



Research Article

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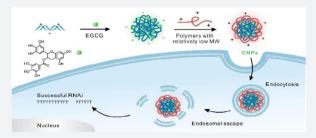
# Green Tea Catechin Dramatically Promotes RNAi Mediated by Low-**Molecular-Weight Polymers**

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Supporting Information

ABSTRACT: Cytosolic delivery is the major challenge that limits the clinical translation of siRNA-based therapeutics. Although thousands of polymers have been developed for siRNA delivery, the efficiency-toxicity correlation is unsatisfactory. Here, we report a facile strategy to fabricate core-shell-structured nanoparticles with robust siRNA delivery efficiency. The nanoparticle is prepared by entropy-driven complexation of siRNA with a green tea catechin to yield a negatively charged core, followed by coating lowmolecular-weight polymers to form the shell. This supramolecular strategy facilitates the polymers condensing siRNA into uniform



nanoparticles. The nanoparticle specifically down-regulates target genes in vitro and in vivo, and efficiently attenuates chronic intestinal inflammation in an inflammatory bowel disease model. Notably, the highly efficient nanoparticles are applicable for various polymers with different topologies and chemical compositions, providing a versatile technique to break down the efficiency—toxicity correlation of cationic polymers. The proposed strategy in this study permits the development of a promising platform for polymer-mediated siRNA delivery.

S mall interfering RNAs (siRNAs) have great potential to specifically down-regulate target genes for the treatment of various diseases. 1-3 However, siRNAs are relatively large, negatively charged, and hydrophilic molecules that are susceptible to nuclease degradation. 4,5 These properties restrict their penetration across cell membrane and decrease the durability of therapeutic effects.<sup>6</sup> An effective and safe delivery system is crucial for clinical translation of siRNA-based therapeutics. 4,7,8 Even with the success of Patisiran, a lipidformulated siRNA nanoparticle, in a phase 3 trial, there is continuing interest in the development of efficient and safe siRNA delivery systems.9

To date, cationic polymers, 10-12 lipid nanoparticles, 13-15 proteins and peptides, 16,17 inorganic nanoparticles, 18,19 ligand-siRNA conjugates, 20,21 and RNA assemblies 22,23 have been designed for RNAi. Among them, cationic polymers were one of the most widely adopted materials. 24-26 They form polyplexes with siRNA via ionic interactions. Considering the rigid and short double-helical structures, it is hard to condense siRNAs, and the polyplex formation lacks cooperativity.<sup>27</sup> The loose siRNA polyplexes are easily destabilized by proteoglycans abundant outside the cells. 25,28 To strengthen the polyplex stability, polymers with high charge densities and molecular weights were used.<sup>29–32</sup> Though siRNA binding affinity and transfection efficiency were improved in these efforts, the efficiency-toxicity correlation for these polymers has been unsatisfactory.<sup>33</sup> To break down the correlation, low-

molecular-weight polycations with minimal toxicity were assembled into nanostructures, or anchored to biocompatible scaffolds to generate hybrid nanostructures. 34-39 In an alternative strategy, siRNAs were prefabricated into nanostructures before complexation with polymers to increase the binding affinity, i.e., concatemerization of siRNAs with thiol groups or commentary overhangs into "genelike" chains, <sup>27,40-42</sup> or hybridization of siRNAs to well-defined oligonucleotide nanoparticles by DNA origami or selfassembly. 23,43-45 Despite these impressive efforts, the current approaches for siRNA delivery still have some limitations such as the need of siRNA chemical modification, sophisticated material syntheses, safety concerns of synthetic polymers, and limited RNA interfering (RNAi) efficiency. 3,46

Here, we reported a general and robust strategy to fabricate nanoparticles for siRNA delivery. The nanoparticle consists of a natural or synthetic low-molecular-weight polymer, a natural polyphenol (-)-epigallocatechin-3-O-gallate (EGCG), and siRNAs. EGCG, a major ingredient of green tea, has strong binding affinity with DNA, RNA, and proteins via hydrogenbond interactions. 47,48 The nanoparticles were fabricated by precomplexation siRNA with EGCG to yield a negatively charged core, followed by coating a low-molecular-weight cationic polymer to form the shell. This supramolecular

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1326



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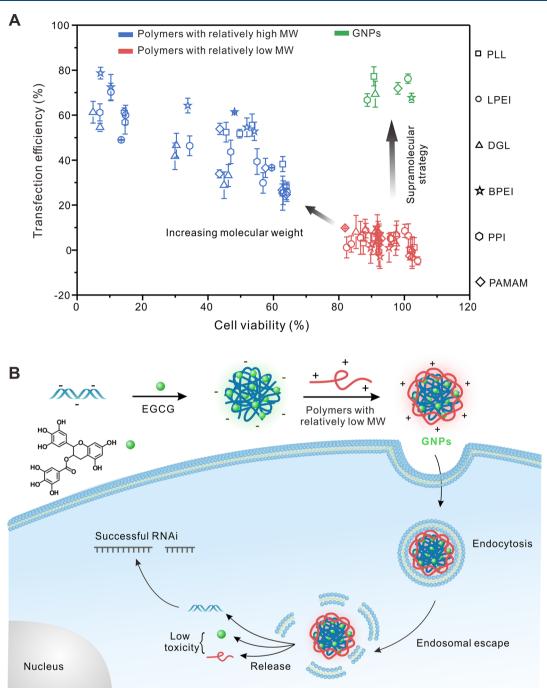


Figure 1. (A) The transfection efficiency—toxicity correlation of cationic polymers in siRNA delivery. Increasing the molecular weight (MW) of polymers improves transfection efficiency, but also induces serious cytotoxicity. The fabricated GNPs by a supramolecular strategy ensure both high efficiency and minimal toxicity. (B) Schematic illustration of GNP formulation and the proposed gene silencing mechanism.

strategy facilitates low-molecular-weight polymers "condensing" siRNA into uniform nanoparticles. The generated nanoparticles successfully achieved high RNAi efficiency with minimal toxicity *in vitro* and *in vivo*. Considering the essential role of green tea catechin in efficient and nontoxic RNAi, the nanoparticle was termed "green" nanoparticles (GNPs).

To confirm the efficiency—toxicity correlation of cationic polymers in siRNA delivery, we tested six types of intact polymers with different molecular weights including poly-Llysine (PLL, 4224 and 25 000 Da), linear polyethylenimine (LPEI, 2500 and 25 000 Da), dendri-graft-L-lysine (DGL, 6800 and 128 000 Da), branched polyethylenimine (BPEI, 1800 and

25 000 Da), poly(propylene imine) dendrimer (PPI, 692 and 7618 Da), and polyamidoamine dendrimer (PAMAM, 1430 and 28 826 Da). Among the polymers, PLL and LPEI belong to linear polymers, and DGL and BPEI are branched polymers, while PPI and PAMAM are hyperbranched polymers. As shown in Figure 1A and Figure S1, all the low-molecular-weight polymers are minimally toxic, but nonefficient at the same time (red symbols), while high-molecular-weight ones show considerable RNAi efficiency, however, accompanied by serious cytotoxicity (blue symbols). To break down this correlation, we fabricate GNPs using the six representative

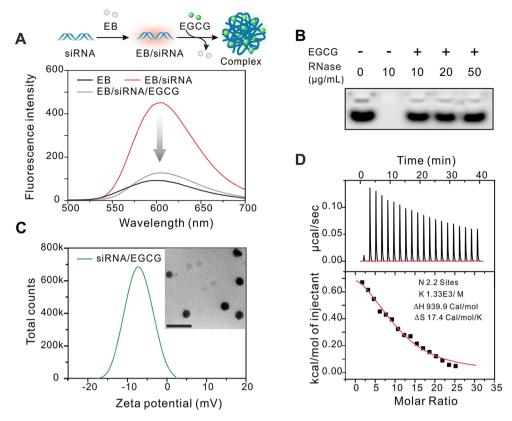


Figure 2. The interactions between EGCG and siRNA. (A) EB competitive binding assay. The binding of EGCG to siRNA causes the exclusion of EB from siRNA and the quenching of EB fluorescence. (B) Stability of EGCG/siRNA complexes against RNase  $(0-50 \,\mu g/mL)$ . (C)  $\zeta$  potential and TEM image of EGCG/siRNA complex prepared at EGCG-to-siRNA weight ratio of 5:1. The scale bar is 200 nm. (D) ITC data for the titration of EGCG into siRNA in 10 mM Tris buffer (pH 7.0). The dilution heat of EGCG was subtracted from released heat.

polymers with relatively low molecular weight, which achieving both high efficiency and biocompatibility (green symbols).

The key problem of low-molecular-weight polymers in siRNA delivery is the difficulty in polyplex formation. <sup>14</sup> Here, a natural polyphenol EGCG was preincubated with siRNA before complexation with polymer (Figure 1B). The polyphenolic structure of EGCG enables strongly affinity to siRNA via cooperative hydrogen-bond and hydrophobic interactions. 47-49 The interactions of EGCG with siRNA were confirmed by ethidium bromide (EB) competitive binding, RNase degradation, and transmission electron microscopy (TEM) experiments. EB intercalates into the grooves of siRNA, yielding a red fluorescent complex. The addition of EGCG into the complex significantly decreases the fluorescence intensity (Figure 2A), suggesting competitive binding of siRNA with EGCG. Similarly, the mixing of EGCG with a fluorescent-labeled siRNA showed partially quenched fluorescence due to EGCG-driven aggregation of siRNA (Figure S2). The EGCG/siRNA complex efficiently prevents the degradation of siRNA by RNase (Figure 2B). The formed complex was characterized to be negatively charged nanoparticles (-8.41 mV, Figure 2C and Figure S3). For an investigation into which type of interaction dominates the complexation, the interaction between EGCG and siRNA was investigated by isothermal titration calorimetry (ITC). As shown in Figure 2D, the binding of EGCG with siRNA is an endothermic reaction. The interaction is entropy-driven and occurs with an increase of entropy. These results suggest the successful complexation of siRNA by EGCG, and the

interaction is likely driven by hydrophobic and hydrogenbond interactions.<sup>50</sup>

The EGCG/siRNA complex was further coated with cationic polymers via electrostatic interactions. EGCG significantly improves the siRNA complexation capability of low-molecular-weight polymers. As shown in Figure 3A, PLL (4224 Da) alone fails to condense siRNA at polymer-to-siRNA weight ratios up to 100:1. In the presence of EGCG, however, it successfully forms nanoparticles within 200 nm even at a weight ratio of 5:1. The yielding nanoparticles are relatively stable in different solutions such as 150 mM NaCl and cell culture medium (Figure S4). The benefit of EGCG in facilitating siRNA condensation is further confirmed by dynamic light scattering (DLS), TEM, and EB competitive binding analysis (Figure 3B, Figures S5 and S6). The successful coating of low-molecular-weight polymers on the EGCG/siRNA complex is confirmed by fluorescence resonance energy transfer (FRET) analysis. EGCG/siRNA complex labeled with carboxyfluorescein shows strong FRET signal after the addition of rhodamine-labeled PLL (PLL-Rho), while PLL and siRNA display a weak FRET signal in the absence of EGCG (Figure 3E). The structure of the formed GNPs consisting of PLL, EGCG, and siRNA is analyzed by TEM and energy-dispersive X-ray spectroscopy (EDX) element mapping. As shown in Figure 3F and Figure S9, the phosphorus (P, represents siRNA) generally locates in the interior of GNPs, while the nitrogen (N) and oxygen (O) distribute throughout the nanoparticle. This result suggests that the ternary complex is a core-shell-structured nanoparticle. Such a supramolecular strategy in GNPs fabrication is

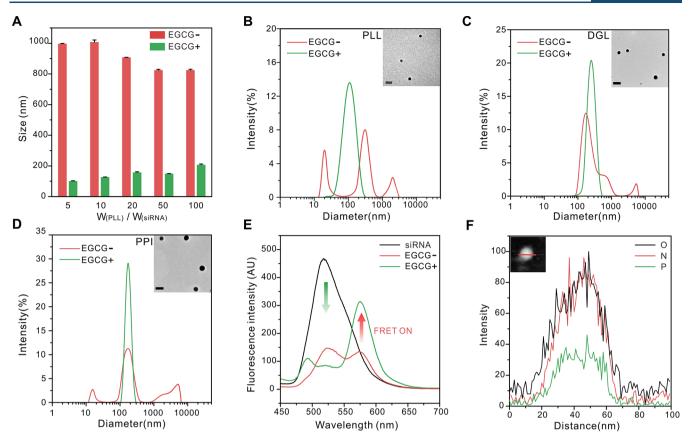


Figure 3. The characterization of GNPs. (A) Sizes of PLL/siRNA polyplexes without and with EGCG at different polymer-to-siRNA weight ratios. DLS and TEM images of GNPs consisting of PLL (B), DGL (C), and PPI (D). The size distribution of polyplexes without EGCG was shown for comparison. Scale bar is 200 nm. (E) Fluorescence spectra of siRNA-FAM, PLL/siRNA-FAM, and GNPs consisting of siRNA-FAM, EGCG, and PLL. PLL was labeled with rhodamine. (F) High-angle annular dark-field TEM (HAADF-TEM) image and corresponding element line-scan of a single GNP consisting of siRNA, EGCG, and PLL. The red scale bar is 100 nm. The EGCG-to-siRNA weight ratios were 5:1 for PLL, and 10:1 for DGL and PPI. The PLL-to-EGCG weight ratio in parts B, E, and F was 1:1. For DGL (C) and PPI (D), the polymer-to-EGCG weight ratio was 1:4.

applicable for all the six types of low-molecular-weight polymers (Figure 3B–D, Figures S6–S8).

We then tested RNAi efficiency of GNPs on HeLa cells stably expressing firefly luciferase (HeLa-Luc). As shown in Figure 4, all the low-molecular-weight polymers show extremely low gene silencing efficiency (<5%) in the absence of EGCG, while the GNPs exhibit high RNAi efficiencies (~80%). In addition, parallel experiments of GNPs containing scrambled siRNA show negative gene silencing (Figure S10), suggesting high specificity for GNPs-mediated RNAi. The chosen polymers in this study include two linear polymers, two branched polymers, and two hyperbranched polymers. Except for a topology difference, the investigated polymers consist of various chemical components. The results suggest that GNPs provide a general and robust method for siRNA delivery.

We further investigated the endocytic pathways for GNPs using specific inhibitors. The results show that the endocytosis of GNPs is mediated by a lipid-raft-dependent pathway (Figure S11). The uptake of GNPs is much more efficient than polyplexes without EGCG (Figure S12). The formulated siRNAs are generally not colocalized with endolysosomes stained by LysoTracker red (Figure S13), and could be released into cytosol after 12 h (Figure S14). These results suggest that GNPs are beneficial for efficient endocytosis and intracellular siRNA release. GNPs also showed high gene silencing efficacy when incubated in cell culture medium or

150 mM salt for 24 h and distilled water for up to 7 days (Figure S15). Decreasing the siRNA dose in GNPs only slightly reduces the efficiency (Figure S16A). Even at a dose of 0.1 nM, the GNPs still show gene knockdown efficiency above 60%. The GNPs also exhibit high efficacies (~80%, 50 nM siRNA) when silencing the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene in PC9 cells, the matrix metallopeptidase 9 (MMP-9) gene in U87 cells, and the prolyl hydroxylase 2 (PHD2) gene in primary murine small intestinal epithelial cells (IECs) (Figure S16B-E). GNPs are even efficient in the delivery of siRNA into hard-to-transfect cells such as Raw264.7 (Figure S17). As described before, lowmolecular-weight polymers are generally nontoxic. The used EGCG in the GNPs is a major constituent of green tea catechins with beneficial properties for various diseases. Daily administration of EGCG at a dose of 300-600 mg per person is proven to be safe in clinical studies. 51 The third component in the GNPs, siRNA, is also an FDA-approved biomolecule.<sup>6</sup> Therefore, all the low-molecular-weight polymers and their GNPs cause negligible toxicity at optimal transfection concentrations (Figure S16F), and the addition of EGCG does not bring additional cytotoxicity. Among the investigated polymers,  $\varepsilon$ -PLL, a natural polymer produced by Streptomyces albus, is especially preferred because it has been used as food additives. The promising results on high efficiency and

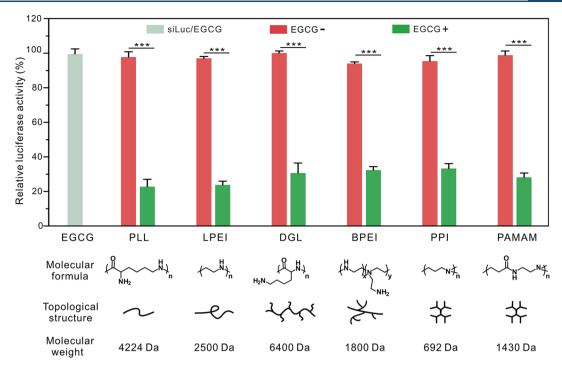


Figure 4. RNAi efficiency of GNPs consisting of polymers with different topological structures and chemical components (green bars) on HeLa-Luc cells for 24 h. The polyplexes without EGCG (red bars) were shown for comparison. The gray bar denotes the EGCG/siRNA complex. The EGCG-to-siRNA weight ratios were 5:1 for PLL and LPEI, and 10:1 for DGL, BPEI, PPI, PAMAM, and EGCG/siRNA complex, respectively. The polymer-to-EGCG weight ratio in the GNPs was 1:1. The siRNA concentration was 50 nM. \*\*\*p < 0.001 analyzed by Student's t test.

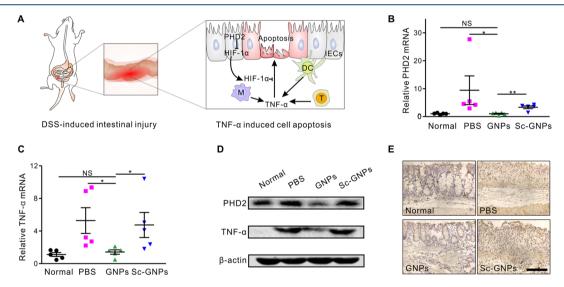
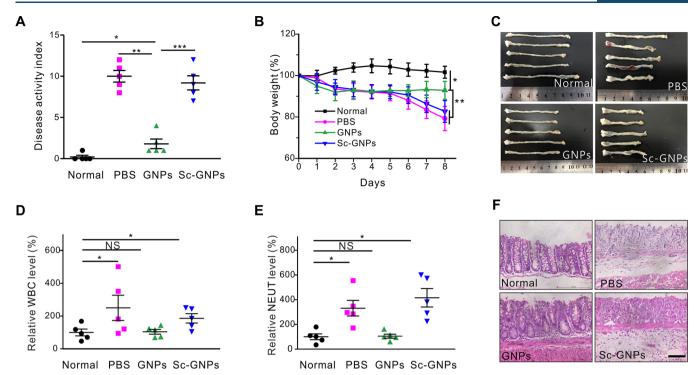


Figure 5. Gene knockdown efficiency of GNPs *in vivo*. (A) A DSS-induced intestinal injury model was established to evaluate the gene knockdown efficiency of GNPs consisting of PLL, EGCG, and siPHD2. The knockdown of PHD2 stabilizes HIF-1 $\alpha$ , and down-regulates TNF- $\alpha$  in the colon tissues, which reduces TNF- $\alpha$ -caused intestinal epithelial damage. The mice with DSS-induced intestinal injury were treated with PBS, GNPs containing siPHD2, or scrambled GNPs containing siNC (Sc-GNPs). The normal mice were not treated during the therapeutic period. PHD2 (B) and TNF- $\alpha$  (C) mRNA levels in the colon tissues measured by quantitative PCR after the treatments. (D) Relative protein levels of PHD2 and TNF- $\alpha$  in the colon tissues analyzed by Western blot. (E) TNF- $\alpha$  in the colon tissues analyzed by immunohistochemistry (scale bar, 100  $\mu$ m). The siRNA dose was 0.75 mg/kg mice, and the weight ratios of EGCG-to-siRNA and EGCG-to-PLL were 5:1 and 1:1, respectively. NS p > 0.05, \*p < 0.05, and \*\*p < 0.01 analyzed by Student's t test.

biocompatibility motivate us to investigate the efficiency of GNPs in vivo.

We further evaluated the therapeutic efficiency of GNPs in a dextran sulfate sodium (DSS) induced intestinal injury model, which is an inflammatory disorder of the gastrointestinal tract. <sup>52</sup> It is reported that prolyl hydroxylase inhibition has beneficial effects in TNF- $\alpha$  induced intestinal

epithelial damage by stabilizing hypoxia-inducible factor  $1\alpha$  (HIF- $1\alpha$ ). Here, one of the prolyl hydroxylases, PHD2, is chosen as the therapeutic target (Figure 5A). Intrarectal administration of GNPs loaded with siRNA targeting PHD2 to mice with DSS-induced intestinal injury led to a significant decrease of PHD2 gene in the colonic biopsies (Figure 5B). The down-regulated PHD2 contributes to HIF- $1\alpha$  stabiliza-



**Figure 6.** Therapeutic efficiency of GNPs in the treatment of DSS-induced intestinal injury. Disease activity index (A), body weight (B), and colon length (C) of mice with DSS-induced intestinal injury after the treatments. Relative white blood cells (WBC, D) and neutrophils (NEUT, E) in the blood of treated mice. (F) Histological staining of colon tissues in mice after the treatments (scale bar, 100  $\mu$ m). NS p > 0.05, \*p < 0.05, \*p <

tion, which further down-regulates the TNF- $\alpha$  gene in the intestinal tissues (Figure 5C). Western blotting results further confirm the decreases in PHD2 and TNF- $\alpha$  proteins (Figure 5D), and reduced HIF-1 $\alpha$  protein degradation (Figure S18). Immunohistochemistry analysis also shows a much decreased TNF- $\alpha$  level in the GNP group compared to the control groups (PBS and GNPs containing scrambled siRNA, Sc-GNPs, Figure 5E). Administration of GNPs to mice with DSSinduced intestinal injury shows an obvious amelioration of intestinal symptoms, i.e., significantly lower disease activity score (Figure 6A and Figure S19), less loss of body weight (Figure 6B), less shortening of the colon (Figure 6C), and lower levels of inflammatory cells such as white blood cells (Figure 6D) and neutrophils (Figure 6E) compared to the control groups. Damage in the intestinal epithelial barrier function is a characteristic feature of intestinal injury. As shown in Figure 6F, GNP-treated mice with DSS-induced intestinal injury show impaired intestinal epithelial structures in histological sections similar to that of healthy mice. The hematological parameters of mice administered with GNPs are similar to those of healthy mice without any treatment (Figure S20). For practical treatment of DSS-induced intestinal injury in the future, the GNPs can be encapsulated within an enteric capsule and administered orally to avoid their disassembly in gastric acid. Considering the beneficial properties of EGCG in antioxidant, anti-inflammation, antibacterial, and anticancer effects, 48 the GNPs can be used for the local treatment of various diseases (e.g., corneoiritis, diabetic wound healing, dermatosis, and local bacterial infections) when combining the therapeutic effect of EGCG and the promising efficiency of GNPs.

In summary, EGCG facilitates siRNA condensation by low-molecular-weight polymers, and the yielding GNPs show

robust efficiency on gene silencing. In addition, GNPs containing a therapeutic siRNA efficiently silenced the target genes *in vivo*, and ameliorated intestinal inflammation in a DSS-induced intestinal injury model. The biocompatible components such as EGCG, siRNA, and PLL in GNPs ensure minimal toxicity on the transfected cells. Considering the chemical similarity of siRNA with microRNAs, antisense oligodeoxynucleotides, DNAzymes, and peptide nucleic acids, the proposed supramolecular strategy for the fabrication of GNPs should be generally applicable to a wide variety of nucleic acids and permits the development of a general and robust platform for gene delivery.

#### ASSOCIATED CONTENT

# **S** Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00363.

Additional experimental details and figures including transfection efficiency and cell viability, fluorescence spectra, size distribution, stability of GNPs, siRNA condensation capability, TEM images, DLS images,  $\zeta$  potentials, elemental distributions, gene knockdown efficiency, cellular uptake pathways, intracellular localization, CLSM images, relative protein levels, treatment effect of GNPs, and relative inflammatory cell levels (PDF)

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#### **Notes**

The authors declare no competing financial interest.

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