Gambogic acid inhibits proliferation and induces apoptosis of human acute T-cell leukemia cells by inducing autophagy and downregulating β-catenin signaling pathway: Mechanisms underlying the effect of Gambogic acid on T-ALL cells

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Received September 21, 2019; Accepted June 11, 2020

DOI: 10.3892/or.2020.7726

Abstract. The main active compound of Garcinia hanburyi (referred to as gamboge) is gambogic acid (GA), which has long been a Chinese herbal medicine for treating several types of cancer. However, the potential therapeutic role and mechanisms of GA in T-cell acute lymphoblastic leukemia (T-ALL) remain unclear. In the present study, the effects of GA on proliferation, cell cycle, apoptosis, and autophagy in T-ALL cell lines were investigated. The possible mechanisms underlying GA activity were also examined. The results showed that GA inhibited proliferation, induced apoptosis, and activated autophagy in T-ALL cell lines (Jurkat and Molt-4 cells). Findings confirmed that GA has an antileukemia effect against peripheral blood lymphocyte cells in patients with ALL. GA inhibited phospho-GSK3β S9 (p-GSK3β S9) protein levels to inactivate Wnt signaling and suppress β-catenin protein levels. In addition, the inhibitory effect of GA on T-ALL was reversed by overexpression of β-catenin. Thus, GA can inhibit the growth and survival of T-ALL cells. GA also had antileukemic activity, at least in part, through the downregulation of the Wnt/β-catenin signaling pathway.

Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous hematopoietic malignancy, characterized by the expansion of lymphoid progenitor cells. Massive proliferation of cells, extensive infiltration, and inhibition of normal hematopoiesis are the main clinical manifestations of ALL. It is the most common neoplasm in pediatric patients. Approximately 85% of ALLs are of the B-cell lineage; the rest are of T-cell lineage (1). T-cell ALL (T-ALL) presents as an uncontrolled malignant accumulation of T-cell progenitors (1,2). T-ALL cases can occur at any age, while the majority of cases occur between 2 and 5 years of age (3). Current treatments focus on intensive chemotherapy, targeted therapy, and bone marrow transplantation (4). Although 80% of cases initially achieve remission (5), most patients show relapse or the cancers resist chemotherapy, resulting in a poor prognosis of patients (1). Therefore, efficient and low-toxicity antitumor compounds are crucial to ameliorate the prognosis of patients with T-ALL.

Currently, several treatment approaches methods for T-ALL are in practice; these include inducing apoptosis, inhibiting proliferation, causing cell cycle arrest and inducing differentiation (6,7). Chinese herbs have been widely used to treat cancers as they are cost-effective and have positive outcomes. More than half antitumor compounds in use today are herbaceous (8).

Gambogic acid (GA) is a natural medicinal compound that is extracted from the traditional Chinese herb Garcinia hanburyi. Its molecular formula is C38H44O8, and it has a molecular weight of 628.75 g/mol. GA is a potential antineoplastic drug against several solid tumors and hematological malignancies (9-11), including hepatocellular (12), malignant (13), breast carcinoma (14), and chronic myeloid leukemia (15). GA has been shown to affect the physiological function and signaling pathways of several tumor cell lines (16,17). Activation of cell apoptosis has been suggested to be the main mechanism underlying the repressive effect of GA on solid tumors. The related molecular mechanisms possibly
include inhibition of telomerase activity or obstructing trans-
ferrin receptor internalization (9,10). In addition, it has been 
found that GA can inhibit multiple cell signaling pathways, 
including the Wnt/β-catenin pathway, and transcription 
factors, including nuclear factor-kappa B, activator protein 
1, NRF2, PPAR-γ, tumor necrosis factor-α, sonic hedgehog, 
and nitric oxide synthase (18,19). However, the potential effect 
of GA on T-ALL is not fully understood, and the underlying 
molecular mechanisms require further investigation.

β-catenin, a transcriptional coactivator of the Wnt/β-catenin 
signaling pathway, is degraded by proteasomes when cell 
function is normal. Once the pathway is activated, β-catenin 
cannot accumulate in the cytoplasm and then translo-
cates to the nucleus. In the nucleus, β-catenin interfaces with 
high-mobility group transcription factors belonging to the T-cell 
factor/lymphoid enhancer factor family to activate downstream 
mechanisms. In the nucleus, β-catenin signaling pathways are 
involved in the transcriptional regulation of target genes, including c-Myb, Axin2, and CyclinD1. All of these 
mechanisms are attractive therapeutic targets (20-23).

The canonical Wnt pathway (Wnt/β-catenin pathway) is 
involved in the regulation of various physiological and 
pathological processes during early-and late-stage embryonic 
development (24) and in carcinogenesis (25,26). Different 
types of tumors cause aberrance of the Wnt/β-catenin pathway. 
Previous studies have shown that Wnt/β-catenin pathway plays 
a crucial role in various leukemias (27-29). This pathway is 
closely associated with self-renewal, overexpression and 
deletion of mutations in hematopoietic stem cell (HSC). 
These factors all play important roles in leukemogenesis, 
drug resistance, and tumor relapse (30). Furthermore, it has 
been demonstrated that Wnt/β-catenin signaling is involved in 
the development of T cells and often the deregulation of 
T-ALL (31,32). Thus, Wnt/β-catenin signaling is a worthy 
target for improving T-ALL therapy results.

In the current study, we investigated the antitumor effects 
of GA using T-ALL cell lines and patient samples. The study 
was expanded to examine the possible mechanisms causing 
the observed effects of GA. GA demonstrated marked potency 
in inducing apoptosis, regulating cell cycle arrest, inhibiting 
proliferation, and inducing autophagy in T-ALL cells. Further 
investigations revealed that the Wnt/β-catenin signaling 
pathway plays an important role in the inhibitory function of 
GA. Thus, GA is a potential antitumor adjuvant agent.

Materials and methods

Cell culture. The human ALL lines Jurkat and Molt-4 were 
obtained from the cell bank of the Chinese Academy of Science 
(Shanghai, China). Jurkat cells and Molt-4 cells were routinely 
cultured in RPMI-1640 (Gibco) medium supplemented with 
10% fetal bovine serum (Gibco) with 1% glutamine-penicillin, 
and 2% non-essential amino acids (Gibco). Cells were cultured in RPMI-1640 medium supplemented with 
20% fetal bovine serum.

Following institutional guidelines, blood samples were obtained 
following informed consent from six patients with T-ALL and 
six healthy patients. Patients were recruited from the Zhejiang 
Provincial People's Hospital in November and December, 2018. 
Lymphocytes were isolated from EDTA-treated blood by density 
gradient centrifugation using Lymphocyte Separation Medium 
(TBD) as per the manufacturer's instructions. Peripheral 
anti-coagulated blood (4 ml) was mixed with calcium-magne-
sium-free phosphate-buffered saline (PBS) (ratio 1:1). Samples 
were layered onto 5-ml lymphocyte separation medium and 
centrifuged at 300 x g for 25 min at room temperature. The 
interface layer was centrifuged at 50 x g for another 10 min at 
temperature, and the pellet was collected. The enriched 
cells were cultured in RPMI-1640 medium supplemented with 
20% fetal bovine serum.

The relevant ethics approval was granted by the Zhejiang 
Provincial People's Hospital Ethics Committee.

Gambogic acid preparation. GA with a purity of >98% was 
purchased from Chengdu Must Bio-Technology Co. Ltd. It was 
dissolved in dimethyl sulfoxide and then stored at -20°C for 
further analyses.

Cell viability assay using Cell Counting Kit-8. Cell viability 
after GA treatment was determined using a Cell Counting 
Kit-8 (CCK-8) assay (Beyotime). Cells were seeded in 
96-well plates (1x10^4 cells/well) and treated with different 
concentrations of GA and immersed overnight. Coverslips 
were treated with 0.2-mg/ml poly-L-lysine for 4 h prior to 
treating them on coverslips. After 1.5 h of incubation, the cells 
were fixed in 0.1% Triton X-100 (Beyotime) and 5% BSA prior 
to incubation. The primary antibody against p-GSK3β (1:200, Cell Signaling Technology, cat. no. 5558) was added, 
and the specimens were incubated overnight at 4°C. The next 
day, the cells were washed with PBS three times and the 
appropriate fluorescent secondary antibody Alexa Fluor 488
was added (1:1,000, Beyotime, cat. no. A0423). The samples were incubated at room temperature for 1 h. Cells were stained with DAPI (Beyotime) to allow visualization of the nucleus. Finally, images were obtained using a fluorescence microscope (Olympus).

Monodansylcadaverine staining. Jurkat cells were plated in 6-well plates and treated with different concentrations (1.2, 2.4 and 4.8 µM) of GA, and controls were treated with 0.1% DMSO. After treatment, the cells were collected and suspended with PBS at a density of 1.5x10^6 cells/ml, followed by co-staining with monodansylcadaverine (MDC) plus DAPI for another 30 min at 37°C. Approximately 10-20 µl of the cell suspension was placed onto a glass slide and covered with a cover glass. The MDC staining was examined and photographed using a fluorescence microscope.
Western blot analysis. Lysed cells in RIPA lysis buffer (Beyotime) were maintained on ice for 30 min and centrifuged at 14,000 x g for 10 min at 4°C. The protein concentration in the supernatant was verified by BCA Protein Assay.
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(Thermo Scientific Inc). Total proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked in 5% de-fatted milk for 1 h at room temperature and then incubated with primary antibodies at 4˚C overnight. The matched secondary antibody (1:3,000, Beyotime, cat. nos. A0208, A0192) was incubated for 2 h at room temperature. Protein levels were detected using an enhanced chemiluminescence reagent and quantified by ImageJ software. The antibodies used in the study were as follows: Anti-CyclinB (1:1,000, Abcam, cat. no. ab32053), anti-CDK1 (1:500, Beyotime, cat. no. AF0111), anti-Beclin1 (1:1,000, Thermo, cat. no. MA5-15825), and anti-LC3-II (1:1,000, Sigma, cat. no. 610832); anti-Bax (1:1,000, Abcam, cat. no. ab32503), anti-Bcl-2 (1:1,000, Abcam, ab182858), anti-cleaved caspase3 (1:1,000, Cell Signaling Technology, cat. no. 9661S),

Figure 3. GA treatment down-regulated Wnt/β-catenin signaling pathways in Jurkat cells. (A) Western blot detected the expression of Wnt signaling pathway proteins in Jurkat cells after GA treatment (β-catenin, p-GSK3β, GSK3β, CyclinD1, c-Myc). Corresponding statistical chart of β-catenin, p-GSK3β, GSK3β, p-GSK3β S9/GSK3β, CyclinD1 and c-Myc is also shown. (B) Single immunocytochemistry of p-GSK3β S9 (green) in Jurkat cells treated with GA at indicated concentrations for 24 h. *P<0.05 and **P<0.01 compared with control, controls were treated with 0.1% DMSO. Bar scale: 20 µm.
anti-cleaved PARP (1:1,000, Abcam, cat. no. ab191217) anti-p-GSK3β S9 (1:1,000, Cell Signaling Technology, cat. no. 5558), and anti-Atg7 (1:1,000, Cell Signaling Technology, cat. no. 8558); anti-β-catenin (1:1,000, Cell Signaling Technology, cat. no. 19807S), anti-c-Myc (1:1,000, Cell Signaling Technology, cat. no. 2278S), and anti-CyclinD1 (1:1,000, Abcam, cat. no. ab134175); and anti-β-actin (1:3,000, Beyotime, cat. no. AF5001).

Statistical analysis. Data are presented as mean ± standard deviation. Data were analyzed using SPSS21.0. The data were collected from at least three independent experiments. Significance of the difference between two groups means of data sets was determined using the Student's t-test. Multiple comparisons test was analyzed by one-way ANOVA (Dunnett's correction). Results with P<0.05 and P<0.01 were considered statistically significant.

Results

GA inhibits the proliferation of human leukemia cell lines. To detect the anti-proliferative effects of GA in vitro, the human leukemia cell lines Jurkat and Molt-4 cells were exposed to increasing concentrations of GA. GA reduced the viability of Jurkat and Molt-4 cells in a dose-dependent manner (Fig. 1A-B). The IC50 values of GA for Jurkat and Molt-4 were 2.23 and 3.03 µM, respectively. Jurkat and Molt-4 cells were treated with GA (1.2, 2.4 and 4.8 µM) for 0, 24, 48 and 72 h. At the higher concentrations (2.4 and 4.8 µM), GA inhibited the growth of cells significantly, and the inhibition rate increased with time. These results suggested that GA cytotoxicity is dose- and time-dependent in leukemia cells.
GA induces G2/M phase cell cycle arrest in Jurkat cells. To test whether GA could induce Jurkat cell cycle arrest, cell cycle distribution was performed. The results demonstrated that the exposure of cells to GA at concentrations of 2.4 and 4.8 µM led to a remarkable increase in G2/M phase, with a corresponding decrease in the S phase compared with exposure of cells to control or 1.2-µM GA (Fig. 1C). To determine the underlying mechanisms causing G2/M phase arrest in Jurkat cells, we examined the effect of GA on the expression of CyclinB and CDK1 (key regulators of G2/M transition). Western blot results revealed that the protein levels of CyclinB and CDK1 were significantly decreased by GA (Fig. 1D). Taken together, these results indicate that GA may inhibit the proliferation of Jurkat cells by inducing cell cycle arrest at the G2/M phase.

GA induces apoptosis of Jurkat cells. It is known that apoptosis is programmed cell death. Thus, we examined whether GA induces apoptosis in cancer cells. Using a flow cytometric analysis the presence of apoptosis in GA-treated cells was identified. GA treatment induced significant apoptosis in Jurkat cells in a dose-dependent manner (Fig. 2A). Apoptosis was detected by western blot analysis (Fig. 2B). It was noted that GA treatment upregulated the expression of c-PARP, c-Caspase-3, and Bax but downregulated the expression of Bcl-2 compared with control treatment. Overall, these results demonstrated that GA upregulated the expression of pro-apoptotic proteins, thus causing apoptosis in Jurkat cells.

GA induced autophagy in Jurkat cells. We observed whether autophagic vacuoles were formed when Jurkat
cells were treated with GA by MDC staining (Fig. 2C). The number of MDC-positive fluorescent points in GA-treated cells was markedly greater than that in the control group. These results demonstrated that the inhibitory effect of GA on cell viability is related to the induction of autophagy. To investigate whether GA regulated the process of autophagy, the protein expression of autophagy-related markers were assessed by western blot analysis (Fig. 2D). Significant upregulation of Atg7, Beclin-1, and LC3-II occurred in GA-treated groups. These results suggest that GA may encourage autophagy of Jurkat cells.

**GA regulates Wnt/β-catenin signaling in Jurkat cells.** To identify other mechanisms involved in the inhibitory effect of GA, we focused on the Wnt pathway (Fig. 3A). The protein levels of β-catenin, as well as downstream proteins CyclinD1 and c-Myc, were significantly decreased in a dose-dependent manner after GA treatment. GSK3β, a highly effective kinase within the β-catenin destruction complex, phosphorylated β-catenin and promoted its destruction by proteasomes. Therefore, GA suppressed the expression of p-GSK3β S9 and subsequently promoted β-catenin degradation to reduce β-catenin nuclear import. Immunostaining results showed that after GA treatment (Fig. 3B), the expression of p-GSK3β S9 decreased. Our findings indicated that GA inhibits the growth of Jurkat cells by regulating the Wnt/β-catenin pathway.

**Overexpression of β-catenin inhibited the apoptosis of Jurkat cells.** To further confirm the role of β-catenin in GA-induced regulation of Jurkat cell proliferation and apoptosis, we transfected the cells with plasmid-carrying β-catenin. We established that the overexpression of β-catenin protects Jurkat cells from apoptosis. Flow cytometry results showed that after β-catenin plasmid transfection, a proportion of the apoptosis (induced by GA) recovered (Fig. 4A). Western blot results revealed that the transfection of β-catenin plasmid reversed the increase of Bax and c-caspase-3 and decreased Bcl-2 induced by GA (2.4 μM) (Fig. 4B). These results confirmed the hypothesis that the overexpression of β-catenin significantly inhibited the apoptosis of Jurkat cells. After transfection of β-catenin plasmid into GA-treated Jurkat cells, the protein levels of β-catenin, CyclinD1, GSK-3β, p-GSK3β S9, and c-Myc were measured using western blot analysis (Fig. 4C). The results showed that the expression of GSK3β significantly decreased in GA-treated Jurkat cells transfected with β-catenin compared with that in cells transfected with the negative control. In the negative control group, the expression of β-catenin, p-GSK3β S9, CyclinD1, and c-Myc are significantly increased.

**Effects of GA on healthy people and T-ALL patients.** GA toxicity was then examined. The results showed that GA is toxic to Jurkat cells. To investigate the potential of GA for cancer therapy, we evaluated the effects of GA on lymphocyte cells from healthy individuals and patients with T-ALL. Apoptotic assays revealed that cell viability of healthy individuals decreased slightly when treated with GA (Fig. 5). Conversely, GA treatment induced significant apoptosis in lymphocyte cells from patients with T-ALL.

**Discussion**

T-ALL is one of the most common types of aggressive hematological malignancies in children, and has high heterogeneity (33). The current treatment options for ALL include chemotherapy (such as VDLP or VILP regimen) and allogeneic bone marrow transplantation. However, the side effects of myelosuppression and central nervous system issues are still severe (34) leading to a poor prognosis (35). Drug resistance and inevitable recurrence also contribute to the poor prognosis of patients (36). Therefore, there is a need to explore new therapeutic targets for T-ALL treatment. GA, obtained from *G. hanburyi*, exhibits cytotoxic abilities in several solid tumors both *in vitro* and *in vivo*. The assumed mechanism of GA in treating solid tumors primarily involves activating apoptotic pathways (9,10). However, the exact role and possible molecular mechanisms underlying GA activity against T-ALL are still poorly understood and require extensive studies. In the current study, we evaluated the potential antileukemic effect of GA on T-ALL cells. We found that GA suppressed cell proliferation, affected cell cycle distribution, induced apoptosis, and activated autophagy in T-ALL cells. We identified that the antileukemic effect of GA on T-ALL cells may involve regulating the Wnt/β-catenin signaling pathway by inactivating p-GSK3β S9 and activating GSK3β. These combined activities promote the degradation of the β-catenin protein.

Apoptosis induction plays an essential role in the antitumor strategies of various therapies. Previous findings have emphasized the fact that traditional Chinese medicines can strengthen the activation of caspase-independent cell apoptosis pathways (37). Flow cytometry analysis revealed that GA induces apoptosis in T-ALL cells. c-Caspase3 is known to activate other caspases, playing a critical role in the execution of the mitochondrial apoptotic pathway. In the present research, GA increased the expression levels of c-caspase 3 in T-ALL cells, indicating that the mitochondrial apoptosis pathway is affected by GA. Bax and Bcl2 are critical regulators of apoptosis, however, Bax is a proapoptotic protein, and Bcl2 is anti-apoptotic protein (38). GA reduced the expression of Bcl2 and increased that of Bax. Therefore, GA's ability to regulate the balance between Bax and Bcl2 provides evidence that GA induces apoptosis in T-ALL cells. The ability of GA to induce apoptosis is one of the mechanisms contributing to the anticancer effects of GA in T-ALL.

The existing literature shows that T-ALL stem cells are highly dependent on the Wnt/β-catenin signaling pathway (39). Downstream target proteins of Wnt/β-catenin signaling play major roles in neoplastic transformation, including cell cycling (c-Myc and CyclinD1), tumor growth, cell invasion, and tumor metastasis (40). Our flow cytometry results show that GA disturbs T-ALL cell cycle and induces G2/M arrest. Cell cycle arrest is closely related to cycle-regulating proteins (41). CyclinBl and CDK1 are critical regulators of cell cycle progression, specifically in the G2/M transition (42). In our experiment, GA regulated the expression levels of CyclinD1, CyclinBl, and CDK1 proteins. The ability of GA to induce cell cycle arrest highlights its antineoplastic role. This demonstrates another mechanism that GA uses to induce anticancer effects on T-ALL.

The Wnt/β-catenin signaling pathway plays an important role in the self-renewal of HSCs, leukemia progression, and BCR-ABL
kinase inhibitor resistance (43-46). In the Wnt signaling pathway, GSK-3β binds to several proteins and undergoes phosphorylation, thereby inducing β-catenin degradation. GSK-3β has been regarded as a tumor suppressor as it negatively regulates the Wnt/β-catenin signaling pathway (47). It can regulate catalytic kinase activity through phosphorylation of different serine/threonine residue sites. Phosphorylation at tyrosine 216 significantly increases the enzymatic activity of GSK3β, whereas phosphorylation at serine 9 decreases enzyme activity (48). Phosphorylation at S9 is the main mechanism causing GSK3β inactivation (49).

In the present study, we revealed that the p-GSK3β S9 level decreases but the catalytic activity of GSK3β increases by GA treatment. Preliminary results demonstrated that decreased β-catenin protein levels in T-ALL cells may result from GSK3β activation. β-catenin overexpression is associated with neoplasm progression, cell proliferation, and cell survival (50). To evaluate our theories, we overexpressed β-catenin and verified the results using western blot analysis. The degree of proliferation and apoptosis in T-ALL cells after GA treatment showed that the inhibitory effect of GA on T-ALL cells was reversed. Overall, our results demonstrated that GA can suppress growth of T-ALL cells by inactivating the Wnt/β-catenin signaling pathway.

It has been reported that the Wnt/β-catenin signaling pathway is aberrantly upregulated during the occurrence and development of cancers. Thus, mature blood cells lack Wnt signaling (30). However, leukemia cells are highly prolific with high Wnt/β-catenin signaling. The deficiency of Wnt ligands is associated with low levels of β-catenin as destruction complexes target β-catenin for degradation. Our research found that GA could exert inhibitory effects on T-ALL cell lines and lymphocyte cells from patients with ALL. These results suggest that GA is more sensitive to lymphocyte cells from patients with T-ALL.

Autophagy is a highly conservative and protective process of cell recycling and degeneration of damaged functional proteins and organelles through lysosomal degradation and digestion (51,52). Autophagy plays an essential role in normal cell stabilization and tumorigenesis, drug resistance, and other pathophysiologial processes (53-55). Currently, there is no uniform classification criterion for autophagy. Autophagy is regulated by autophagy-related genes. LC3 acts as an autophagosomal marker for monitoring autophagy (56). Beclin1 and Atg7 also belong to autophagy markers as they participate in the formation and maturation of autophagosomes (57). MDC is localized and mainly restricted to the lysosomal membrane (it is also used to assess autophagy activity). Recent research has shown that autophagy and the Wnt/β-catenin pathway are closely related. Autophagy negatively regulates Wnt signaling (58). The tumor inhibitor GABARAPL1 suppresses the Wnt/β-catenin pathway by activating autophagy (59). In our study, autophagy-related proteins (LC3-II, Beclin1, and Atg7) were upregulated, and MDC fluorescence intensity increased significantly after GA treatment. This suggests that GA induces autophagy in T-ALL cells. Activation of autophagy can inhibit the Wnt/β-catenin pathway, further encouraging cell damage. However, the specific mechanisms require further research.

In summary, our study demonstrates that GA exhibits effective antileukemia characteristics in T-ALL cells. The proliferation, apoptosis, and autophagy regulation effects of GA on T-ALL cells seem to result from downregulating Wnt/β-catenin signaling and upregulating GSK3β activity, thereby inducing β-catenin degradation. Collectively, our experimental results indicate that GA is a promising antileukemia agent for the treatment of T-ALL.

Acknowledgements

Not applicable

Funding

This study was supported by grants from the National Natural Science Foundation of China (no. 81570198); National Science and Technology Major Project of China (no. SQ2017ZX09030110); Medical and Health Science and Technology Project of Zhejiang Province (grant no. 2017KY209).

Availability of data and materials

All relevant raw data in our trial will be made freely available to any researchers who wish to use them for non-commercial purposes.

Authors' contributions

YL, YW, TTW designed the study. JD, DK, GY, QZ performed the clinic trials. DK and GY analyzed the data. TTW and JD wrote the paper, and were responsible for the images. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethics approval was granted by the Zhejiang Provincial People's Hospital Ethics Committee. Informed consent was obtained from the subjects included in the present study.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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