

## Research Article

# A linker approach to phospholipopeptides

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Peptides and phospholipids are important macromolecules in both the chemistry and biology fields, but phospholipopeptides are barely found in nature and have not been studied. Here, we report a novel and efficient approach to the synthesis of phospholipopeptides. By utilizing cross-linkers, phospholipopeptides from peptides with varied sequences and lengths were produced from phosphatidylethanolamine at high (>90%) yield, mild conditions and good applied scopes for multiple substrates. Synthetic phospholipopeptides containing bioactive peptide sequences may serve as therapeutics for a wide range of biomedical applications.

**Practical applications:** Phospholipopeptides are prone to hydrolysis by the endogenous phospholipase activity in cells. Synthetic phospholipopeptides containing a bioactive peptide sequences may serve as therapeutics for a wide range of biomedical applications.

**Keywords:** Conjugation / Cross-linker / Lipopeptide / Liposomes / Peptides

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## 1 Introduction

Lipopeptides have received considerable attention recently for their antimicrobial, antitumor, immunosuppressant and surfactant properties [1–3]. It has been found that lipidation greatly improves the efficiency and potency of peptides because of altered pharmacokinetic properties and enhanced metabolic resistance [4]. An obvious example is daptomycin, a lipopeptide consisting of 13 amino acids produced by *Streptomyces roseosporus* (Actinobacteria). Daptomycin is one of the most effective antibiotics for methicillin-resistant *S. aureus* and soft tissue infections [5].

Biosynthesis of lipopeptides happens in a number of bacterial species by large non-ribosomal peptide synthetases via a thiotemplate process [6]. Naturally occurring

lipopeptides consist of short linear chains or cyclic structures of amino acids, linked to a fatty acid, in most cases, either fatty acyl groups or cholesterol. However, phospholipopeptides are barely found in nature. Cleavage of the membrane proteins with enzymes yielded phospholipopeptides [7]. Although phospholipopeptides were found to play an important role in the formation of extracellular proteins [8], broad biological activities and functions of phospholipopeptides have not been well studied [9]. Among the established strategies, synthesis of phospholipopeptides through native chemical ligation requires multiple steps of modification on the phospholipid head group prior to ligation [10], while the enzymatic ligation strategy employing phospholipase D provides only moderate yields even with its most favorable peptide substrates [11].

## 2 Materials and methods

### 2.1 Materials

All solvents, which include dichloromethane, methanol, *n*-hexane, ethyl acetate, and *N,N*-dimethylformamide were purchased from Pharmco-Aaper.

All chemical reagents, which include 4-aminobenzoic acid, maleic anhydride, trifluoroacetic anhydride, *N*-

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**Abbreviations:** **DMPE**, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine; **DPPE**, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; **DSPE**, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; **NHS**, *N*-hydroxysuccinimide

hydroxysuccinimide (NHS), 2,4,6-trimethylpyridine, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride, 4-(*N*-maleimidomethyl) cyclohexanecarboxylic acid *N*-hydroxysuccinimide ester (SMCC), *N*-ethyl-diisopropylamine (DIPEA), cysteine, and L-glutathione (GSH), were from Sigma–Aldrich.

All peptides for this study (>90% in purity), which include CHHH, LIRHGEC, and YSTCDFIMLPETGK, were synthesized by Genescript Corporation (Piscataway, NJ).

All phospholipids, which include 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), were purchased from Sigma–Aldrich.

## 2.2 NMR and MASS analysis

NMR spectra were measured on Bruker AV-400 High Performance Digital NMR Spectrometer ( $^1\text{H}$  at 400 MHz,  $^{13}\text{C}$  at 100 MHz). The  $^1\text{H}$  NMR spectra were calibrated against the peak of tetramethylsilane (TMS, 0 ppm) and the  $^{13}\text{C}$  NMR spectra were calibrated against the peak of  $\text{CDCl}_3$  (77.0 ppm). Data for  $^1\text{H}$  NMR spectra were reported as follows: chemical shift (ppm), peak shape (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, dd=doublet of doublets, br=broad), coupling constant (Hz), and integration. Data for  $^{13}\text{C}$  NMR were reported in terms of chemical shift (ppm). Mass spectra were performed on Agilent 1100 Series Capillary LCMSD Trap XCT Spectrometer, or Agilent 1100 Series LCMSD VL MS Spectrometer.

## 2.3 General procedure for the preparation of cross-linkers

Three cross-linkers, 4-maleimidobenzoic acid *N*-hydroxysuccinimide ester (4-MBS), 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (3-MBS), and SMCC, were used in this study (Fig. 1).

SMCC were purchased from Sigma, but 3- and 4-MBS were synthesized [12, 13] in this lab (Scheme 1) (see Supporting Information).

**1a:** yellow solid, 99% yield,  $^1\text{H}$  NMR (400 MHz,  $d_6$ -DMSO):  $\delta$  6.32 (d, 1H,  $J=12.0$  Hz), 6.48 (d, 1H,  $J=12.0$  Hz), 7.33 (d, 2H,  $J=8.4$  Hz), 7.90 (d, 2H,  $J=8.4$  Hz), 10.59 (s, 1H), 12.82 (br, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $d_6$ -DMSO):  $\delta$  118.8, 125.6, 130.3, 130.5, 131.7, 142.8, 163.7, 166.9, 167.0. Calculated MS (ESI) for  $\text{C}_{11}\text{H}_9\text{NO}_5$  [ $\text{M}-\text{H}$ ] $^-$  234.1, found 233.9.

**2a:** yellow solid, 99% yield,  $^1\text{H}$  NMR (400 MHz,  $d_6$ -DMSO):  $\delta$  6.31 (d, 1H,  $J=12.0$  Hz), 6.47 (d, 1H,  $J=12.0$  Hz), 7.44 (m, 1H), 7.65 (dd, 1H,  $J=1.2$  Hz, 6.4 Hz), 7.82 (dd, 1H,  $J=1.2$  Hz, 6.4 Hz), 8.27 (s, 1H), 10.5 (s, 1H), 12.8 (br, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $d_6$ -DMSO):  $\delta$  120.3, 123.6, 124.6, 129.1, 130.3, 131.4, 131.8, 138.9, 163.5, 166.9,

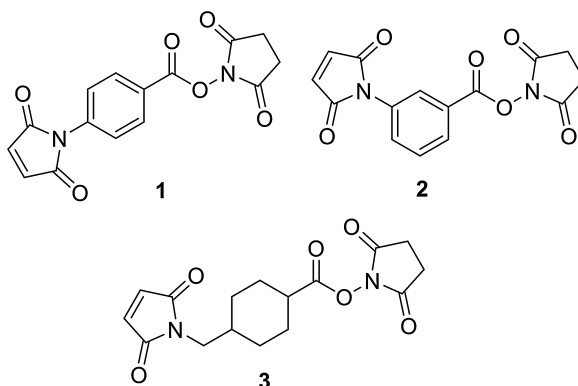


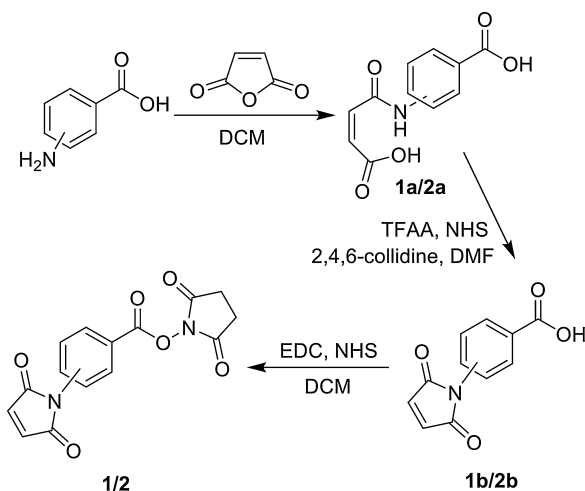
Figure 1. Cross-linkers used for amino and thiol group linkage.

167.1. Calculated MS (ESI) for  $\text{C}_{11}\text{H}_9\text{NO}_5$  [ $\text{M}-\text{H}$ ] $^-$  234.1, found 233.9.

**1b:** yellow solid, 65% yield,  $^1\text{H}$  NMR (400 MHz,  $d_6$ -DMSO):  $\delta$  6.99 (s, 2H), 7.52 (d, 2H,  $J=5.2$  Hz), 8.11 (d, 2H,  $J=5.2$  Hz);  $^{13}\text{C}$  NMR (100 MHz,  $d_6$ -DMSO):  $\delta$  116.8, 121.3, 125.7, 127.3, 158.9, 160.8. Calculated MS (ESI) for  $\text{C}_{11}\text{H}_7\text{NO}_4$  [ $\text{M}-\text{H}$ ] $^-$  217.0, found 216.9.

**2b:** yellow solid, 62% yield,  $^1\text{H}$  NMR (400 MHz,  $d_6$ -DMSO):  $\delta$  7.21 (s, 2H), 7.63 (m, 2H), 7.95 (m, 2H), 13.1 (br, 1H);  $^{13}\text{C}$  NMR (100 MHz,  $d_6$ -DMSO):  $\delta$  127.3, 128.3, 129.2, 130.9, 131.6, 131.9, 134.7, 162.3, 166.6, 169.7. Calculated MS (ESI) for  $\text{C}_{11}\text{H}_7\text{NO}_4$  [ $\text{M}-\text{H}$ ] $^-$  217.0, found 216.9.

**1:** yellow solid, 89% yield,  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  2.82 (s, 4H), 6.82 (s, 2H), 7.55 (d, 2H,  $J=8.8$  Hz), 8.14 (d, 2H,  $J=8.8$  Hz);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  25.7, 123.8, 125.2, 131.4, 134.5, 137.3, 161.1, 168.6, 169.0. Calculated MS (ESI) for  $\text{C}_{16}\text{H}_{13}\text{N}_2\text{O}_7$  [ $\text{M}+\text{CH}_3\text{OH}-\text{H}$ ] $^-$  345.1, found 344.9.



Scheme 1. Synthesis of cross-linkers 3-MBS and 4-MBS.

2: yellow solid, 80% yield,  $^1\text{H}$  NMR (400 MHz,  $d_6$ -DMSO):  $\delta$  2.91 (s, 4H), 6.89 (s, 2H), 7.62 (m, 1H), 7.72 (m, 1H), 8.13–8.17 (m, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $d_6$ -DMSO):  $\delta$  126.3, 127.6, 129.6, 129.7, 131.9, 132.1, 134.4, 161.1, 168.8, 169.0. Calculated MS (ESI) for  $\text{C}_{16}\text{H}_{13}\text{N}_2\text{O}_7$   $[\text{M}+\text{CH}_3\text{OH}-\text{H}]^-$  345.1, found 345.1.

## 2.4 General procedure for the synthesis of phospholipids-peptides

Step 1: To a solution of dichloromethane (5 mL), 0.02 mmol phosphatidylethanolamine (DPPE = 13.8 mg, DMPE = 12.7 mg or DSPE = 15.0 mg) was added and mixed well. Then 0.02 mmol cross-linker (4-MBS/3-MBS = 6.3 mg or SMCC = 6.7 mg) and DIPEA (0.03 mmol, 1.5eq) were added into the solution. Reactions were kept at room temperature under nitrogen atmosphere. After 12 h, the solution was washed by 1% ammonium chloride solution and water, dried by anhydrous magnesium sulfate. Then the solution was evaporated in vacuum for next step use.

Complex of DPPE and 4-MBS (1): Calculated MS (ESI) for  $\text{C}_{49}\text{H}_{82}\text{N}_2\text{O}_{12}\text{P}$   $[\text{M}+\text{CH}_3\text{OH}-\text{H}]^-$  921.6, found 921.7.

Complex of DPPE and 3-MBS (2): Calculated MS (ESI) for  $\text{C}_{48}\text{H}_{78}\text{N}_2\text{O}_{11}\text{P}$   $[\text{M}-\text{H}]^-$  889.5, found 889.8.

Complex of DPPE and SMCC (3): Calculated MS (ESI) for  $\text{C}_{49}\text{H}_{86}\text{N}_2\text{O}_{11}\text{P}$   $[\text{M}-\text{H}]^-$  909.6, found 909.8.

Complex of DMPE and SMCC (3): Calculated MS (ESI) for  $\text{C}_{45}\text{H}_{79}\text{N}_2\text{O}_{11}\text{P}$   $[\text{M}-\text{H}]^-$  853.5, found 853.8.

Complex of DSPE and SMCC (3): Calculated MS (ESI) for  $\text{C}_{53}\text{H}_{95}\text{N}_2\text{O}_{11}\text{P}$   $[\text{M}-\text{H}]^-$  965.7, found 965.8.

Step 2: To a solution of methanol (5 mL), the intermediate on step 1 was added, peptides (1.0eq, 0.02 mmol) was added in one portion. The mixture was reacted for 5 h and purified using flash chromatography column, eluted with chloroform/methanol/water (100:10:1 to 60:15:1). Phospholipopeptide peaks with UV absorbance at 215 nm were collected (see Supporting Information).

Complex of DPPE-SMCC-cysteine (entry 5): Calculated MS (ESI) for  $\text{C}_{52}\text{H}_{94}\text{N}_3\text{O}_{13}\text{PS}$   $[\text{M}+\text{H}]^+$  1032.6, found 1032.7.

Complex of DPPE-SMCC-GSH (entry 6): Calculated MS (ESI) for  $\text{C}_{59}\text{H}_{104}\text{N}_5\text{O}_{17}\text{PS}$   $[\text{M}-\text{H}]^-$  1216.7, found 1217.0.

Complex of DPPE-SMCC-CHHH (entry 7): Calculated MS (ESI) for  $\text{C}_{79}\text{H}_{115}\text{N}_{12}\text{O}_{16}\text{PS}$   $[\text{M}-\text{H}]^-$  1441.8, found 1442.0.

Complex of DPPE-SMCC-LIRHGEC (entry 8): Calculated MS (ESI) for  $\text{C}_{83}\text{H}_{146}\text{N}_{14}\text{O}_{21}\text{PS}$   $[\text{M}-\text{H}]^-$  1737.0, found 1737.2.

Complex of DMPE-SMCC-GSH (entry 9): Calculated MS (ESI) for  $\text{C}_{55}\text{H}_{96}\text{N}_5\text{O}_{17}\text{PS}$   $[\text{M}-\text{H}]^-$  1160.6, found 1160.3.

Complex of DMPE-SMCC-CHHH (entry 10): Calculated MS (ESI) Calcd. for  $\text{C}_{66}\text{H}_{107}\text{N}_{12}\text{O}_{16}\text{PS}$   $[\text{M}-\text{H}]^-$  1385.7, found 1385.3.

Complex of DMPE-SMCC-LIRHGEC (entry 11): Calculated MS (ESI) for  $\text{C}_{79}\text{H}_{138}\text{N}_{14}\text{O}_{21}\text{PS}$   $[\text{M}-\text{H}]^-$  1682.0, found 1682.1.

Complex of DMPE-SMCC-YSTCDFILMPETGK (entry 12): Calculated MS (ESI) for  $\text{C}_{116}\text{H}_{188}\text{N}_{17}\text{O}_{34}\text{PS}_2$   $[\text{M}+2\text{H}]^{2+}$  1230.1, found 1230.4.

Complex of DSPE-SMCC-GSH (entry 13): Calculated MS (ESI) for  $\text{C}_{63}\text{H}_{112}\text{N}_5\text{O}_{17}\text{PS}$   $[\text{M}-\text{H}]^-$  1272.7, found 1272.3.

Complex of DSPE-SMCC-CHHH (entry 14): Calculated MS (ESI) for  $\text{C}_{74}\text{H}_{123}\text{N}_{12}\text{O}_{16}\text{PS}$   $[\text{M}-\text{H}]^-$  1497.8, found 1497.3.

Complex of DSPE-SMCC-LIRHGEC (entry 15): Calculated MS (ESI) For  $\text{C}_{87}\text{H}_{154}\text{N}_{14}\text{O}_{21}\text{PS}$   $[\text{M}-\text{H}]^-$  1794.1, found 1794.2.

Complex of DSPE-SMCC-YSTCDFILMPETGK (entry 16): Calculated MS (ESI) for  $\text{C}_{124}\text{H}_{204}\text{N}_{17}\text{O}_{34}\text{PS}_2$   $[\text{M}+2\text{H}]^{2+}$  1286.2, found 1286.4.

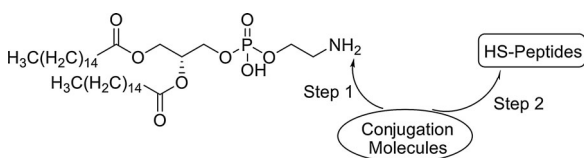
## 2.5 SEM analysis of phospholipopeptide

Phospholipopeptide solution in water was applied on pieces ( $1.5 \times 1.5 \text{ cm}^2$ ) of silicon wafer. Silicon wafers were washed with water and dried with nitrogen. Samples were then coated with gold. SEM images were taken using an Auriga Modular CrossBeam workstation (Carl Zeiss, Inc.) operating at 5 kV [14].

## 3 Results and discussion

Originated from polymer sciences, cross-linking, the process in which a covalent linkage forms between two polymer molecules [15], has found its importance in the field of life sciences. The concept of cross-linking has expanded to include the bonding formed between a biopolymer and a small molecule, or two small molecules. For instance, functional groups specifically reactive to thiol (maleimide, alkyl halide) or amine (NHS ester, isothiocyanate) have been developed, allowing site-specific labeling of proteins or peptides with DNA [16], fluorophore [17], spin labels [18], or bioactive small molecules as therapeutic warheads [19, 20].

Herein, we synthesized phospholipopeptides using a cross-linker bearing a thiol-reactive maleimide and an amine-reactive *N*-hydroxysuccinimide (NHS) ester (Fig. 2). The



**Figure 2.** Design of phospholipids (e.g., DPPE) and thiol-containing peptides.

amine group in phosphatidylethanolamine is a strong nucleophile which reacts with NHS completely, while the maleimide is very specific for the thiol group of cysteine residue in peptides [21–23]. Such synthesis requires neither protection of nucleophilic groups on peptides nor specific positioning of the cysteine in peptide sequences.

### 3.1 Conjugation by small molecules

Based on the above notions, we tested three amine-thiol bidental cross-linker **1–3** (see Fig. 1). As an initial test, linking of cysteine or a thiol containing reduced GSH to DPPE was tried (see Table 1 and Supporting Information). The cross-linkers were added in slight excess (1.05eq.) and the reaction was carried out in dichloromethane. Stoichiometric amount of DIPEA was included to neutralize the *N*-hydroxysuccinimide generated during the coupling. Overnight stirring at room temperature resulted in quantitative conversion of DPPE. Then, the reaction was washed with aqueous solution of ammonium chloride, and chloroform was evaporated from the organic phase. The residual, DPPE-coupled cross-linker was re-dissolved in methanol. After 5 h reaction at room temperature, little progression was detected for the addition of either thiol to the maleimideon cross-linkers **4-MBS** (entries 1 and 2) or **3-MBS** (entries 3 and 4). In contrast,

**Table 1.** Ligation test of phospholipids and peptides using three different cross-linkers

Entry	Cross-linkers	Cysteine/GSH	Yield (%)
1	<b>1</b>	C	Trace
2	<b>1</b>	GSH <sup>a</sup>	Trace
3	<b>2</b>	C	Trace
4	<b>2</b>	GSH	Trace
5	<b>3</b>	C	96
6	<b>3</b>	GSH	90

<sup>a</sup>GSH is reduced L-glutathione.

cross-linker **SMCC** (entries 5 and 6) exhibited excellent reactivity (>90%) to both cysteine and GSH. The incapability of the maleimide groups in **1** and **2** to receive thiol addition might result from their reactions with an aromatic ring, leading to diminished electrophilicity of the C=C double bond on the maleimide groups. On the contrary, the C=C bond on cross-linker **3** was highly reactive because the saturated cyclohexyl group lacks  $\pi$ -electrons.

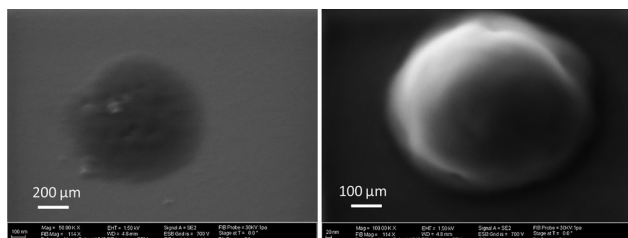
Since cross-linker **3** demonstrated the most potent efficiency in linking DPPE to simple thiol-containing molecules, it was tested further in the conjugations involving a series of PEs (DPPE, DMPE, and DSPE) and peptides with varied chain lengths and different peptide sequences (see Table 2 and Supporting Information). It was reported that the biological activities of these peptides could potentially be strengthened upon lipidation as a result of either enhanced bilayer-targeting affinity or membrane permeability [24–26]. Encouragingly, phospholipopeptides were formed all tested phospholipids and bioactive peptides at satisfactory to excellent yields, indicating the reactivity

**Table 2.** The application scope tests for different peptides and phospholipids

Entry	<i>n</i>	Cysteine/peptides	Yield <sup>a</sup> (%)
7	14	CHHH	92
8	14	LIRHGEC	75
9	12	GSH <sup>b</sup>	96
10	12	CHHH	90
11	12	LIRHGEC	80
12	12	YSTCDFIMLPETGK	86
13	16	GSH	89
14	16	CHHH	93
15	16	LIRHGEC	68
16	16	YSTCDFIMLPETGK	90

<sup>a</sup>Isolated yields, all products were characterized by LCMSD Trap XCT Spectrometer.

<sup>b</sup>GSH is reduced L-glutathione.



**Figure 3.** SEM images of distinguished lipid vesicles from phosphatidylethanolamine (micelles, became flat on silicon wafer) and phosphatidylethanolamine-CHH (liposomes, maintained round shapes on silicon wafer).

of cross-linker **3** to cysteine residues on either the N- or C-terminus of the peptide sequences and underscoring the remarkable versatility of this strategy.

Compared to naturally occurring and well-studied lipopeptides, phospholipopeptides are prone to hydrolysis by endogenous phospholipases. This “bio-degradable” property is particularly attractive since it allows the peptide portion, once reaches the inner leaflet of the cell membrane, to be removed from the lipid anchor—a mechanism that potentially enriches the peptide “warhead” in the cytosol. Furthermore, the plasma membrane of eukaryotic cells is composed of structurally rigid “rafts” enriched of cholesterol and glycolipids floating within a phospholipid-rich, high-fluidity surroundings [27, 28]. We envision phospholipopeptides to preferentially target the high-fluidity domain due to its better miscibility to phospholipids. There, the structural flexibility may lead to high levels of two-dimensional diffusion rate and flipping rate of the conjugate. To test these speculations, we studied self-assembly of low concentrations of phospholipid and phospholipopeptide in water solutions. Phospholipid and phospholipopeptide formed lipid vesicles with different structures. For example, phosphatidylethanolamine form lipid micelles but a small peptide containing phosphatidylethanolamine, phosphatidylethanolamine-CHHH, formed liposomes (Fig. 3).

## 4 Conclusions

We validated a new synthesis approach for phospholipopeptides. By using cross-linkers, phospholipopeptides of peptides with varied sequences and lengths were produced from phosphatidylethanolamine at high (>90%) yield, mild conditions, and good applied scopes for multiple substrates. Synthetic phospholipopeptides may have important biological significance and wide biomedical applications.

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*The authors declare that they have no conflict of interest.*

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