

Anti-Metastatic Effect of Boiled Garlic Extract on A549 Lung Cancer Cells Line by FAK Mechanism

Efek Anti-Metastase Ekstrak Rebusan Bawang Putih terhadap Pertumbuhan Sel Kanker Paru A549 Lung Melalui Mekanisme FAK

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ABSTRACT

According to epidemiological and animal study, the daily intake of garlic have important role in decreasing of cancer prevalence and tumor metastasis. However, mechanism of anti-metastatic of garlic is not fully understood. In the present study, we examined the anti metastatic effect of a brief exposure to boiled garlic extract (BGE) on A549 Lung cancer cells. Cytotoxicity BGE on A549 lung cancer cells was tested using MTT Assay. Migration and invasion scratching wound assay A549 lung cancer cells was tested using scratching wound assay and agarose dropt explants assay. The growth of A549 lung cancer cell was measured using Colony forming assay. To explore the molecular mechanism and effect of BGE on migration and invasion, western blotting was used to analyze the FAK/Paxillin pathway. We found that BGE inhibits the growth, migration and invasion of A549 lung cancer cells at 300 µg/mL. Interestingly, BGE inhibits migration and invasion of A549 lung cancer cells by decreasing the total of FAK and P-Y397 expression at 6 hours after a brief exposure, but not influence of the paxillin expression. We think the present finding demonstrate a novel anti-metastatic effect of garlic and suggests a certain garlic-derived compounds, BGE may be potential agent for inhibiting migration and invasion through decreasing the total of FAK and P-Y397 expression in the cancer cell.

Keywords: A549 lung cancer cells, boiled garlic extract, FAK mechanism, migration cell

ABSTRAK

Berdasarkan studi epidemiologi dan hewan coba menunjukkan bahwa asupan harian bawang putih memiliki peran penting dalam menurunkan prevalensi dan metastasis kanker. Mekanisme anti-metastasis bawang putih tidak sepenuhnya dipahami. Pada penelitian ini, kami meneliti efek anti metastasis ekstrak rebusan bawang putih (ERBP) secara in vitro pada sel kanker paru A549. Uji toksisitas ERBP terhadap sel kanker paru A549 menggunakan MTT Assay. *Scratching wound Assay* dan *Agarose dropt explants Assay* untuk menguji migrasi dan invasi sel kanker paru A549. *Colony forming assay* untuk mengukur pertumbuhan sel kanker paru A549. Untuk mengetahui lebih jauh efek dan mekanisme molekuler ERBP terhadap migrasi dan invasi sel, kami menggunakan teknik *western blotting* dengan menganalisa jalur FAK/ Paxillin. Hasil menunjukkan bahwa ERBP menghambat pertumbuhan, migrasi dan invasi sel kanker paru A549 pada konsentrasi 300 mg/mL. Menariknya, ERBP menghambat migrasi dan invasi sel kanker paru A549 dengan cara menurunkan ekspresi total FAK dan P-Y397 pada 6 jam setelah paparan, tapi tidak mempengaruhi ekspresi paxillin. ERBP mungkin menjadi agen potensial anti-metastasis dengan cara menghambat migrasi, invasi dan pertumbuhan sel kanker. Migrasi dan invasi sel kanker mungkin sebagian dihambat melalui penurunan ekspresi total FAK dan P - Y397.

Kata Kunci: Ekstrak rebusan bawang putih, mekanisme FAK, sel kanker paru A549, sel migrasi

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INTRODUCTION

The beneficial effect of garlic (*Allium sativum*) and their constituent include hypolipidaemic, hypoglycaemic, chelating agents, immune stimulants, antihypertensive, antimicrobial, and possibly anticancer effect. Consumption of fresh garlic may be linked to potential cytotoxicity of intestinal cells, not boiled garlic. Therefore, proteinaceous cytotoxic component of garlic has change their functional properties such as solubility, mobility and toxicity, during short boiling (1). Initial evidence for the anticancer effect of garlic was provided by epidemiological study, that high intake of garlic may be associated with a protective effect against stomach and colorectal cancers. Preclinical animal studies have indicated that garlic is highly effective in affording protection against cancer induced by a variety of chemical carcinogens. For instance, Belman demonstrated that topical application of garlic and onion oil inhibited the incidence of tumor promoted by phorbol-myristate acetate (2). Cancer chemoprevention by garlic constituents has been observed against benzo[a]pyrene-induced forestomach and pulmonary cancer in mice, *N*-nitrosomethyl-benzylamine induced esophageal cancer in rats, azoxymethane-induced colon carcinogenesis in rats, and 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine induced mammary tumorigenesis in rats (2-5).

Elucidation of the mechanisms by which garlic may offer protection against cancer has been a passionate subject of research for the past 20 year. There are summaries of molecular targets of cancer chemoprevention by garlic, including modulation of carcinogen activation, induction of phase 2 enzyme and modulation of anti-oxidative enzymes, inhibition of post-translational modification of oncogenic Ras, inhibition of cell cycle progression, histone modification, induction of apoptosis, as well as inhibition angiogenesis and metastasis. Focal adhesion kinase (FAK) is an important regulator of cell migration in which a function required for the invasion and metastasis of cancer cell (6). Because of its major role as kinase upstream of a number of signaling pathways involved in the processes metastasis, manipulation of FAK is likely to be more effective than manipulation of downstream targets (7). The adapter protein, paxillin, is important in scaffolding a large number of proteins to the focal adhesion kinase. Moreover, overexpression and/or increased activity of FAK are common in a wide variety of human cancers, implicating a role for FAK in carcinogenesis. Given the important role of FAK in a large number of processes involved in tumorigenesis, metastasis and survival signaling FAK should be regarded as a potential target in the development of anti-cancer drugs. Therefore, selective inhibitors of FAK need to be developed. Combination of these selective FAK inhibitors with cytotoxic agents could be a very promising anti-cancer therapy. In the present study, the precise mechanism whether non toxic dose of boiled garlic extract could inhibit migration of A549 lung cancer cell line *in vitro* was analyzed.

METHOD

Rabbit Polyclonal Anti-FAK Antibody

Rabbit polyclonal anti-FAK antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit

polyclonal anti-FAK [pY³⁹⁷] antibody was purchased from Biosource International (Camarillo, CA, USA). Monoclonal anti-GAPDH antibody (clone 6G5) was purchased from Biogenesis Ltd. (England, UK). All other reagents used were of the purest grade available.

Preparation of Boiled Garlic extract

Garlic cultivated as an environmental-friendly agricultural product was purchased from local supermarket near Jember last July 2013. Fresh garlic cloves (250 g) were homogenized in 500 ml ice-cold distilled water using kitchen blender for 30 sec. The homogenates were filtered through four layers of medical gauze and the filtrate was lyophilized. The yield of extract (23.5 g powder) is stored at -20 °C until use. For experiments, one gram of the lyophilized garlic powder was extracted with 10 ml PBS, by vigorous vortexing (30 sec for 5 times with intermittent cooling on ice for 1 min) and centrifugation (10,000 ×g, 10 min). Aliquot of the above fresh garlic extract was heated in boiling water for 30 min, then centrifuged at 10,000 ×g for 10 min and the supernatant was used as boiled garlic extract (BGE). Protein concentration of the supernatant was determined using Bradford assay (8).

Cytotoxic Effect of Boiled Garlic Extract

A549 lung cancer cells were seeded in 24-well plates at a density of 2×10^4 cells/well in 1 ml MEM containing 10% FBS overnight at 37 °C. Non-adherent cells were removed by gentle washing. Then cells were treated for 24 hr with various concentrations of boiled garlic extract. To assess cell viability, 100 µl of MTT solution (5 mg/ml) was added to each well and incubated for another 4 hr at 37 °C. After the removal of supernatant, the generated formazan crystal was dissolved by adding 125 µl/well of DMSO and the absorbance was detected at 540 nm using ELISA reader (Bio-Rad, USA). The percentage of cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{absorbance}_{540\text{ nm}} \text{ of experimental well}}{\text{absorbance}_{540\text{ nm}} \text{ of control well}} \times 100\%$$

Scratching Wound Assay

A549 lung cancer cells were seeded into six well culture plates and allowed to grow to approximately 90-95% confluence. Similar sized wounds were introduced to monolayer cells using a sterile yellow pipette tip. Wounded monolayer cells were washed three times by PBS to remove cell debris and then cultured with or without BGE. The speed of wound closure was monitored and photographed every 8-12 hr using a phase-contrast microscope until complete wound closure was observed in the untreated control (9).

Agarose Drop Migration Assay

A549 lung cancer cells (10^6 cells) in 50 µl of free serum medium DMEM were mixed with 150 µl of 0.2% (w/v) SeaPlaque agarose (Cambrex Bio Science Rocklan, Inc., ME, USA) in free serum medium DMEM. One or two microliter size drops of cell suspension were gently delivered onto the pre-cooled surface of 48-well plates. The agarose was allowed to solidify for 10 min at 4 °C. Covered with 200 µl of complete medium (DMEM+FBS 20% + Ab 1%) with BGE 75, 150, and 300 µg/mL or vehicles were added, and centrifuge. It did incubate at 37 °C for 24 hr. Take a picture using phase contrast and 100 x. Cells that migrated out of the rim of the drop were counted for

quantitative determination of migrating cells. The distance of edge of the agarose drop to the leading edge of migrating cells was determined on 4 sides of each droplet. Since test in quadruplicate, 16 reading were obtained from each measurement. The migration of cells out of the agarose drop produced a uniform, expanding corona of the cells around the agarose drop. While the majority of the cells migrated out of agarose drop in the corona, few cells migrated much farther out the major population of cells. The number of cells that migrated beyond the corona of cells was counted (9,10).

Colony forming assay

The effects of BGE on colony formation on plates were measured as previously described (11,12). 500 cells of A549 lung cancer cells in 2 ml of DMEM complete media were plated into each well of 6-well plates. The cells were cultured in the absence or presence of BGE concentrations of 150 and 300 $\mu\text{g/mL}$. After 7 days incubation, stop experiment and stain by crystal violet. Total number of colony was counted by ImageJ open software (National Institute of Health, Bethesda, Maryland, USA).

BGE Treatment and Western Blotting

Serum-starved A549 lung cancer cells were treated with or without BGE 300 $\mu\text{g/mL}$ for indicated times. Effects of various kinds of inhibitors were examined by the preincubation of cells with an inhibitor for 1 hr before BGE treatment. The cells were washed with PBS and treated with lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 1 $\mu\text{g/mL}$ leupeptin, 5 $\mu\text{g/mL}$ aprotinin and 2 μM pepstatin A) on ice. Lysates were scraped from the plates with a rubber policeman and the supernatants were obtained after centrifugation at 15.000 rpm for 10 min at 4 °C. Protein concentrations of the clarified cell lysates were determined using Bradford assay (8). The cell lysates were added with (5x) SDS sample buffer and heated at 95 °C for 5 min. The proteins were separated by 6–16% SDS-PAGE, and then transferred to nitrocellulose membranes. Blocking was performed with TTBS buffer (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20) containing 5% skim milk powder. The membranes were then incubated with primary antibody for 4 hr at room temperature. Immunoblots were washed for 2 hr with changing TTBS buffer for every 20 min, and then incubated with horseradish peroxidase-linked secondary antibody for 30 min at room temperature. Immunoblots were washed for 1 hr with changing TTBS buffer for every 10 min, and developed with horseradish peroxidase dependent chemiluminescence (ECL) (Amersham Corp.).

Statistical Analysis

The values are represented as means standard deviation (S.D.). A paired Student's *t*-test was used to assess the significance of differences between two mean values or one-way analysis of variance (ANOVA). Results were considered to be statistically significant at $P < 0.05$.

RESULT

Cytotoxic Effect of Boiled Garlic Extract

Various concentrations of BGE were treated A549 lung cancer cells for 24 hr. The cytotoxicity of BGE was determined by MTT assay. Compared to that of controls, the remaining cell viability was not significantly altered by boiled garlic extracts, even at a concentration as high as 5 mg/mL (Fig. 1.). It was therefore clear that a 24 hr treatment of boiled garlic extracts, at the concentration ranging from 0 to 5 mg/mL, has no cytotoxicity to A549 lung cancer cells, highly metastatic cancer cells. This concentration range was then applied in all subsequent experiments.

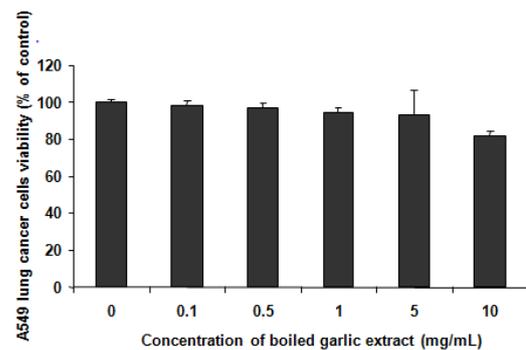


Figure 1. Cytotoxic effect of boiled garlic extract on A549 lung cancer cells

Note: Exponentially growing A549 lung cancer cells were exposed to boiled garlic extract in DMEM complete media, and then incubated for 24 hr. Cell viability of each treatment was assessed using MTT assay. The results are expressed as mean \pm SD for three independent experiments. **= $p < 0.01$ when compared to control.

Boiled Garlic Extract Inhibits Cell Migration of A549 Lung Cancer Cells

In order to determine the inhibitory effect of boiled garlic extracts on cell migration of A549 lung cancer cells, analyses were carried out using two distinct cell migration assays, such as scratch wound assay and agarose drop migration assay. The results indicate that boiled garlic extract inhibits the cell migration of A549 lung cancer cells in a dose dependent manner using scratch wound assay (Fig. 2; Fig. 3); and agarose drop migration assay (Fig. 4; Fig. 5)

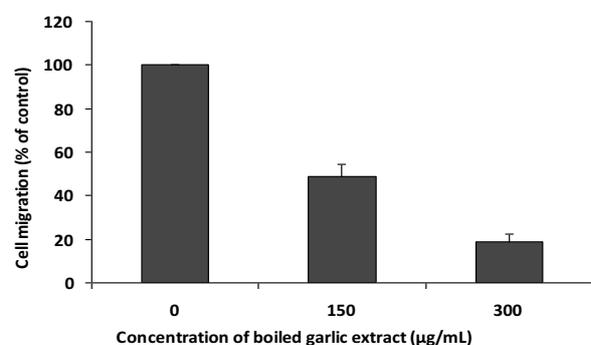


Figure 2. Statistical analysis of scratch wound assay

Note: Statistical analysis was used image open software (National Institute of Health, Bethesda, Maryland, USA). The results are expressed as mean \pm SD ($n=3$). ** $P < 0.01$ when compared to control.

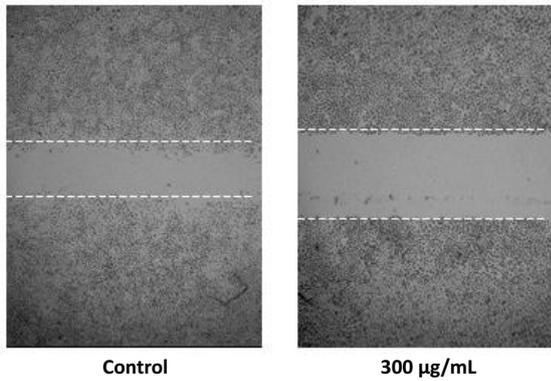


Figure 3. Microscope photograph of scratch wound assay

Note: Representative microscope photograph low magnification (50x) of A549 lung cancer cells by scratch wound assay after 24 hr incubation was shown.

Boiled Garlic Extract Inhibits Colony Forming of A549 Lung Cancer Cells

In order to determined inhibition effect of boiled garlic extracts on colony forming of A549 lung cancer cells, 500 cells of A549 lung cancer cells in 2 ml of DMEM complete media were plated into each well of 6-well plates. The cells were cultured in the absence or presence of BGE concentrations of 150 and 300 µg/mL. After 7 days incubation, stop experiment and stain by crystal violet. Colony forming was counted by ImageJ open software (National Institute of Health, Bethesda, Maryland, USA). The results indicate that boiled garlic extract inhibits colony forming of A549 lung cancer cells at dose dependent (Fig. 4).



Figure 4. Statistical analysis of agarose drop migration assay

Note: Cells that migrated out of the rim of the drop were counted for quantitative of cells migration, using ImageJ open software (National Institute of Health, Bethesda, Maryland, USA). The results are expressed as mean ± SD (n=3).

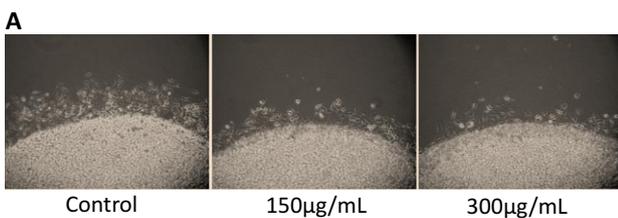


Figure 5. Agarose drop migration assay

Note: A, Representative microscope photograph of cell migration out of agarose barrier ring; B, Schematic illustration of agarose drop migration assay using 12-well plates.

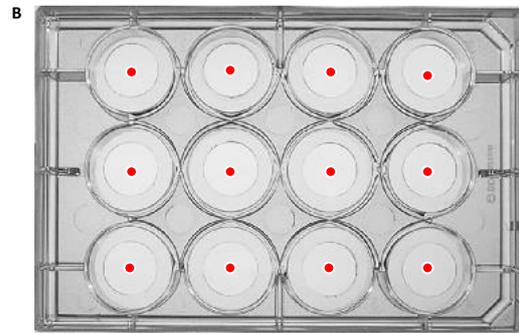


Figure 5. Agarose drop migration assay (Lanjutan)

Note: A, Representative microscope photograph of cell migration out of agarose barrier ring; B, Schematic illustration of agarose drop migration assay using 12-well plates.

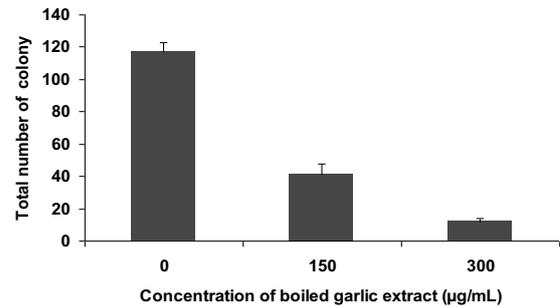


Figure 6. Statistical analysis of colony forming assay

Note: Briefly, 500 cells of A549 lung cancer cells in 2 ml of DMEM complete media were plated into each well of 6-well plates. The cells were cultured in the absence or presence of BGE concentrations of 150 and 300 µg/mL. After 7 days incubation, stop experiment and stain by crystal violet. Total number of colony was counted by ImageJ open software (National Institute of Health, Bethesda, Maryland, USA). **P < 0,01 when compared to control.

Western Blotting Analysis

Activation of FAK on cancer cells related with increasing cell migration and proliferation (18). Paxillin is an adaptor protein down-regulation of FAK that related with cell motility. Therefore, the western blotting analysis was performed to see the effect boiled garlic extracts on FAK and its phosphorylation. The result was shown that boiled garlic extracts inhibited migration A549 lung cancer cell by decreasing the level of FAK and its phosphorylation (P-Y³⁹⁷) in cancer cells after 3 hr (Figure 7).

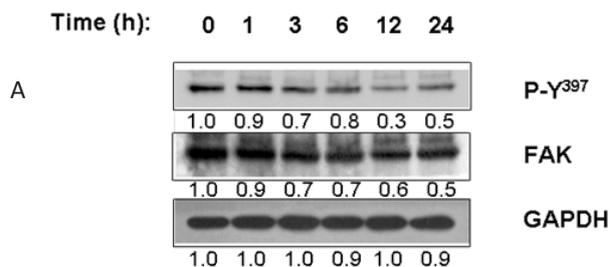


Figure 7a. Western blotting analysis

Note: A. Effect of BGE on protein expression of FAK and its phosphorylation (P-Y³⁹⁷);

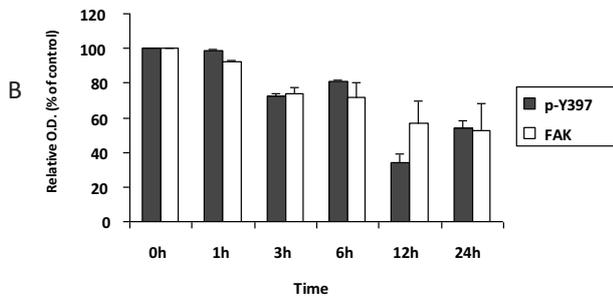


Figure 7b. Western Blotting Analysis

Note: B. Analysis results shown that BGE inhibits cell migration of A549 lung cancer cell by decreasing the level (Relative of optical density) of FAK and its phosphorylation (P-Y³⁹⁷) from 3 hr after the treatment.

DISCUSSIONS

Cancer metastasis the leading cause of cancer deaths. It is a multi-step process that a tumor cell migrates to distant organ as new colony. There are several important event, including infiltration, invasion, migration, angiogenesis, escape from immune surveillance as well as forming new colony. In addition, cell migration plays an important role in diverse biological processes, such as development, the immune response, and cancer metastasis. The actin cytoskeleton is key component in cell migration. In which tumor cell attachment to the extra-cellular matrix components, the gradation of the matrix by tumor cell-associated proteases, and tumor cell progression into the region where the matrixes were modified by proteolysis. Evidence from several investigations suggests that some components of the diet supplements, including garlic, have shown anti-metastatic effect on cancer cells (13,14). The mechanism of anti-metastatic of garlic extract was not fully understood yet and therefore, this study attempts to elucidate the mechanism an anti metastatic effect of a brief exposure to boiled garlic extract (BGE) on A549 Lung cancer cells line in vitro.

In the present study, the effects non-toxic concentrations of boiled garlic on cell migration of A549 lung cancer cells were analyzed. Instead of using colony forming assay for analysis capacity of individual cancer cell forming new colony, were carried out by two distinct cell migration assays, using scratch wound assay and agarose drop migration assay. It was found that boiled garlic extract inhibited not only the cell migration, but also the colony formation of A549 lung cancer cells at the concentration of 300 µg/mL. This result may be can answer some

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problem on anti cancer agent, which it generally using toxic dose with some side effect. BGE was not containing toxic compound or decreasing beneficial compound. The bioactive compound such as selenium and copper content of rawgarlic is not altered by boiling (1,15-17). Therefore, BGE can be used as nutraceutical compound for chemopreventive of cancer metastasis. In addition, the mechanism of migration and colony formation inhibitory effect of boiled garlic were confirmed by Western blotting analysis. Interestingly, BGE inhibits migration and colony forming of A549 lung cancer cells by decreasing the level of FAK and its phosphorylation (P-Y³⁹⁷) from 3 hr after the treatment. Cell migration is a complex process that involves rapid change in dynamic of actin filaments, together with the formation and disassembly of cell adhesion site. FAK is crucial signaling component that is activated by numerous stimuli and function as biosensor or integrator to control cell motility. FAK can influence the cytoskeleton structure of cell adhesion site and membrane protrusion to regulate cell movement. Activation of FAK on cancer cells related with increasing cell migration and proliferation (18). The adapter protein, paxillin, is important in scaffolding a large number of proteins to the focal adhesion kinase (19-22).

For decades, the fighting cancer has been an ongoing battle, and until now, there is no exact way to treat this disease. Conventional treatments include surgery and chemotherapy, nevertheless, these methods have several limitation including physical pain, increased relapse and lower survival rate (2,6). One of the main reasons of cancer treatment is to eliminate cancer cells with minimal or no side effect; it is also favorable that it inhibits the metastatic process as well. Natural products have an important role in search for new drugs, even some of the most famous widely used drugs are discovered from natural sources. BGE that used in this study, was still crude extract. Its not clear which compound of BGE is responsible for the metastatic observed in the study. It can be one of the above or an unidentified one yet. To clarify this, further studies will be needed in the future.

Taken together, the results suggested that the present finding demonstrates a novel anti-metastatic effect of garlic and a certain garlic-derived compounds of BGE may be a potential agent for inhibiting cell migration and colony forming by the modulation of FAK in cancer cells.

CONFLICT OF INTEREST

No competing financial interests exist.

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