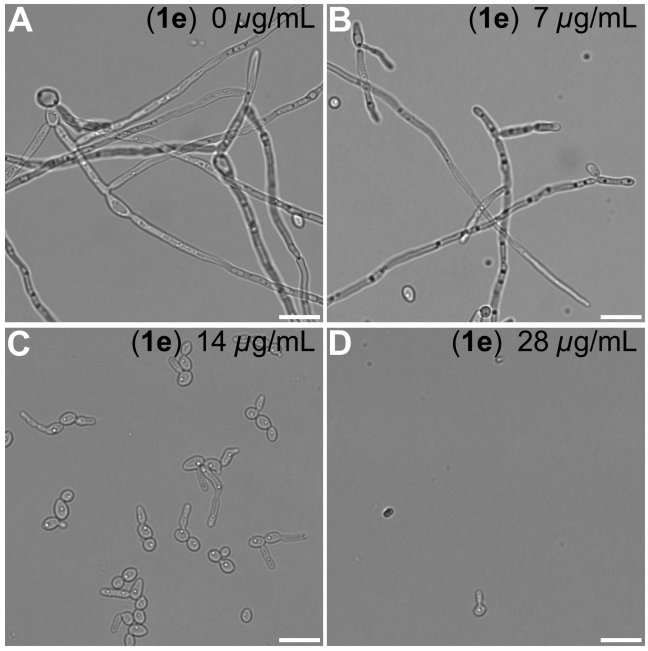

Figure 2. Effect of different concentrations of 3,5-dimethoxyresveratrol (**1e**) on the yeast-to-hyphae morphogenetic transition of *C. albicans* SC5314 after 48 h of incubation at 32 °C in RPMI medium; untreated control cells (A) and 7 (B), 14 (C), and 28 μg/mL (D) 3,5-dimethoxyresveratrol (**1e**).

showed that **1e** is able to inhibit hyphal formation for this strain at 14 μg/mL. The antifungal activity of **1e** and its capacity to disrupt hyphal formation of *C. albicans* SC5314 are in agreement with recent results.[21]

Although *C. albicans* remains the most frequent agent of candidiasis, non-*albicans Candida* (NAC) species now account for a substantial proportion of clinical isolates collected worldwide. NAC species of particular clinical importance include *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*, as well as the emerging species *C. guilliermondii*, *C. lusitaniae*, *C. kefyr*, *C. famata*, *C. inconspicua*, *C. dubliniensis*, and *C. norvegensis*.[22] In a second series of experiments, the antifungal activity was tested for compounds **1b**−**1e** and **3** against these 11 NAC species (Table 2). The results showed antifungal activities for compounds **1b** and **1e** against all the tested *Candida* species including *C. albicans*.

Therefore, the two dimethoxy-resveratrol derivatives **1d** and **1e** displayed interesting antifungal activities against a broad spectrum of yeasts of the genus *Candida*. The pharmacomodulation of **1a** by selective methoxylation showed that candidacidal activity is position-dependent, with the best activity at the 3,5-position. Although several oligomers of **1a** have shown significant activity against various phytopathogenic fungi,[23] they were not potently active against *C. albicans*, thereby suggesting the ability of this yeast to detoxify **1a** oligomers.

EXPERIMENTAL SECTION

General Experimental Procedures. CPC was carried out on an FCPC Preparative 200 Kromaton Technologies apparatus (Annonay, France). Semipreparative HPLC was conducted on a Dionex UHPLC U3000RS system using a 3 μm column (250 × 10 mm, Multospher 120 RP-18 HP; Chromatographie Service, Langerwehe, Germany). TLC was performed on silica gel sheets (silica gel 60 F254, Merck, Germany). NMR experiments were performed at 300 MHz (^{1}H) and 75 MHz (^{13}C) on a Bruker-Avance 300 MHz spectrometer, with TMS as internal reference. Mass spectra were obtained on a Varian MAT 311 and a Micromass ZAB SpecTOF mass spectrometer. Compounds **1b**−**d**, **1f**−**j**, **2a**−**2c**, **3**, **4**, and **5** were identified by comparison of their physicochemical properties (Supporting Information) with reported values.[24−32] *trans*-Resveratrol (**1a**), *trans*-resveratrol 5-*β*-monoglucoside (piceid, **1k**), and 3,5-dimethoxyresveratrol (**1e**) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Purification of Resveratrol Oligomers. Grape canes of *Vitis vinifera* L. variety "Malbec" (Vitaceae) were collected from vineyards in the wine school of Amboise (France). Grape canes were ground with a cooled analytical mill (IKA-Werke, Germany) and extracted in ethanol−water (6:4; 24 h) in a Soxhlet apparatus. After filtration, the solution was concentrated at 40 °C under reduced pressure and lyophilized. Purification procedures for **2a** and **3**−**5** were adapted from published procedures.[18,19] Dried extract (4 g) was dissolved in 20 mL of the organic/aqueous phase mixture (1:1) of Arizona L system (*n*-heptane−EtOAc−MeOH−H_2O, 3:2:3:2) and injected into the CPC apparatus. The extract was injected at 3 mL/min at low rotation speed (600 rpm), and then the rotation speed was increased to 1300 rpm. Separation was performed at 1300 rpm, with a 6 mL/min flow rate (with 5% cocurrent flow) and using a step gradient procedure in the ascending mode. The Arizona L organic phase was used as starting mobile phase for 75 min; then the mobile phase was switched to the organic phase of the Arizona K solvent system (*n*-heptane−EtOAc−MeOH−H_2O, 1:2:1:2) for an additional 75 min. Fractions were collected every minute and combined on the basis of HPLC analysis, providing a total of three fractions of interest. Fraction 1 (101 mg) corresponded to 90% pure **2a** as a yellow solid, mp 258 °C. Fraction 2 (55.8 mg) contained one tetramer of interest, presumed as **3**, along with other compounds, and was purified by semipreparative HPLC. The mobile phase consisted of aqueous trifluoroacetic acid (0.1% w/v; eluent A) and acetonitrile (eluent B) pumped at 3.5 mL/min into the HPLC system. The elution program was as follows: 10% to 40% B (from 0 to 15 min), 40% to 42% B (from 15 to 50 min), followed by washing and reconditioning the column. The isolated compound was identified by HPLC-DAD and ^{1}H and ^{13}C NMR spectroscopy analysis in acetone-d_6. However, it was possible to obtain only a mixture of the 70% *cis* and 30% *trans* forms because **3** was subject to isomerization during purification. Fraction 3 (264.3 mg) was fractionated with a second CPC step using the Arizona H solvent system (*n*-heptane−EtOAc−MeOH−H_2O, 1:3:1:3) in the ascending mode, at 1300 rpm at a flow rate of 6 mL/min. Fraction 3 was injected at 3 mL/min, at low rotation speed (600 rpm), in a mixture of 10 mL of organic and aqueous phase (1:1, v/v). Fractions were collected every 30 s and combined on the basis of HPLC analyses, providing a total of two fractions of interest. Fraction 3A (60.4 mg) corresponded to 90% pure

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Table 2. Antifungal Activity (MIC95 μg/mL) of Selected Stilbenoid Compounds against *Candida* Species[a]

		MIC$_{95}$[b] (μg/mL)					
Candida sp.	strain	5-FC[c]	**1b**	**1c**	**1d**	**1e**	**3**
C. albicans	ATCC MYA-2876 (SC5314)	2	150	300	37	28	300
C. dubliniensis	ATCC MYA-646	2	150	150	18	14	150
C. famata	VKMY-9	2	18	18	9	7	18
C. glabrata	ATCC 90030	4	150	300	37	28	300
C. guilliermondii	ATCC 6260	2	150	150	18	7	150
C. inconspicua	clinical isolate	4	300	300	37	28	300
C. kefyr	clinical isolate	4	150	150	18	14	150
C. krusei	ATCC 6258	4	150	300	37	28	300
C. lusitaniae	CBS 6936	2	300	300	37	28	300
C. norvegensis	clinical isolate	4	75	300	37	28	300
C. parapsilopsis	ATCC 22019	4	300	300	18	14	300
C. tropicalis	ATCC MYA-3404/T1	4	300	na[d]	27	28	na[d]

[a]Data are mean values of two independent experiments, each with three replicates. [b]Minimum inhibitory concentration (the lowest compound concentration causing 95% growth inhibition). [c]No activity at the highest test concentration, 300 μg/mL. [d]5-Fluorocytosine.

4. Fraction 3B (12.8 mg), containing one tetramer of interest with other compounds, was purified by semipreparative TLC. The TLC plates were developed with $CHCl_3$−MeOH−acetic acid (80:20:3, v/v). The detection was achieved at 254 nm. The eluted compound (8.7 mg) corresponded to 90% pure **5**.

Synthesis of Methyl Derivatives of *trans*-Resveratrol (1b−1f). To a suspension of **1a** (1g, 4.38 mmol) in anhydrous DMF (20 mL) under an inert atmosphere was added portionwise NaH (157 mg, 1.5 × 4.38 mmol) at −5 to 0 °C. The mixture was stirred for 30 min under continued cooling. CH_3I (0.4 mL, 1.5 × 4.38 mmol) was then added, and the mixture was warmed to ambient temperature naturally. After 18 h, the mixture was diluted with EtOAc (40 mL), washed with brine (3 × 30 mL), dried, filtered, concentrated under vacuum, and purified by flash column chromatography (light petroleum−EtOAc, 95:5 to 70:30), giving **1f** (176 mg) in 24.5% yield as a white solid, **1e** (345 mg) in 48% yield as a white solid, **1d** (97 mg) in 13.5% yield as a white solid, **1c** (30 mg) in 4.5% yield as a white solid, and **1b** (68 mg) in 9.5% yield as a white solid.

Synthesis of Acetyl Derivatives of *trans*-Resveratrol (1g−1j). A solution of **1a** (1 g, 4.38 mmol) in a mixture of Et_3N−THF (10 mL) under an inert atmosphere was added to a solution of acetyl chloride (0.47 mL, 1.5 × 4.38 mmol) in anhydrous THF (5 mL) at 0 °C. The mixture was then stirred for 18 h under continued cooling, basified using aqueous 10% NaOH until pH 9, and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine (3 × 30 mL), dried, filtered, concentrated under vacuum, and then separated by flash column chromatography (light petroleum−EtOAc, 90:10 to 70:30), affording **1j** (103 mg) in 17.3% yield as a white solid, **1i** (290 mg) in 48.8% yield as a white solid, **1h** (40 mg) in 6.7% yield as a white solid, and **1g** (119 mg) in 20.8% yield as a white solid.

Synthesis of *trans*-ε-Viniferin Pentamethyl Ether (2b). NaH (78 mg, 6 × 0.33 mmol) was added dropwise at −5 to 0 °C to a magnetically stirred solution of **2a** (150 mg, 0.33 mmol) in anhydrous DMF (5 mL) under an inert atmosphere. Then, CH_3I (120 μL, 6 × 0.33 mmol) was added, and the mixture was warmed to ambient temperature naturally. After 18 h, the mixture was diluted with EtOAc (20 mL), washed with brine (3 × 20 mL), dried, filtered, concentrated under a vacuum, and purified by flash column chromatography (light petroleum−EtOAc, 80:20), giving **2b** (100 mg) in 58% yield as a white solid.

Synthesis of *trans*-ε-Viniferin Pentaacetate (2c). A solution of acetyl chloride (60 μL, 6 × 0.13 mmol) in anhydrous THF (5 mL) at 0 °C was added dropwise to a solution of **2a** (60 mg, 0.13 mmol) in a mixture of Et_3N−THF (2 mL) under an inert atmosphere. The mixture was then stirred for 18 h under continued cooling, basified using aqueous 10% NaOH until pH 9, and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (3 × 10 mL), dried, filtered, concentrated under vacuum, and purified by flash column chromatography (light petroleum−EtOAc, 70:30), giving **2c** (55 mg) in 63% yield as a white solid.

In Vitro Antifungal Assays. Minimum inhibitory concentration values (Tables 1 and 2) were determined using the broth microdilution method (serial 2-fold dilutions) as recommended by the Clinical Laboratory Standards Institute.[33] Strains were grown in RPMI AutoMod medium (Sigma). Yeasts were added by inoculation a 0.5 McFarland standard solution (10^6 cfu/mL) in a final volume of 0.3 mL. After incubation for 48 h at 32 °C, the MIC was read following the OD at 630 nm at the lowest compound concentration causing 95% growth inhibition compared to the control. The *C. lusitaniae* strain was from Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), and *Candida guilliermondii*, *C. parapsilopsis*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. dubliniensis*, and *C. albicans* strains were from the American Type Culture Collection (ATCC, Manassas, VA, USA). The *C. famata* strain was kindly provided by Prof. Andriy Sibirny (National Academy of Sciences, Kiev, Ukraine). *Candida kefyr*, *C. norvegensis, and C. inconspicua* clinical isolates were from the Angers University Hospital (Angers, France). The antifungal agent flucytosine (5-fluorocytosine, Sigma-Aldrich, St. Louis, MO, USA) was used as positive control.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR spectroscopic data of compounds **1b−d**, **1f−j**, **2a−2c**, and **3−5**. ^{1}H NMR spectra of **3** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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