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Baicalein, a flavonoid extracted from a methanolic extract of *Oroxylum indicum* inhibits proliferation of a cancer cell line *in vitro* via induction of apoptosis

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A methanolic extract of the fruits of *Oroxylum indicum*, which is widely used in traditional Chinese herbal medicine for its anti-inflammatory, anti-pyretic and anti-hypersensitivity effects, inhibited in vitro proliferation of HL-60 cells. The flavonoid baicalein was found as an active component in the extract. Analysis of freeze-dried fruits of the plant indicated that this component comprised about 4% of the material by dry weight. In this study, we investigated the *in vitro* effects of baicalein on the viability and induction of apoptosis in the HL-60 cell line. The cell viability after treating with baicalein for 24 h was quantified by counting viable cells using trypan blue staining. The results showed that baicalein caused a 50% inhibition of HL-60 cells at concentrations of 25–30 μM. The inhibition of proliferation of HL-60 cells due to 36–48 h exposure to 10 or 20 μM baicalein was associated with the accumulation of cells at S or G2M phases. However, proliferation inhibition at a higher dose may be associated with induction by apoptosis, as evidenced by the typical nuclear fragmentation using DNA fragmentation assay and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The results indicate that baicalein has anti-tumor effects on human cancer cells, and *Oroxylum indicum* extract could be used in supplementary cancer therapy.

1. Introduction

Oroxylum indicum, is a deciduous tree which is found in many parts of the world, particularly in South Asia, Southeast Asia and China (Tomimori et al. 1988). The plant has been used for a very long time in Indian Ayurvedic medicine and Chinese folk medicine for curing stomach problems, diarrhea, dysentery and rheumatism (Kizu et al. 1994). The fruits of this tree are consumed as a part of the diet in north and northeastern Thailand. Recently, phytochemical studies of the tree have revealed the presence of flavonoids, such as chrysin, oroxylin, baicalein, baicalein glycosides, benzoic acid and fatty acids (Chen et al. 2003; Chen et al. 2005; Joshi et al. 1977). The best known biologically active compounds are the phenolic compounds baicalein, and chrysin identified in the seeds of the plant.

The anti-mutagenic properties of *Oroxylym indicum* extract have been investigated against mutagenicity of Trp-P-1 in an Ames test (Nakahara et al. 2001), and its toxicological properties in a Brine Shrimp Lethality Bioassay. In studying the mechanism of anti-mutagenicity, Nakahara et al. (2001) identified baicalein in the methanolic extract of the edible part of this tree and showed that the antimutagenic effect mainly involved the inhibition of *N*-hydroxylation catalyzed by P450 monooxygenase in S-9.

In spite of these pharmacological properties of *Oroxylum indicum* extract, little is known about its anticancer properties, because no extensive cytological investigations have yet been conducted, so far we know. We found that the extract from *Oroxylum indicum* caused inhibition of cell proliferation. The aim of this paper is to further investigate the biological action (survival, cell cycle, apoptosis) of the active component present in the extract of *Oroxylum indicum* on promyelotic leukemia cells (HL-60 cell line) to define the anticancer effect of the extract.

2. Investigations and results

2.1. HPLC analysis

Analysis of the retention times and UV spectra of the purified compound and comparison of standard substance with the same data, showed the presence of baicalein ($> 99.5\%$) in the final active preparation.

2.2. Effects of baicalein on cell viability

The doubling time of HL-60 was about 24 h. The results from trypan blue exclusion experiments indicated that the viability of vehicle control cells (0.2% DMSO) was $100 \pm 2\%$ of control cultures. However, viability was decreased when cells were treated with baicalein, as the number of stained cells increased. The result suggests that baicalein induced cell death in HL-60. Increase in the concentration of baicalein results in greater decrease in numbers of viable HL-60 cells ($^{*}P < 0.05$; Fig. 1). At a con-

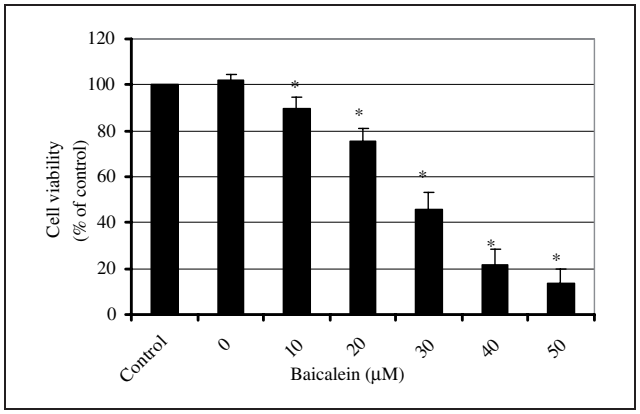

Fig. 1: Dose response activity of baicalein on proliferation of HL-60 cells. Cells were exposed for 24 h to various concentrations of baicalein. Analysis was performed as described in Materials and Methods. Results are expressed as mean percentage of control cultures containing 0.1% DMSO (vehicle control). Each point represents a mean of three independent experiments. Bars represent SD; $^{*}P < 0.05$

centration of 10 μM, baicalein was able to inhibit the growth of HL-60 cells significantly. A 40 μM concentration inhibited the cell growth by 78% at 24 h.

2.3. Cell cycle analysis

Cell cycle analysis by flow cytometry was used to estimate quantitatively the number of cells in each phase of the cell cycle. To assess the effect of baicalein on the cell cycle, HL-60 cells were exposed to 10 or 20 μM baicalein for 24, 36 and 48 h. We observed that when cells were exposed to 10 μM baicalein for 36 h, the proportion of cells in the G2M phase increased accompanied by a reduced proportion of G1 cells (Fig. 2B). This effect was also observed when cells were exposed to 20 μM baicalein for a longer period, 48 h (Fig. 2D). In both treatments, an increase in S-phase cells was also observed.

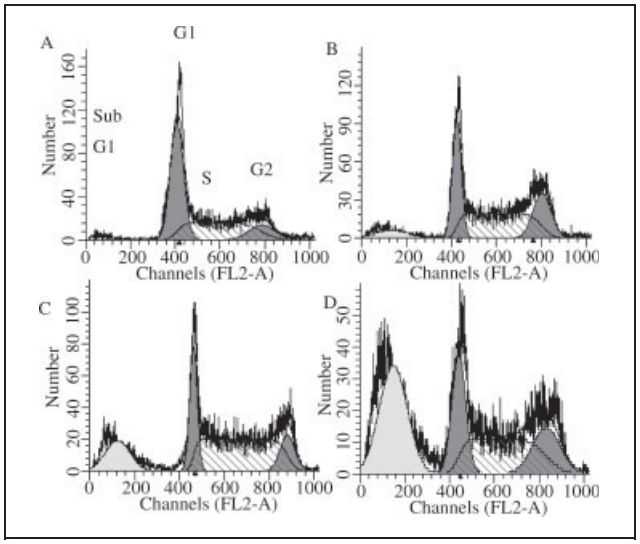

Fig. 2: Cell cycle analysis of HL-60 cells. Cells were cultured with 0.1% DMSO (vehicle control, panel A) or baicalein at 10 μM for 36 h (panel B) and 48 h (panel C); at 20 μM for 48 h (panel D). Analysis was performed as described in Materials and Methods. Horizontal and vertical axes represent DNA content and cell number respectively. The data represent one representative of three separate experiments

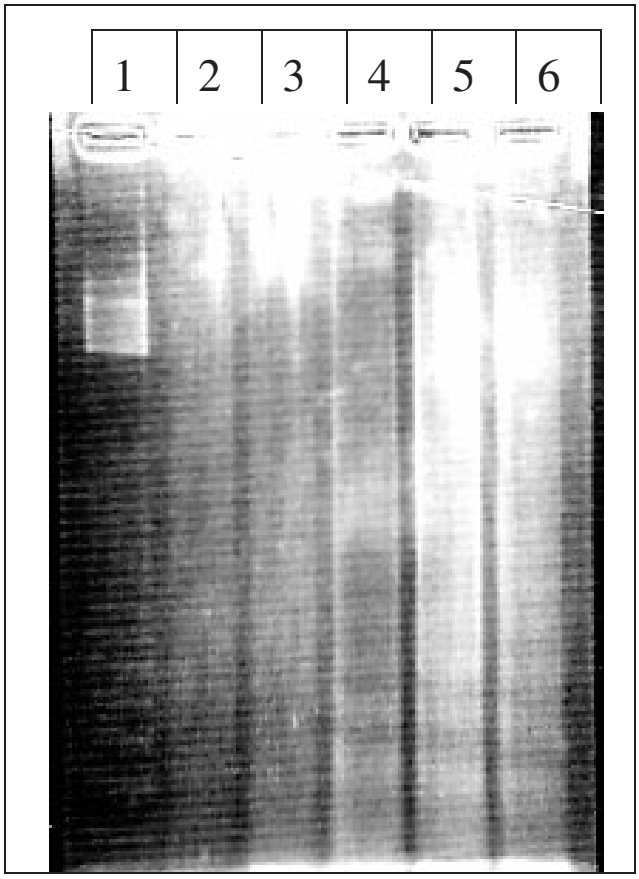

Fig. 3: Baicalein induced DNA fragmentation in HL-60 cells. HL-60 cells were incubated with either 0.1% DMSO (lane 2) or 5, 10, 20 and 40 μM (lanes 3, 4, 5 and 6, respectively) baicalein for 48 h. DNA fragmentation was analyzed by agarose gel electrophoresis as described in 'Materials and Methods' section. Lane 1 represents marker DNA. The result is one representative of three separate experiments

2.4. Effect of baicalein on DNA fragmentation

DNA fragmentation is a typical feature of apoptosis (Wyllie 1980). A dose dependent occurrence of cellular DNA fragmentation was studied by gel electrophoresis, and a ladder

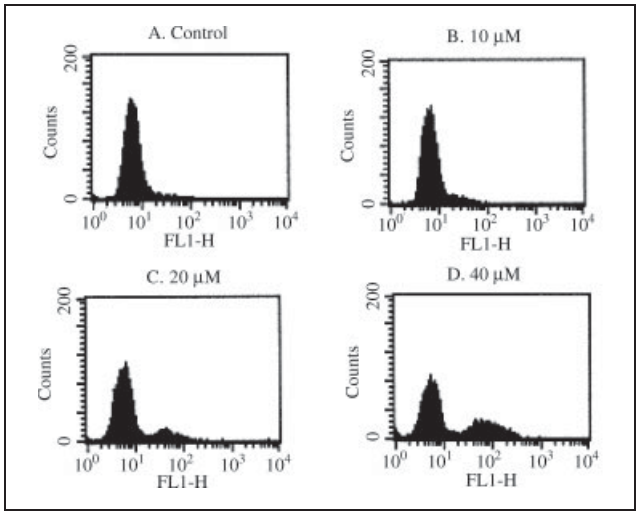

Fig. 4: DNA fragmentation analysis by TUNEL assay. HL-60 cells were incubated with indicated amount of baicalein for 24 h. Cells were collected and fragmentated DNA was marked by TUNEL assay and analyzed by flow cytometry as described in 'Materials and Methods' sections. Results are one representative of three separate experiments

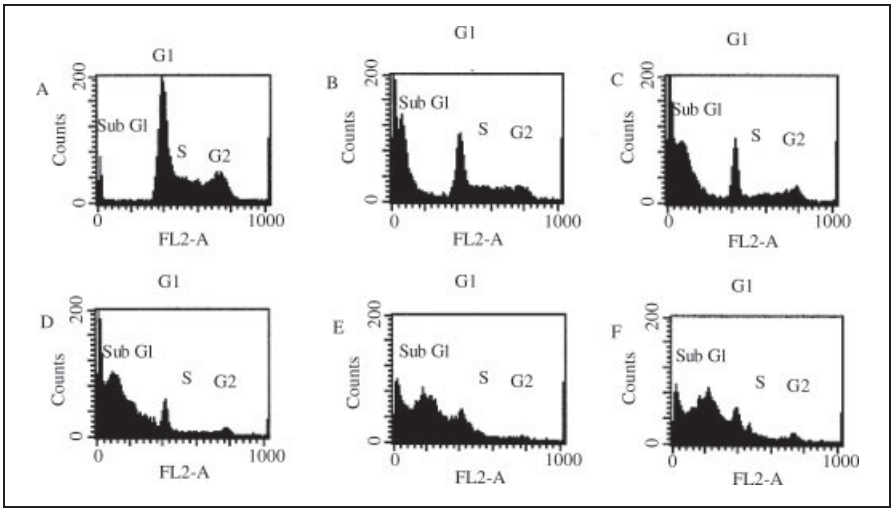

Fig. 5:
Effects of baicalein on DNA level in HL-60 cells. Cells were incubated with either 0.1% DMSO (vehicle control, panel A) or 40 μM baicalein for 3, 6, 12, 24 and 48 h (panel B, C, D, E and F, respectively). Cells were collected permealized with 70% ethanol at −20 °C. After marking DNA with PI, cells were analyzed by flow cytometry as described in 'Experimental' section. Results are one representative of three independent experiments

like pattern, typical of DNA cleavage between nucleosomes, was visible in cells treated with 10, 20 or 40 μM baicalein for 48 h (Fig. 3). TUNEL assay, based on flow cytometry was used to detect DNA breaks and total cellular DNA in cells exposed to baicalein. A substantial increase in the fluorescence of labeled DNA breaks in baicalein (20 μM) treated cells was observed at 24 h compared to the control cells (Fig. 4), an indication of the occurrence of apoptosis. Cells with a lower DNA content can also be estimated using PI staining followed by flow cytometry analysis. Here, in analyzing DNA content, we observed that untreated cells (Fig. 5A) showed a typical pattern of DNA content. However, baicalein treatment changed the DNA profile, and an increase in the number of cells with reduced DNA content (sub-diploid fraction) was observed, a characteristic feature of apoptosis. We observed that cells treated with 40 μM baicalein for 3 h (Fig. 5B) showed 45% of cells in the sub diploid fraction, while when cells were treated for a longer time the number of apoptotic cells was increased (Fig. 5: C, D, E and F).

3. Discussion

In many recent reports, plant extracts have been investigated for their possible therapeutic role against diseases affecting human health. Effective plant extracts are desired as a "natural" way to combat diseases including cancer. In some previous reports, *Oroxylum indicum* extract has been used to study various phytotherapeutic actions (Costa-Lotufo et al. 2005; Laupattarakasem et al. 2003; Nakahara et al. 2002; Palasuwan et al. 2005). Thus, studying the anti-tumor properties of the extract of *Oroxylum indicum* has, not surprisingly, come into our research interests, investigating the therapeutic action of plant products. To clarify the action of the methanolic extract of *Oroxilum indicum*, we purified a major component, designated as baicalein, using HPLC analysis. Baicalein is also found as one of the major flavonoids in many other plant extracts, such as Sho-saiko-to (Nishioka et al. 1998; Rossi et al. 2001), a Japanese herbal preparation widely used for the treatment of chronic liver diseases; PC-SPES (Chen et al. 2001), an eight herb mixture effective against prostate cancer; and Huangqin-tang (Tsang et al. 2000), a preparation of four Chinese herbs traditionally used for treating abdominal pain, dysentery and pancreatic cancer. It is noteworthy that a flavonoid baicalin (baicalein-7-D-glucuronide) is also present as one of the major components in the above plant preparations. However, we were not able to detect the compound in the most active fraction when analyzing the methanolic extract of *Oroxylum indicum* fruits, because the compound, although a significant component of the herbal preparations, is often described as a weaker component than baicalein in inhibiting the proliferation of various cell cultures (Chen et al. 2001; Huang et al. 1994).

In this study, we showed that baicalein effectively inhibited the proliferation of a leukemic cell line, HL-60 by demonstrating the roles of baicalein in decreasing the number of cells, in the induction of apoptosis, and in interfering with cell cycle arrest at the S and G2/M phases of the cell cycle.

At a concentration of 10 μM, baicalein had a modest inhibitory effect on the growth of HL-60 cells, which however reduced to 13% of control cells when cells were exposed to 50 μM baicalein for 24 h. Specific inhibition of cell growth was also apparent in cell cycle distribution when cells were exposed to 40 μM baicalein for 3, 6, 12, 24 and 48 h, as demonstrated by a dramatic decrease in the S and G2M phase cell population, with few cells able to return to the G1 phase. Thus, at a higher dose, increased exposure times led to an increase in the number of apoptotic cells as determined by the loss of low molecular weight DNA during the staining procedure, a feature characteristic of apoptotic cells (Fig. 5).

It is well acknowledged that the efficacy of a compound in controlling the cell cycle regulatory points can be better assessed when the cells are exposed to lower doses than to doses exhibiting maximum growth inhibition. It is also widely accepted that the cell cycle point which is blocked by a lower dose of a compound is the same point where a higher concentration induces apoptosis (Bhuyan and Groppi 1989; Gorczyca et al. 1993). Our observation was that cells initially blocked at the S or G2/M phases due to exposure to 10 or 20 μM baicalein for 36 or 48 h may result in a rapid disappearance of cells from the cell cycle after treatment with a higher concentration, 40 μM baicalein. Thus, increase in the frequency of apoptotic cells occurred at the expense of cells in the S and G2M phases which had begun to increase following 10 μM baicalein exposure for 36 h or 48 h. By 48 h, the frequency of apoptotic cells increased to 83% with only 6.4% in the S and 1.5% in the G2M phase (Fig. 5F), and a reduced G1 population presumably due to the inability of cells to divide successfully.

As noted above, changes in cell proliferation observed in baicalein treated cells may be due to apoptotic cell death in addition to cell cycle arrest. Cells failing to progress to mitosis may be destined for apoptosis. The degradation of nuclear DNA into nucleosomal units is one of the features of apoptotic cell death. In dying cells, DNA is cleaved by an endonuclease that fragments the chromatin into nucleosomal units, which are multiples of about 100–180 bp oligomers and appear as a DNA ladder when run on an agarose gel. This feature, fragmentation of DNA into oligonucleosomal size fragments, has been described as a hallmark of apoptosis. In this study, we have established that baicalein induced cell death is characteristic of apoptosis, as cleavage of DNA in the cells was investigated by different methods, which measure the extent of fragmentation in the cells. In the ladder study, we have seen that fragmentation of nucleosomal DNA appeared in cells treated with 10, 20 or 40 μM Baicalein after 48 h. TUNEL assay and related *in situ* nick translation technique confirmed the presence of fragmented oligonucleosomal DNA within apoptotic cells for the addition of labeled nucleotides by either terminal transferase or DNA polymerase. TUNEL assay, thus, can serve as a high conformity indicator of the occurrence of apoptosis. In our study, a major fraction of the cell population became TUNEL positive by the end of 24 h incubation (Fig. 4).

In conclusion, the present study demonstrated that baicalein was the main component in the methanolic extract of *Oroxylum indicum* active against the proliferation of HL-60 cells. The study further shows that cell proliferation inhibition was concentration dependent. At a lower concentration baicalein may interfere with cell cycle progression, by arresting cells at the S or G2M phases; however at a higher concentration cells in the S or G2M phases dramatically reduced, becoming apoptotic as confirmed by multiple DNA fragmentation assays. The cytostatic and antiproliferative properties of baicalein can thus confirm that *Oroxylum indicum* extract can be used in supplementary cancer therapy.

4. Experimental

4.1. Extract preparation

The fruits of this plant were purchased from a local Thai super store, in Tsukuba, Ibaraki, Japan. The plant material (about 1 kg) was cut into small pieces and immersed in 3 L of methanol for three days at room temperature. The extract was filtered and the filtrate obtained was used as a crude extract to determine the antiproliferative activity against the proliferation of HL-60 cells.

4.2. HPLC analysis

Chemical investigation was carried out as described earlier by Nakahara et al. (2001). The crude extract was evaporated at 35 °C, under reduced pressure to 250 mL, and then partitioned twice with 500 mL of diethyl ether. The diethyl ether layer, which showed most activity against the proliferation of HL-60 cells, was evaporated to dryness. The dried residue was dissolved in 250 mL ethanol and mixed with 250 ml water containing 1% formic acid. The mixture was immediately applied to a reverse-phase column (Wakosil C18LP40) and the column was eluted by a stepwise gradient of 50%, 60%, 70%, 80% and 90% methanol with 1% formic acid. The eluates were collected and analyzed by HPLC, which was performed on a Tosoh PX-8020 system, equipped with a photodiode array detector. Separation was done on a TSK gel super-ODS column maintained at 40 °C. The mobile phase consisted of 0.5% formic acid (A), acetonitrile (B) used in linear gradient elution with a flow rate of 1 $cm^3\ min^{-1}$ and detection at 275 nm. Activity was detected in the fraction eluted in 70%–80% methanol. The active fraction was concentrated to about 300 ml and stored at 4 °C. Amber crystals were formed and after purification by repeated recrystallization was confirmed by HPLC (>99.5%) using standard baicalein as purified previously (Nakahara et al. 2001). The peak was assigned by spiking the sample with standard substance and comparing the retention time and UV spectra.

4.3. Cell culture and exposure to Oroxylum indicum extract

Promyelotic leukemia cells of the HL-60 line were obtained from Riken Gene Bank (RCB0041), Tsukuba, Ibaraki, Japan. The cells were cultured in RPMI 1640 (Invitrogen Corporation, Grand Island, NY) medium supplemented with 10% inactivated fetal bovine serum (FBS, ICN Biomedicals, Aurora, Ohio) in antibacterial antimycotic solution (Sigma Chemical Co., St Louis, MO) at 37 °C in air supplemented with 5% CO_2. For cytological investigation, 10^5 cells per ml of RPMI medium were used. Cells were treated with various concentration of baicalein for 3, 6, 12, 18, 24, 36 or 48 h. Control culture received an equal amount of DMSO (Wako Pure Chemicals, Ltd., Osaka, Japan), which resulted in a final concentration of 0.1% in the media. After each time interval of incubation, cells were collected for further cytological investigations.

4.4. Cell viability assay

Cell proliferation was measured by counting viable cells using trypan blue dye. Briefly, HL-60 cells (1×10^6 cells) in 5 mL culture media were plated onto 60 × 15 mm culture dishes (Falcon 3002, Beckton Dickinson and Co., Franklin Lakes, NJ). Plates containing medium without drugs were used for measuring control cell viability. Five concentrations of baicalein ranging from 10 to 50 μM were added to the dishes. The cultures were incubated in a humidified incubator in 5% CO_2 for 24 h at 37 °C. At the end of treatment, cells were collected by low speed centrifugation and the number of viable cells was determined by trypan blue dye exclusion under a light microscope.

4.5. Measurement of sub-diploid cells and cell cycle analysis by flow cytometry

Formation of a sub-G1 peak in baicalein induced apoptosis was investigated by flow cytometry. For this, cells (1×10^6 cells/5 mL) treated with or without baicalein were washed with PBS, and fixed in suspension in 70% ethanol on ice and then stored at −20 °C for more than 12 h. After three washes with PBS, fixed cells were incubated with 1 μg/mL RNAse at 37 °C for 30 min and stained with propidium iodide (Sigma) (20 μg/mL) in the dark at room temperature for 20 min. The stained cells were analyzed on a FACSort flow cytometer (Becton Dickinson, Palo Alto, CA) for relative DNA content.

4.6. DNA extraction and electrophoresis

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis. DNA was extracted from the control and treated cells using an apoptosis ladder detection kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Briefly, after treatment with baicalein at 5, 10, 20 or 40 μM concentrations for 48 h, the total cells were collected by low speed centrifugation. DNA was extracted according to the manufacturer's instructions, and fractionated on 1.5% agarose gel and visualized by SYBR™ green 1 staining.

4.7. Measurement of apoptosis

DNA fragments in control and baicalein treated cells were measured by terminal deoxynucleotidyl transferase mediated fluorescent isothiocyanate deoxyuridine triphosphate (FITC-dUTP) nick end labeling (TUNEL) assay (APO-DIRECT, Phoenix Flow System, Inc., San Diego, CA). The cells were fixed with 1% parafolmaldehyde, followed by fixation with 70% ethanol. The DNA was subjected to an *in situ* tailing reaction in which residues of fluorescent deoxyuridine triphosphate nucleotides (F-dUTP) were added catalytically to the 3′-OH sites of DNA strand breaks by terminaldeoxynucleotidyl transferase (TdT). Cells were analyzed by flow cytometry on a FACsort (Beckton Dickinson)

4.8. Analysis of data

All data in this report are presented as means of three separate experiments, and one-way analysis of variance (ANOVA) with Student-Newman-Keuls post-hoc comparison was used for statistical significance with $P \leq 0.05$. All the figures shown in this paper were obtained from at least three independent experiments with a similar pattern.

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