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Trapping of Methylglyoxal by Sieboldin from *Malus baccata* L. and Identification of Sieboldin-Methylglyoxal Adducts Forms

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Abstract – The methylglyoxal (MGO) trapping constituents from *Malus baccata* L. were investigated using incubation of MGO and crude extract under physiological conditions followed by HPLC analysis. The peak areas of MGO trapping compounds decreased, and their chemical structures were identified by HPLC-ESI/MS. Sieboldin was identified as a major active molecule representing MGO-trapping activity of the crude extract. After reaction of sieboldin and MGO, remaining MGO was calculated by microplate assay method using imine (Schiff base) formation of 2,4-dinitrophenylhydrazine (DNPH) and aldehyde group. After 4 h incubation, sieboldin trapped over 43.8% MGO at a concentration of 0.33 mM and showed MGO scavenging activity with an RC_{50} value of 0.88 mM for the incubation of 30 min under physiological conditions. It was also confirmed that sieboldin inhibited the production of advanced glycation end products (AGE) produced by bovine serum albumins (BSA)/MGO. Additionally, MGO trapping mechanism of sieboldin was more specifically identified by ^{1}H-, ^{13}C-, 2D NMR and, confirm to be attached to the position of C-3' (or 5').
Keywords – *Malus baccata* L., Methylglyoxal trapping, Sieboldin

Introduction

In organisms, methylglyoxal (MGO) is built as a byproduct of metabolic pathways. It can be also made from lipid peroxidation, but the most important component is glycolysis.[1] Because of increased blood glucose levels, MGO is a higher concentration in diabetic patients and has also been associated with arteriosclerosis. MGO damage to low-density lipoproteins by glycation causes a 4-fold increase in atherogenesis in diabetic patients.[2] Advanced glycation end products (AGEs), produced as the result of the non-enzymatic reaction between the carbonyl group of sugars and a free amino group of plasma proteins (Maillard reaction), are associated with multiple features of diabetes and related complications.[3-4] The mechanisms involved in AGEs inhibition include sugar auto-oxidation inhibition, scavenging of free radicals and carbonyls, and breaking of sugar-protein cross-links.[1] Aminoguanidine is a synthetic compound with profound antiglycation activity, but it has some adverse effects *in vivo*.[1] Therefore, natural products have gained more attention due to their higher efficacy and relative safety. Phenols from natural such as quercetin,[5] catechins,[6,7] curcumins,[8] anthocyanins,[9] and gingerols[10] have the capability to scavenge free radicals and trap the carbonyls by forming adducts. Sieboldin, dihydrochalcone, has been reported in certain wild *Malus* species,[11,12] which exerted free radical scavenging activities and cytotoxicity in cancer cell lines.[13] Sieboldin obtained from aerial parts of the *Malus baccata*, which trapped MGO confirmed by pre-column reaction with MGO. In addition, it was refined into a single compound by centrifugal partition chromatography (CPC), and the trapped-MGO adducts were revealed using HPLC-ESI/MS analysis and NMR spectra interpretations.

Dedicated to Prof. Jinwoong Kim of the Seoul National University for his pioneering works on Pharmacognosy.

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Experimental

Materials – methylglyoxal (40% aqueous solution), bovine serum albumins (lyophilized powder), 2,4-dinitrophenylhydrazine (reagent grade), analytical grade organic solvents, formic acid, MeOD-d_4 and, DMSO-d_6 for NMR solvent were purchased from Merck KGaA (Darmstadt, Germany). HPLC grade acetonitrile and water were obtained from Daejung Chemical (Gyonggi-do, Korea). All other chemicals used were of analytical grade and from commercial sources.

Preparation of crude extracts and organic solvent fractions – The branches and leaves of *M. baccata* were collected from a Hanyang University Herbarium (Hanyang University, Ansan, Gyeonggi-do 15588, Republic of Korea). The voucher specimen (HYUP-MB-001) was deposited in the laboratory. Dried branches and leaves (359 g) were extracted with ethanol under the reflux for 3 h. The filtered solution was concentrated by a rotary evaporator under vacuum then, lyophilized to yield 46.2 g crude extract. Afterward, ethanolic extracts were fractionated with the organic solvent (*n*-hexane, ethyl acetate, *n*-butanol) dispersed in distilled water to afford four fractions (combined aqueous fraction). These four fractions were concentrated by a rotary evaporator *in vacuo* and stored at -20°C.

Selection of solvent system – The Partition coefficient (K), *K* values were calculated as the following method. Briefly, 2 mg of ethyl acetate fractions were percolated with 1.8 mL of each phase (1:1, v/v) of two-phased immiscible solvent in a 2 mL tube. After shaking the tube, the equal volume of the upper and the lower phase was analyzed individually by HPLC to evaluate the optimal biphasic solvent. The *K* values of target compounds were calculated by the following equation:
K value = the peak areas of mobile $_{\text{upper layer}}$ phase / the peak areas of stationary $_{\text{lower layer}}$ phase.

Purified sieboldin using centrifugal partition chromatography – Sieboldin was purified using CPC and the method was as follows: CPC separation was performed with an unmixed two-phase solvent system composed of ethyl acetate and water with a volume ratio of 1:1 and operated as follows: the aforementioned solvents were prepared when the solvent was completely mixed and fully equilibrated. The 1000 mL volume of the CPC rotor was filled with upper phase solvent as the stationary phase at a flow rate of 50 mL/min at a speed of 500 rpm rotation. Then, the rotation speed of the rotor was accelerated to 900 rpm, and the lower phase as mobile phase was carried into the rotor in descending mode at a 10 mL/min flow rate. When the CPC rotor reached hydrostatic equilibrium, the sample solution (3.01 g of ethyl acetate fraction dissolved in 25 mL of mixed upper and lower phase) was subjected to the Armen CPC system. The effluent from the outlet of the CPC rotor was continuously monitored at the wavelength of 254 and 280 nm. The fractions were concentrated and analyzed by analytical HPLC.

Trapping of MGO in physiological conditions – The crude extract and sub-fractions (10 mg/mL) were dissolved in phosphate-buffered saline (PBS; 100 mM, pH 7.4) and centrifuged to remove undissolved materials. This sample was incubated with 0.33 mM MGO in PBS at 37°C and kept for 12 h. After incubation, the remaining MGO is identified and subjected to HPLC analysis. Control was congruently carried out for comparison. The chromatographic peaks were identified by HPLC-UV-ESI/MS analysis.

Determination of MGO using DNPH derivatization – This method was modified according to our circumstances by referring to the method specified in the literature.[14] A simple plate assay was devised using imine (Schiff base) formation. Experimental progress was as follows: 20 μl of the reaction solution obtained in *section 2.2* and, 180 μl of 0.74 mM DNPH were dispensed on a 96-well plate, and then the plate was incubated in a dark place at 42°C for 30 min. Absorbance at 432 nm was measured. All determinations were performed in triplicate.

Effects of sieboldin on the AGEs formation – BSA (10 mg/mL) was incubated with MGO (6.49 μM) in PBS pH 7.4, in the presence or absence of sieboldin (serial concentration: 1, 0.5, 0.25, 0.125 mM) at 37°C. Streptomycin and penicillin (1:1000, v/v) were added to the solution to prevent bacterial growth. The amount of AGEs was determined using fluorescence intensity with excitation/emission wavelengths at 370/440 nm. All determinations were performed in triplicate.

HPLC-ESI/MS analysis – An Agilent 1260 HPLC (Agilent Technologies, Waldbronn, Germany) equipped with an Advion expression CMS mass spectrometry (Advion, Ithaca, NY) was used to identify crude sample and reaction mixture. In the same condition, sieboldin and MGO adducts were carried out on a Waters 2695 HPLC equipped with Waters 2996 photo array detector (PAD), Micromass ZQ electrospray ionization-mass spectrometry (ESI-MS; Micromass, Manchester, UK), and MassLynx Software version 3.5 (Micromass). The HPLC conditions were as follows: an HPLC system with an INNO column C18 (2.0 mm × 150 mm, YoungJin Biochrom, Gyeonggi-do, South Korea) was performed at a flow rate of 0.2 ml/

min at 40°C. The mobile phase consisted of acetonitrile and water (solvent A and B) containing both 0.1% formic acid. A linear gradient system was used as follows: 0–10 min, 5–15% A; 10–35 min, 15–40% A; 35–45 min, 40–100% A; 45–50 min, 100% A. The chromatographic profile of the effluents was recorded at 280 nm with a spectrum ranging from 210 to 400 nm. The ESI-MS spectra conditions were as follows: positive and negative ion mode; mass range, *m/z* 100-1200; capillary temperature, 250°C; capillary voltage, 180 V; source voltage offset, 32–45 sources voltage span, 10; source gas temperature, 250°C; ESI voltage, - 2.5 kV.

NMR analysis – ^{1}H- and ^{13}C-NMR were acquired on a Bucker AVANCE III 400 MHz instrument at 25°C (Bruker, Inc., Silberstreifen, Rheinstetten, Germany). A sieboldin was analyzed in MeOD-d_4 or DMSO-d_6.

Result and Discussion

Considering purification of a major compound in *M. baccata*, the removal of nonpolar- and polar-part impurity such as chlorophylls, lipids, polysaccharides, inorganic compounds, and amino acids are required for high resolution during the centrifugal partition chromatography. For this reason, ethyl acetate fractions were prepared by conventional solvent fractionation methods, yielding 18.82 g (out of 46.22 g in total). According to calculation *K* values of major peaks (Table 1), ethyl acetate/water (1:1, v/v) was operated with ascending mode for 180 min. As shown in Fig. 1, a peak at 90–120 min was collected and evaporated to dryness. Sieboldin (1.06 g), a major component, was purified from the 3.01 g of the ethyl acetate fraction by the CPC method. The purified sieboldin was verified by HPLC-ESI/MS data and NMR analysis.

The two adjacent reactive carbonyl groups of MGO are reactive electrophilic species and are generally known to bind to unsaturated carbon sites in phenolic compounds.[15] These compounds can be considered to have an MGO scavenging effect. After the reaction of MGO and crude extract, as the amount of MGO and target active com-

Table 1. The partition coefficients (*K* values) of peaks **1** and **2** in *M. baccata* extracts by HPLC analysis. (H: *n*-hexane; E: ethyl acetate; M: methyl alcohol; W: water)

No	Solvent system	Volume ratio	*K* values		
			Peak **1**	Separation factor	Peak **2**
1	H/E/M/W (v/v)	2:8:2:8	0.50	1	0.49
2		1:9:1:9	0.42	1.5	0.65
3		0:10:0:10	**1.09**	1.6	**1.79**

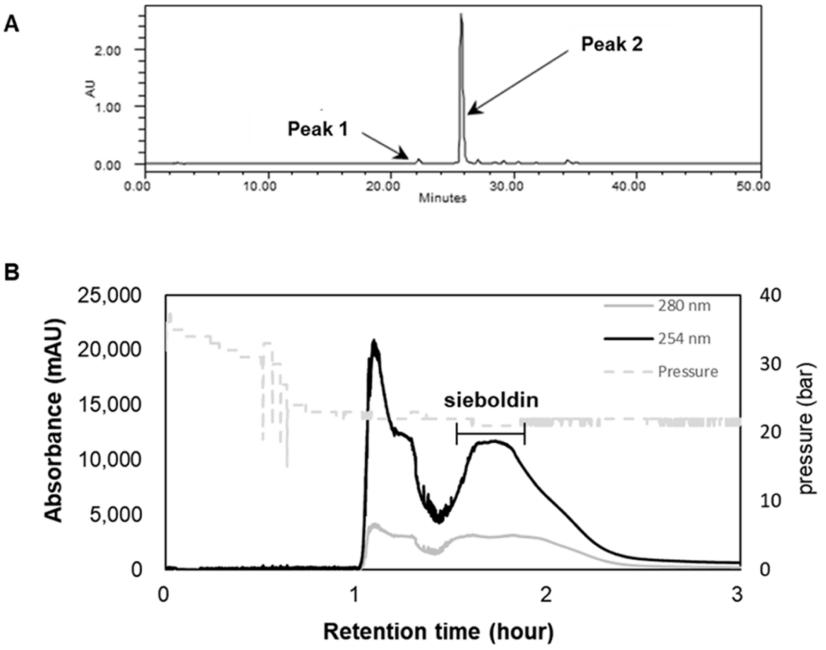

Fig. 1. HPLC (A) and CPC (B) chromatograms of M. *baccata* extract. Detailed methods in described in Experimental.

Table 2. ^{1}H NMR chemical shift of sieboldin and MGO-adducts

Moiety	Proton position	Sieboldin	MGO adduct-**1**	MGO adduct-**2**
		^{1}H NMR (δ_H)		
Chalcone	2, 5	6.69 (m, *J* = 5.1, 2.8 Hz, 2H)	6.72 (m, *J* = 5.1, 2.8 Hz, 2H)	
	6	6.56 (dd, *J* = 8.1, 2.1 Hz, 1H)	6.61 (dd, *J* = 8.1, 2.1 Hz, 1H)	
	3', 5'	6.11 (s, 2H)	6.26 (brs, 1H)	
	β	2.83 (dd, *J* = 9.8, 5.7 Hz, 2H)	2.86 (t, *J* = 7.6 Hz, 2H)	
Glucose	α, 2", 3", 4", 5"	3.50-3.29 (m, overlapped H-α; 2"; 3"; 4"; 5", 6H)		
	1"	4.94 (d, *J* = 7.3 Hz, 1H)	5.12 (brs, 1H)	
	6"-α	3.93 (dd, *J* = 12.1, 2.0 Hz, 1H)	3.92 (dd, *J* = 12.1, 2.0 Hz, 1H)	
	6"-β	3.72 (dt, *J* = 12.1, 5.2 Hz, 1H)	3.75 (dt, *J* = 12.1, 5.2 Hz, 1H)	
MGO	2"'		4.79 (s, 1H)	Overlapped
	3"'		1.72 (brs, 3H)	1.59 (brs, 3H).

pounds would be decreased, the amount of the newly generated MGO adducts would be proportionally increased. Based on this, MGO scavengers can be screened in the crude extract under physiological conditions. In this study, *M. baccata* extract was incubated with MGO or without MGO (to compare) for 12 hours under physiological conditions, and the reacted products were analyzed using HPLC-UV-ESI/MS. In the chromatogram incubated without MGO, the peak with the highest intensity was expected sieboldin (*m/z* 452) and had a retention time of 25.39 min. Compared with the foregoing, the retention time of peak (*m/z* 524) for the mono-MGO adduct was 25.71 min. As a result, it was confirmed that the major component of the crude extract was the active component, and its molecular weight was found to be *m/z* 452, which indicated that the mono-MGO adduct was formed (Fig. 2).

In previous studies, MGO was quantitated by HPLC analysis after derivatization with 1,2-diaminobenzene to form 2-methylquinoxaline.[16] It is time-consuming; analysis time of one sample requires at least 1 hr. Therefore, the quantitative method using DNPH was adopted as a more reasonable method, and that was fast and efficient.[14] DNPH has a hydrazino group and easily reacts with a carbonyl group to form MGO-bis-2,4-DNPH. As the absorption maximum of DNPH was 360 nm and the absorption maximum of MGO-bis-2,4-DNPH was red-shifted to 432 nm.[14] MGO-bis-2,4-DNPH could be monitored at 432 nm of its absorption maximum, which is generally recorded in a microplate reader. To confirm the previously identified sieboldin as MGO-scavenger, the amount of MGO was observed at various serial concentrations and kinetic curves of the remaining MGO during reaction time (up to 4 hr). First, sieboldin decreases MGO level in a concentration-dependent manner, and the concentration that reduces MGO concentration by half is 0.88 mM. Next, MGO concentration was rapidly decreased in the range of 0-0.5 hr with 1 mM of sieboldin, and 56% of MGO remained after 4 hr (Fig. 3).

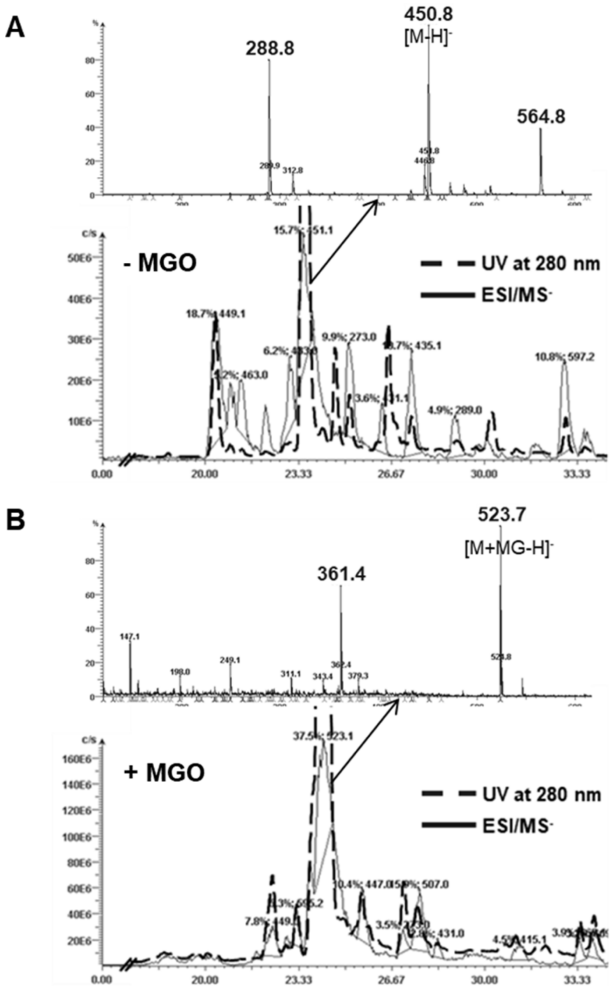

Fig. 2. Identification of MGO scavenger using pre-column incubation. Chromatograms and Mass spectra of crude extract incubated without MGO (A) and with MGO (B) in PBS.

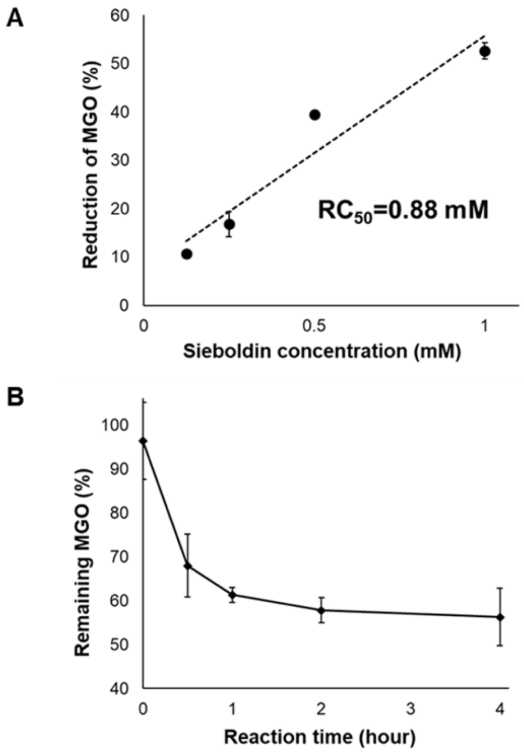

Fig. 3. MGO scavenging ability of sieboldin by concentration (A) and time (B).

The structure of sieboldin was confirmed by comparing ^{1}H NMR data with the literature.[13] As mentioned above, sieboldin trapped one molecule of MGO by HPLC-ESI/MS analysis, and the chemical structures of MGO-adducts were also identified through NMR analysis. The most prominent point is a proton-missing of 3' (or 5'-position) in the chalcone molecule. The proton chemical shift of 6.26 ppm singlet peak was reduced to one proton compared to the previous integral of the proton peak. Based on these facts, a molecule of MGO binds to the carbon-3' (or 5' position). The next thing to notice is that there are two types of compounds.[17] In ^{1}H NMR spectrum of MGO-adduct, two CH_3 peaks of MGO-adducts were identified at δ 1.59 and 1.72 ppm (Fig. 4), which indicates that MGO-adduct is a mixture of two tautomers, and the ratio of major and minor is around 2:1.

The AGEs generated in the BSA/MGO, it was measured using fluorescence and compared with 2,4,6-trihydroxybenzoic acid as a known positive control.[18] Sieboldin was treated with 125, 250, 500, 1000 μg/ml and inhibited the production of AGEs in a concentration-dependent manner. The same concentration of positive control and sieboldin showed similar inhibition (Fig. 5).

In conclusion, sieboldin was identified as a major active molecule representing the MGO-trapping activity

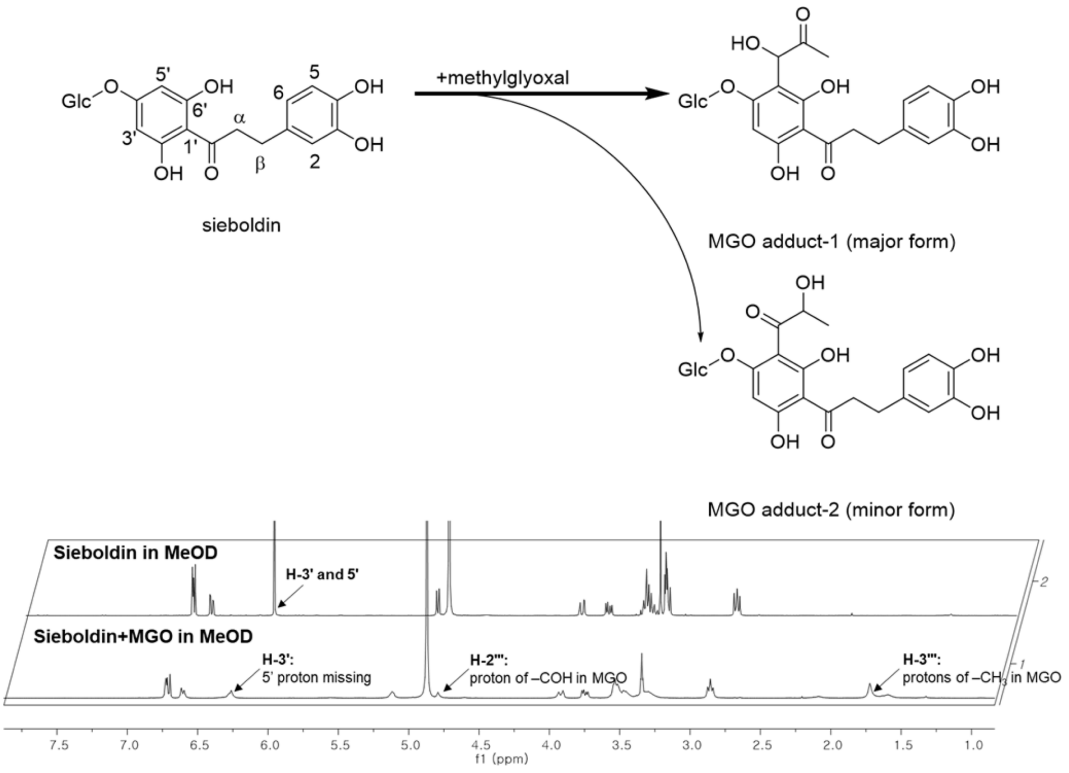

Fig. 4. The chemical structures of sieboldin and mono-MGO adducts of sieboldin (A) and ^{1}H NMR spectra (B). MGO adduct is a mixture of two tautomers (ratio of 2:1).

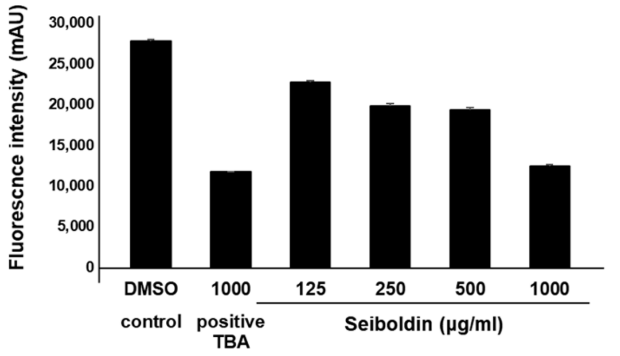

Fig. 5. Inhibitory effect of the formation of AGEs by sieboldin in the BSA-MGO assay. Data are presented as the means ± SD of three replications.

of *Malus baccata.* After 4 hr incubation, sieboldin trapped over 43.8% MGO at a concentration of 0.33 mM and showed MGO scavenging activity with an RC_{50} value of 0.88 mM for the incubation of 30 min under physiological conditions. It was also confirmed that inhibited formation of AGEs generated in the BSA/MGO. Thus, sieboldin could prevent the development of diabetic complications but further studies require, especially under *in vivo* conditions.

Conflicts of interest

All authors declare no conflicts of interest.

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