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Research Article

CLEISTANTHIN B SHOWS A POTENT CYTOTOXIC ACTIVITY AGAINST COLORECTAL CANCER CELLS

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ABSTRACT

Objective: The aim was to find out the cleistanthin B sensitive cancer cell type among a panel of cancer cell lines.

Methods: The 50% inhibitory concentrations (IC50) of cleistanthin B against different cancer cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The cell death caused by cleistanthin B in colorectal cancer (CRC) cells was evaluated by acridine orange and ethidium bromide (AO-EB) dual staining. Using short exposure, we generated the 5-fluorouracil+oxaliplatin (5-FU+Ox) surviving cells from the parental HT-29 CRC cell lines. These surviving CRC cells were further treated with cleistanthin B either alone or combined with 5-FU. Annexin V apoptosis assay was used to determine the combined effect of cleistanthin B with 5-FU against HT-29 cells.

Results: The IC50 values of cleistanthin B were found to be 3.6±0.55, 5.2±0.51, 8.6±1.02, 10.5±1.50, 18.3±3.71, 25.8±5.50, and 26.7±5.90 μg/mL against HT-29, SW-480, HCT-15, HELA, MDA-MB-231, A549, and DU145, respectively. The IC50 value of cleistanthin B against L132 cells was >100 μg/mL. The cleistanthin B treated HT-29, SW-480, and HCT-15 CRC cells showed apoptotic changes such as chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies in the AO-EB dual staining method. Flow cytometry analysis revealed that cleistanthin B enhances the 5-FU induced apoptosis against 5-FU+Ox surviving HT-29 CRC cells.

Conclusion: Cleistanthin B is relatively more potent against CRC cells than other cancer cells, and it induces apoptosis mediated cell death in CRC cells. Cleistanthin B enhances the anticancer activity of 5-FU against HT-29 CRC cells.

Keywords: *Cleistanthus collinus*, Cleistanthin B, *In vitro,* Cytotoxicity, Apoptosis, Flow cytometry, Anticancer, Colorectal cancer.

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INTRODUCTION

Cancer is a second significant health problem with high mortality worldwide, and the incidence continues to rise in India [1]. Cytotoxic chemotherapy is considered a standard treatment modality for cancer apart from surgery and radiotherapy. The development of adverse effects is one of the major obstacles with cytotoxic chemotherapy. Therefore, targeted therapy has been developed to precisely kill cancer cells with minimal adverse effects on normal cells [2]. However, targeted therapy with cytotoxic chemotherapy is routinely used for better clinical outcomes. Although several cytotoxic drugs are in use, the development of adverse effects and drug resistance limits the treatment options. Therefore, identifying new cytotoxic agents with distinct mechanisms of action is incessant.

Plants, a rich source of natural compounds, have provided many successful anticancer drugs like vincristine, etoposide, paclitaxel, and irinotecan. Natural compounds mainly belong to bioactive substances such as alkaloids, phenols, and glycosides [3]. *Cleistanthus collinus*, a herb, is primarily located in countries of the Southeast Asia region. Intentional consumption of large quantities of *C. collinus* leaves decoction is fatal up to 30% [4,5]. However, the *C. collinus* plant extract and its compounds showed diverse pharmacological activities in experimental studies. These biologically active compounds include diphyllin and its derivatives such as cleistanthin A and cleistanthin B belonging to arylnaphthalene lignan glycosides [6,7]. Arylnaphthalene lignan glycosides primarily belong to natural compounds found in many plant species. Their structural similarity with an anticancer podophyllotoxin prompted many researchers to evaluate such compounds for cytotoxic properties [8,9].

Cleistanthin B is an arylnaphthalene lignan glycoside found in *C. collinus* and other Euphorbiaceae family plants [6,10]. The antiplatelet, antihypertensive, diuretic, and cytotoxic properties of cleistanthin B have been reported in experimental animal models [6,11-13]. Furthermore, cleistanthin B was reported to cause DNA damage in cervical cancer cells [14]. However, studies on the cytotoxic effect of cleistanthin B against multiple cancer cells are limited. Identifying tissue-specific cytotoxicity of compounds is possible when a broad spectrum of cancer cells is tested simultaneously. In earlier studies, the selective inhibition of cleistanthin B against particular cancer cells was not identified. Furthermore, reports on the combined effect of cleistanthin B with conventional anticancer drugs were not available. Therefore, the present study aimed to investigate the cytotoxic effect of cleistanthin B against a panel of solid cancer cell lines to determine the sensitive cancer type.

METHODS

Drugs and chemicals

Dulbecco's Modified Eagle Medium, fetal bovine serum (FBS) (Hyclone, UK), penicillin-streptomycin antibiotic solution, dimethyl sulfoxide (DMSO), and trypsin-ethylene diamine tetra acetic acid (Trypsin-EDTA) were purchased from Hi-Media (USA). 5-fluorouracil (5-FU), oxaliplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange (AO), and ethidium bromide (EB) were purchased from Sigma-Aldrich (USA). Annexin V-APC conjugate, binding buffer, and propidium iodide (PI) from BD bioscience (USA). All other consumables used in the study were purchased locally.

Plant material and compound isolation

C. collinus plant leaves were collected from rural areas of Puducherry, India. The plant specimen was authenticated and deposited (Accession No: HIFP 27059) at the French Institute of Pondicherry and in the Pharmacology department, JIPMER, Puducherry, India. Cleistanthin B

was isolated from *C. collinus* plant leaves, as described in our previous study [15]. Briefly, the shade dried *C. collinus* leaves were powdered and defatted with a 1:1 ratio of petroleum ether and n-hexane. The sediment was then resuspended in acetone (85%): methanol (15%) solvent mixture. Finally, the extract was subjected to column chromatography using neutral alumina and was eluted using various fractions with benzene, ethyl acetate, chloroform, and methanol. Several solvent fractions of chloroform and methanol were collected and identified by the thin-layer chromatography. The cleistanthin B structure was predicted by nuclear magnetic resonance (Bruker Avance-II, USA).

Cell lines and culture conditions

All the human cell lines, colorectal (HT-29, HCT-15, and SW-480), cervical (HeLa), breast (MDA-MB-231), prostate (DU-145), lung (A-549) cancer cells, and embryonic lung (L-132) cells used in this study, were procured from National Centre for Cell Sciences, Pune, India. All the cell lines were usually cultured in DMEM medium with 10% FBS, antibiotics and maintained in a humidified incubator at 37°C with the provision of 5% CO2.

Cell viability assay

The standard MTT assay was used for evaluating the percentage of a viable cell population treated with cleistanthin B [16]. Briefly, cells were seeded in 96 well plates at a density of 5×10^4 cells per well and kept overnight in an incubator (ESCO CLS-170T-8, Singapore). On the following day, cells were treated with various concentrations of cleistanthin B and further incubated at 37°C for 48 h. After the treatment, a freshly prepared MTT solution (5 mg/mL) was added to each well and incubated for 3–4 h. At the end of incubation, DMSO was added to dissolve the formazan crystals, and the absorbance of the plate was recorded at 570 nm using a microplate reader (BIO-RAD 680 XR, USA). The inhibitory concentrations 50 (IC50) of cleistanthin B were calculated against each cell line from three independent assays, each done in triplicates using the following formula.

$$\text{Percentage of cell death} = \frac{(\text{OD of control cells} - \text{OD of treated cells})}{(\text{OD of control cells}) \times 100}$$

AO-EB dual staining

Cleistanthin B induced morphological changes in colorectal cancer (CRC) cells were assessed by AO-EB dual staining described previously [17]. Approximately 5×10^5 cells/well was seeded in six well plates. The CRC cells were incubated with DMSO (vehicle control) and indicated doses of cleistanthin B for 12 h. Then, a mixture (1:1) of AO-EB staining solution (100 μg/mL in PBS) was added to cells after a PBS wash. The morphological changes due to apoptosis were observed, and images were captured using a fluorescent microscope (Thermo Fisher Scientific EVOS Imaging Systems, USA). All experiments were performed in triplicate.

Generation of 5-FU+Ox-surviving CRC cells

We adopted a previously reported protocol to generate 5-FU+Ox surviving CRC cells [18]. Briefly, HT-29 CRC cells were added to a new culture vessel at 30–40% confluence and incubated. When optimal cell growth was reached, cells were treated with a combination of 50 μM of 5-FU and 1.25 μM of oxaliplatin for 48 h. The cells which survived the 5-FU+Ox insult were considered 5-FU+Ox surviving HT-29 cancer cells and used for further experiments.

Annexin V-PI flow cytometry assay

The assay was performed following a previously published method and following the manufacturer's instructions [19]. Briefly, HT-29 cells that survived from 5-FU+Ox treatment were seeded in 6 well plates at a density of 1×10^6 cells/mL and incubated overnight. On the following day, the cells were treated with DMSO (vehicle control), 5-FU, cleistanthin B alone, and in combination with 5-FU for 12 h. After the treatment period, the cells were washed with PBS and centrifuged for 5 min at 1500 RPM at room temperature. The cell pellet was washed with PBS and resuspended in 1× binding buffer. Annexin V-APC solution and PI (5 mg/mL) were added to 100 μL cell suspensions and incubated for 15 min at room temperature in the dark. After incubation, cells were washed and resuspended in 1× binding buffer and analyzed by flow cytometry within 30 min. The percentage of live, apoptotic, and dead cells was assessed by gating strategy. The apoptotic changes in 5-FU+Ox survived HT-29 cells were differentiated based on their Annexin V and PI uptake, and the percentage of live, apoptotic, and dead cells was calculated. Viable cells were both annexin-V and PI negative. Early apoptotic cells were annexin-V positive, and PI negative and dead cells were both annexin-V, and PI-positive was considered for analysis.

Data analysis

The IC50 values of cleistanthin B were obtained using OD values from the MTT assay, and the cell viability graph was prepared in Microsoft Excel 2016. The IC50 values of cleistanthin B were calculated from the inhibitor versus response curves plotted using the non-linear regression analysis with GraphPad Prism version 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Cell viability assay

Cytotoxic activity of cleistanthin B on colorectal, cervical, breast, lung, prostate cancer cells, and non-cancerous human embryonic lung epithelial (L132) cells was evaluated by MTT assay. The IC50 values (Fig. 1) of cleistanthin B against each cell type were calculated based on the percentage of viable cells and expressed as mean±SEM of three independent experiments, each done in triplicates. The IC50 values of cleistanthin B were found to be 3.6±0.55, 5.2±0.51, 8.6±1.02, 10.5±1.50, 18.3±3.71, 25.8±5.50, and 26.7±5.90 μg/mL against HT-29, SW-480, HCT-15, HELA, MDA-MB-231, A549, and DU145, respectively. The IC50 value of cleistanthin B against L132 cells could not be derived due to submaximal cell inhibition even at higher concentrations (>100 μg/mL) used in the assay.

AO-EB dual staining

All viable cells with organized nuclei emitted green fluorescence. On the other hand, early apoptotic cells with disorganized nuclei emitted green-orange color. Late apoptotic cells appeared orange-red and expressed highly condensed or fragmented nuclei with characteristic apoptotic body formation. The morphology (Fig. 2) of cleistanthin B treated HT-29, SW-480, and HCT-15 cells showed distinctive apoptotic features such as cell shrinkage, chromatin condensation, nuclear fragmentation, and apoptotic body formation. Thus, morphological changes indicated that cleistanthin B might promote apoptotic cell death in CRC cells.

Annexin V-PI flow cytometry assay

The Annexin V staining method assessed the apoptosis-inducing effect of cleistanthin B either alone or in combination with 5-FU against 5-FU+Ox surviving HT-29 cells. According to this method, 5-FU+Ox surviving HT-29 cells were evaluated by flowcytometry. When compared to the percentage of dead and apoptotic cells (0.5 % and 1.2%) in the control group (Fig. 3a), the percentage of cells treated with cleistanthin B that underwent apoptosis (41.0%) and death (10.8%) eventually increased (Fig. 3b). Furthermore, the 5-FU+Ox surviving HT-29 cells treated with 5-FU alone were also dead (8.2%) (Fig. 3c). The combination of cleistanthin B and 5-FU has driven more cells to death (54.4%) (Fig. 3d).

DISCUSSION

In the current investigation, we assessed the antiproliferative activity of cleistanthin B against colorectal, cervical, breast, lung, prostate, and non-cancerous human embryonic lung epithelial cells. The compound showed different degrees of cytotoxicity against human cancer cell lines in this study. We found that CRC cells were more sensitive to cleistanthin B compared to other cancer cell lines. The compound did not show significant cytotoxicity against nonmalignant cells in the concentrations used. The cell death caused by cleistanthin B in CRC

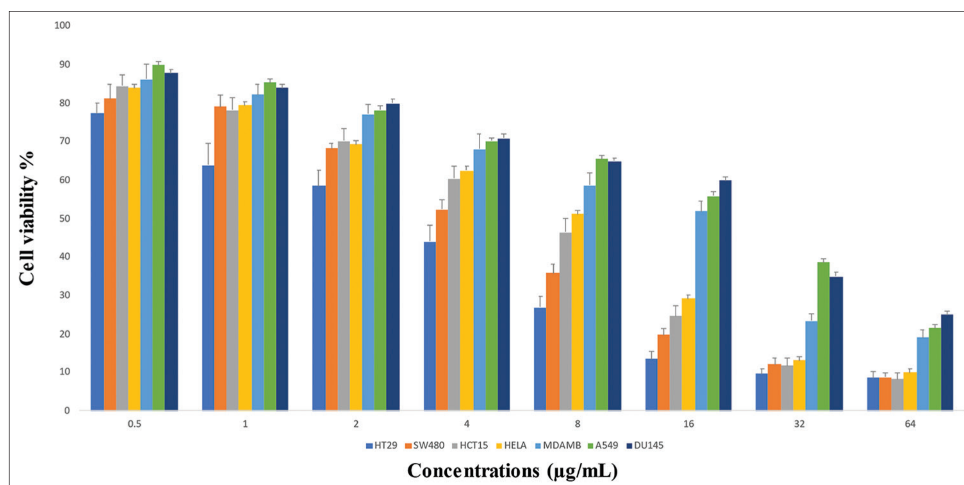

Fig. 1: Effect of cleistanthin B on the viability of cancer cells. The bars represent the percentage of viable HT-29, SW-480, HCT-15, HELA, MDA-MB-231, A549, and DU145 cancer cells

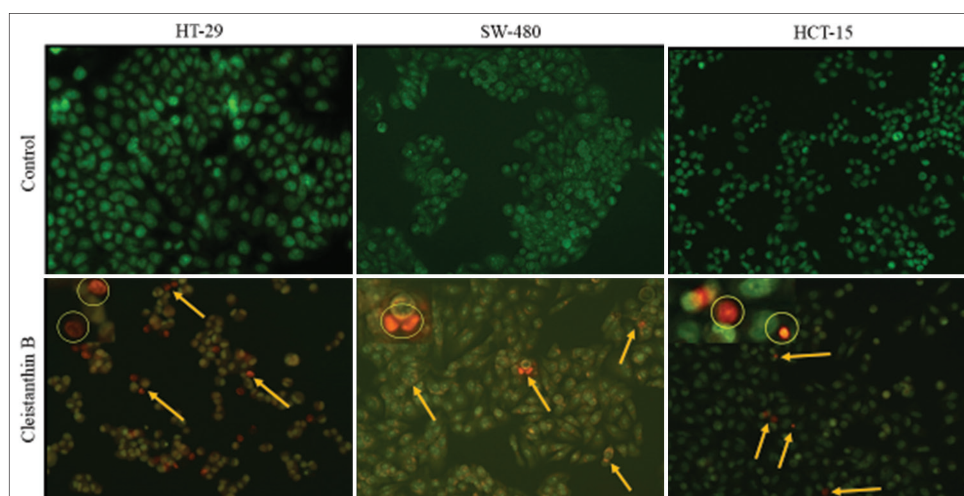

Fig. 2: Assessment of cleistanthin B apoptotic morphological changes by acridine orange and ethidium bromide dual staining. Arrows indicate apoptotic cells emitting orange-red color fluorescence. For better visualization of cellular apoptotic morphology, the cells were magnified and encircled

cells was due to induction of apoptosis. We also observed cleistanthin B synergistically acted with 5-FU to induce cell death in 5-FU surviving CRC cells.

We chose a panel of solid cancer cell lines to determine the most sensitive cancer type for cytotoxic action of the glycosidic compound, cleistanthin B. The IC50 values of cleistanthin B were relatively low against HT-29, HCT-15, and SW-480 CRC cells. We found all three CRC cell lines were more sensitive, followed by cervical, breast, lung, and prostate cancer cells. Cleistanthin B is a derivative of diphyllin, a vacuolar-ATPase (V-ATPase) inhibitor with anticancer activity [20]. Previously, a study that screened a series of arylnaphthalene lignan lactones with diphyllin as part of their structure also found CRC (HT-29) cell line as the most sensitive one than ovarian and breast cancer cell lines [7,21]. Many plant-derived or synthetic glycosides showed cytotoxicity against multiple cell lines, especially against gastrointestinal tract cancers [22,23]. The reason for specific toxicity towards CRC cells is unknown but might be due to the expression of specific cellular targets. The test compound did not induce optimal cell death in normal lung epithelial cells within the dose range that killed cancer cells. Failure to obtain an IC50 value within the concentrations range could be due to the poor cytotoxicity of the compound towards nonmalignant cells. It has been suggested that an efficient anticancer drug should not significantly affect nonmalignant cells within cytotoxic concentrations inhibiting cancer cells [24]. Hence, the compound has the advantage of being non-toxic to normal cells.

Based on cytotoxicity data, we decided to study the effect of cleistanthin B on three CRC cell lines (HT-29, SW-480, and HCT-15). Fluorescence staining of cleistanthin B treated CRC cells revealed that HT-29, SW-480, and HCT-15 produce typical apoptotic features as evident from chromatin condensation and marginalization, nuclear fragmentation, and formation of apoptotic bodies in treated cells. Apoptosis is a natural phenomenon to maintain cell homeostasis, and disturbances in this pathway lead to many pathological conditions, including cancer [25]. Our results are in accordance with an observation reported in cervical cancer cells [14]. Several plant-derived conventional anticancer drugs such as vinca alkaloids, taxanes, epipodophylotoxins, and camptothecins act as DNA damaging agents in malignant cells.

Anticancer agent 5-FU based chemotherapy is essential for neoadjuvant and adjuvant treatment for CRC [26]. The ability of CRC cells to adopt resistance mechanisms may affect the treatment outcomes. Many natural compounds reported synergistic or additive cytotoxic effects when combined with 5-FU [27-29]. The potent DNA damaging agents drive cancer cells to apoptosis [30]. Cleistanthin B was reported to damage the DNA in various cancer cells by arresting the G1 phase of the

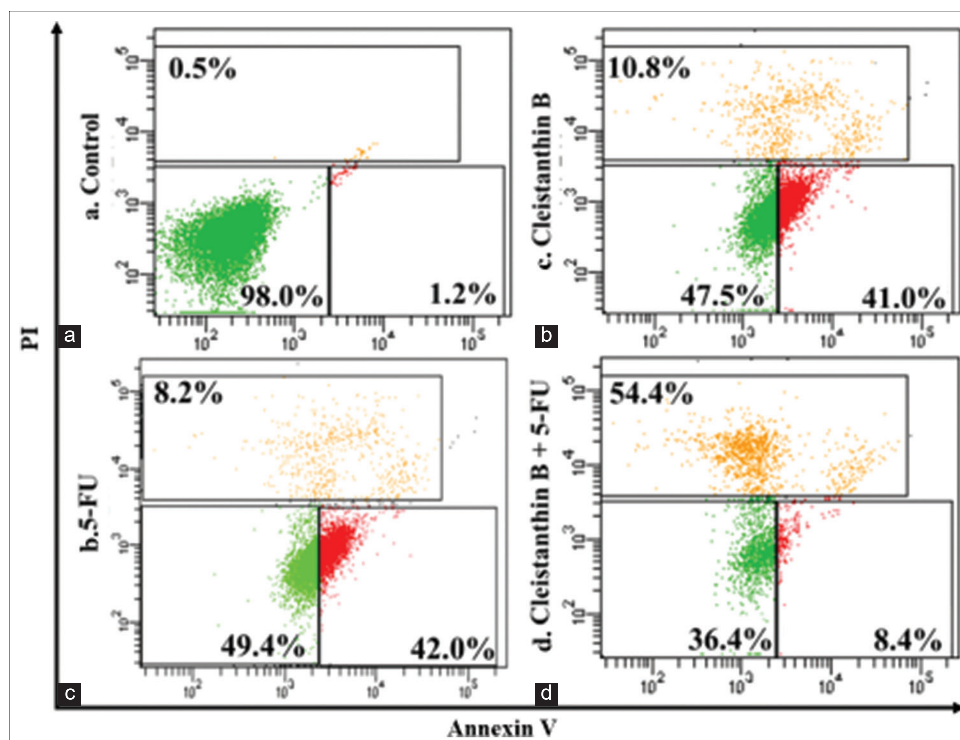

Fig. 3: Cleistanthin B induced apoptosis detection in 5-fluorouracil (5-FU) and oxaliplatin survived HT-29 cells (a) Control (DMSO), (b) 5-FU, (c) cleistanthin B, (d) cleistanthin B and 5-FU combination. The lower left quadrants show viable cells, and the lower right quadrants, early apoptotic cells. Upper quadrants show dead cells, including late apoptotic cells

cell cycle [14,20,31]. The HT-29 line is a resistant counterpart derived from a primary CRC tumor [32]. This study observed that cleistanthin B could inhibit HT-29 cells at low concentrations. Keeping this in view, we initially exposed HT-29 cells to a standard 5-FU+Ox regimen. The cells surviving the insult were treated with cleistanthin B alone or combined with 5-FU. We observed cleistanthin B alone drive 5-FU+Ox survived HT-29 cells to apoptosis. Furthermore, combining cleistanthin B with 5-FU increased the percentage of apoptotic cells compared to cleistanthin B alone. This synergistic property of cleistanthin B may be beneficial against cancer cells that escape 5-FU treatment initially.

Moreover, cytotoxic chemotherapy with standard anticancer drugs is known to cause myelosuppression as an adverse effect [33,34]. The neutrophil and platelet sparing effect of cleistanthin B has been reported in a tumor-bearing mouse model [13]. This diverse mechanism of cleistanthin B could be advantageous when combined with conventional anticancer treatments.

The trigger for apoptosis-mediated cell death may arise naturally from various sources or be induced by anticancer drugs. The ability of cancer cells to adopt mechanisms against apoptosis-inducing anticancer drugs leads to resistance. In the present study, cleistanthin B glycoside was more effective against naturally resistant HT-29 cells and 5-FU+Ox surviving HT-29 CRC cells. A recent study reported that inhibition of V-ATPase function led to increased colon cancer cell sensitivity to 5-FU [35]. Proton pumps like V-ATPase play an essential role in tissue homeostasis, but their dysfunction results in various pathological conditions, including cancer. It has been reported that *C. collinus* plant extract inhibits proton pump activity in Chang's liver and human embryonic kidney 293 [36]. The inhibition of vacuolar-type H+ -ATPase (V-ATPase) activity of renal tubules was observed in patients who consumed *C. collinus* plant extract [37]. The finding from our study and the existing knowledge is interesting as it provides new insights to further evaluate the cleistanthin B activity on CRC cells.

CONCLUSION

Among all cancer cells, cleistanthin B shows relatively potent cytotoxicity against CRC cells, evidenced by low cytotoxic concentrations. Cleistanthin B induces apoptosis-mediated cell death in all three CRC cell lines (HT-29, SW-480, and HCT-15). The L-132 cell line (nonmalignant cells) shows low sensitivity to the compound, and 50% cell growth inhibition (IC50%) was not found be within the concentrations that induce cell death in cancer cells. The compound causes apoptosis-mediated cell death in CRC cells and enhances the anticancer activity of 5-FU against HT-29 CRC cells.

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AUTHORS' CONTRIBUTIONS

Puli Sagar designed, performed the experiments, and drafted the article with inputs from and under the supervision of Ramasamy Raveendran.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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