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ORIGINAL ARTICLE

Taxifolin inhibits the development of scar cell carcinoma by inducing apoptosis, cell cycle arrest, and suppression of PI3K/AKT/mTOR pathway

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Summary

Purpose: *Plant metabolites have gained considerable attention as the anticancer agents over the last few decades. Previous studies have indicated the potential of taxifolin as an anticancer agent. However, the information regarding the anticancer activity of taxifolin against skin scar cell carcinoma (SSCC) as well as several other types of cancers is limited. Against this background, the present study was designed to investigate the anticancer activity of taxifolin against a panel of SSCC lines.*

Methods: *Proliferation rate of the cells was monitored by MTT assay. DAPI and annexin V/PI assay were used to investigate the induction of apoptosis. Flow cytometry was employed for cell cycle analysis. Transwell assay was used to check the invasion of the cancer cells.*

Results: *The results indicated that taxifolin inhibits the development of the SSCC lines. However, the anticancer effects were more evident on the SSCC cancer cells (IC_{50}, 20 μM). In contrast, the anticancer effects of taxifolin on the non-cancer skin cells were minimal. Taxifolin caused apoptosis and cell cycle arrest of the SSCC cells. Moreover, taxifolin inhibited the invasion of SCCC cells which was associated with down-regulation of the matrix metalloproteinases (MMP) MMP-2 and MMP-9 expression.*

Conclusion: *It is was found that taxifolin inhibited the development of SSCC by triggering apoptosis and cell cycle arrest, as well as inhibiting cell invasion capacity.*

Key words: *skin scar cell carcinoma, taxifolin, apoptosis, invasion*

Introduction

Cancer is a devastating group of diseases and several factors have been attributed for the development of cancer [1]. Although there are different factors responsible for the development of cancer, scars caused by vaccines, burns, and other injuries may also lead to development of cancers. Such cancers are known as scar carcinomas and generally occur in skin tissue [2]. Among scar carcinomas, the burn scar carcinomas are the most prevalent types of scar carcinomas [3,4]. The chemotherapeutic agents for scar cell carcinomas are limited and associated with several adverse effects. Over the years, natural products have shown tremendous potential as chemo-preventive and chemotherapeutic agents [5]. Besides being effective in the treatment of several diseases, they exhibit relatively lower side effects [6,7]. Among the natural products, flavonoids constitute a group of plant metabolites that are structurally diverse and exhibit the capacity to interact with several bio-molecules including enzymes [8]. They are common ingredients of human diet and studies have shown that consumption of flavonoids lowers the risk of cancer development [9,10]. Taxifolin is one of the most important flavonoids prevalent across the plant kingdom [11]. Taxifolin has been reported to exhibit antiprolifera-

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tive effects against the cancer cells. For instance, Taxifolin has been shown to inhibit the growth of prostate cancer cells by inducing apoptosis and cell cycle arrest [12]. Similarly, taxifolin has also been reported to inhibit the proliferation of cervical cancer cells [13]. However, the anticancer effects of taxifolin have not been investigated against the skin scar cell carcinoma (SSCC) and the underlying mechanism of its anticancer activity is yet largely unknown. The current study was therefore designed to evaluate the anticancer effects of taxifolin against the human SSCC cells. Herein, we report for the first time that taxifolin inhibits the development of SSCC cells by inducing apoptosis and G2/M cell cycle arrest, and modulating the PI3K/AKT/mTOR signaling pathway. Having been involved in the proliferation and tumorigenesis of many cancer types, PI3K/AKT/MTOR pathway has been considered as an essential therapeutic target [13]. Therefore, the effects of taxifolin were also examined on PI3K/AKT/mTOR signalling pathway.

Methods

Cell lines and culturing conditions

SSCC cell line and the non-cancer skin cell line BJ-5TA were procured from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium in CO_2 incubator (Thermo Scientific, Waltham, Mass, USA) with 37°C, 98% humidity and 5% CO_2.

Proliferation assay

The effects of taxifolin on the viability of SSCC cell lines was assessed by MTT assay as described previously by Mosmann [14]. The SSCC cells were subjected to treatment with varied concentrations of taxifolin.The proliferation rate was determined by absorbance, measured at 570 nm.

Apoptosis assay

The apoptosis inducing effects of taxifolin were determined by DAPI staining as described previously [15]. In brief, the SSCC cells (0.6×10^6) were grown in 6-well plates. Following 12 h of incubation, the cells were subjected to taxifolin treatment for 24 h at 37°C. The cell cultures were then centrifuged and the pellets were washed with phosphate buffered saline (PBS). Thereafter, the cells were stained with DAPI, centrifuged, and washed in PBS. Finally, the nuclear morphology of the stained cells was examined by fluorescence microscopy. The percentage of the apoptotic cells was estimated by annexin V/propidium iodide (PI) staining as described in the literature [16].

Cell cycle analysis and cell invasion assay

The distribution of the SSCC cells in different cycle phases was performed by flow cytometry after PI staining by following the method reported in literature. In brief, the SSCC cells were grown in 6-well plates and treated with taxifolin for 24 h. The cells were then collected and washed in PBS followed by fixation in ethanol (70%). After overnight incubation at 4°C, the cells were subjected to PI staining and then subjected to flow cy-

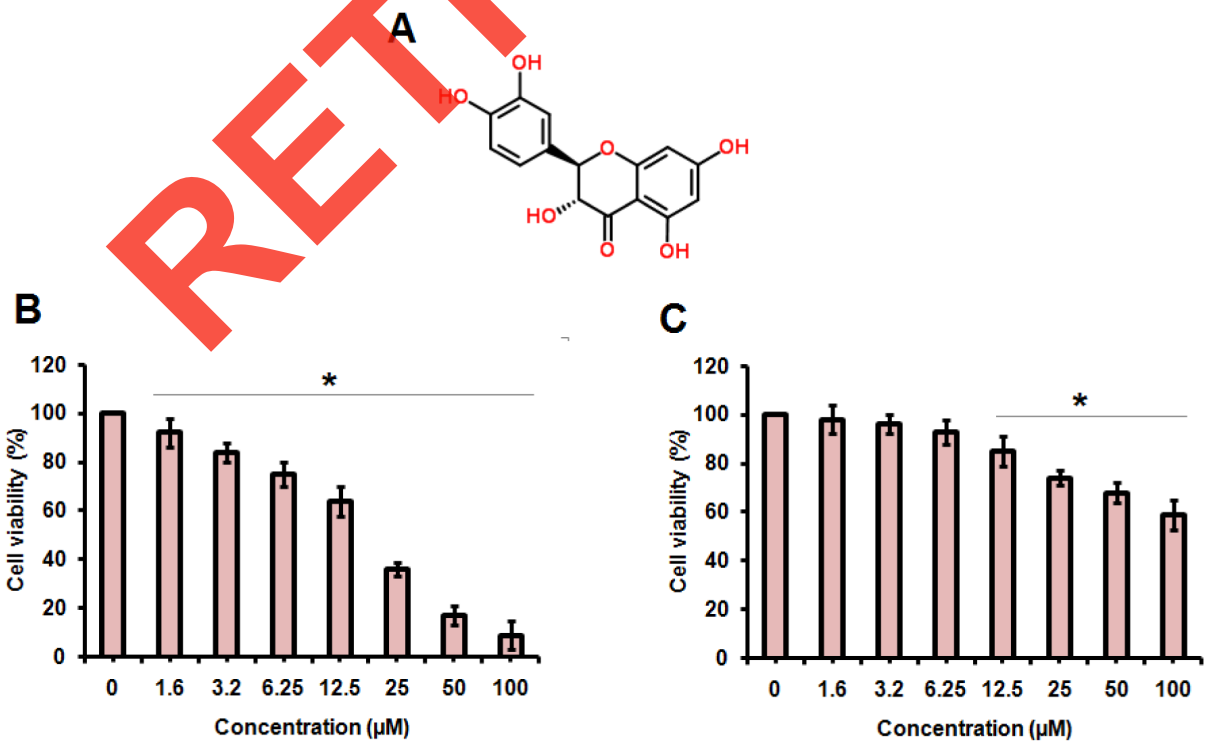

Figure 1. (A): Chemical structure of taxifolin. **(B):** effect of taxifolin on the proliferation of cancer SSCC cells. **(C):** Effect of taxifolin on the normal BJ-5TA cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.01).

tometry. Cell invasion assay was performed as described previously by Wang et al. [17].

Western blotting

After lysis of the SSCC cells in RIPA lysis buffer, the protein content of the each lysates was estimated by bicinchoninic acid (BCA) assay. The samples were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for 24 h. After this, the membranes were incubated with HRP-conjugated secondary antibody (1:1000) for 50 min at 25°C. Enhanced chemi-luminescence reagent was used to visualize the protein bands.

Statistics

All experiments were performed in triplicate and presented as mean±SD. Student's t-test was employed for statistical analysis using Graph Pad prism 7 software and $p<0.01$ was taken as statistically significant difference.

Results

Taxifolin inhibits the growth of SSCC cell lines

The anticancer effects taxifolin (Figure 1A) were examined against the human SSCC cell lines by MTT assay. It was found that taxifolin concentration-dependently inhibits the viability of SSCC (Figure 1B). The IC_{50} of taxifolin against the human SSCC cells was found to be 20 µM. Interestingly, taxifolin was found to exert minimal cytotoxicity against the normal BJ-5TA cells, with an IC_{50} of 110 µM (Figure 1C), indicating that taxifolin selectively inhibits the growth of SSCC cells.

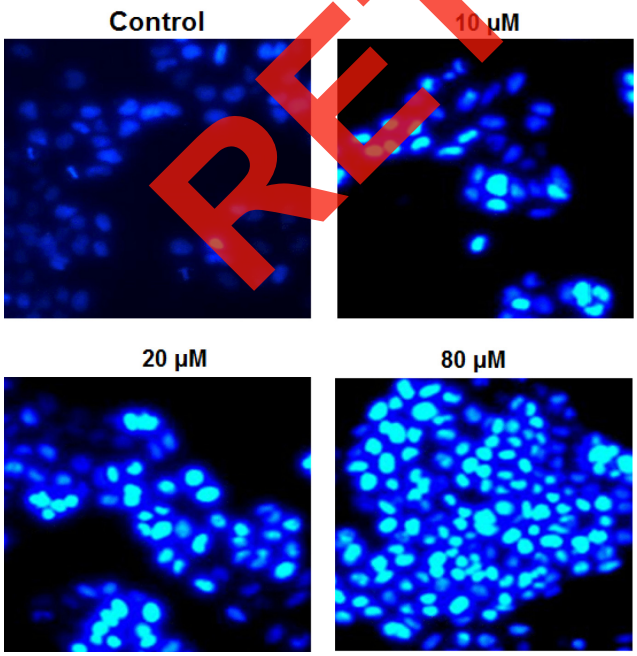

Figure 2. DAPI staining showing that taxifolin induces apoptosis in SSCC cells in a concentration-dependent manner. The experiments were performed in triplicate.

Taxifolin triggers apoptotic death of the SSCC cells

Previously, taxifolin was reported to trigger the apoptotic cell death of the cervical cancer cells [12]; therefore, DAPI and annexin V/PI staining

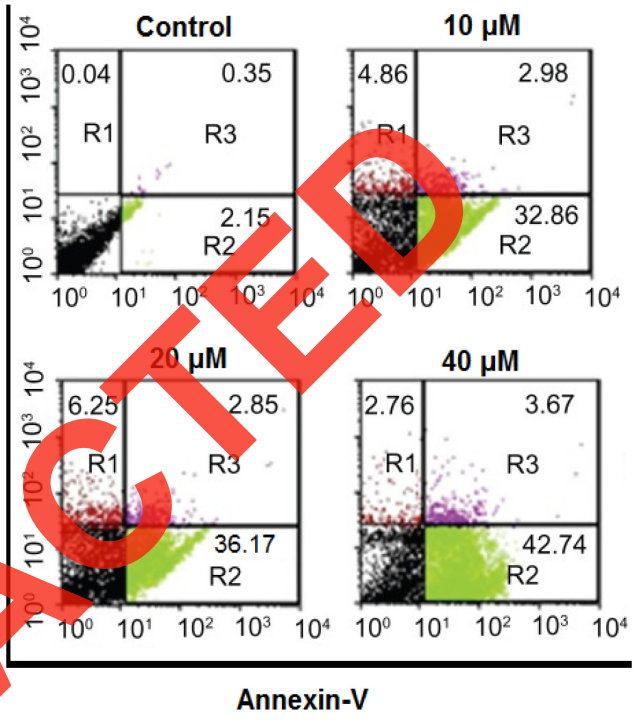

Figure 3. Annexin V/PI assay showing the percentage of apoptotic cells at increasing concentrations of taxifolin. The Figure shows that the apoptotic SSCC cell percentage increases concentration-dependently. The experiments were repeated thrice.

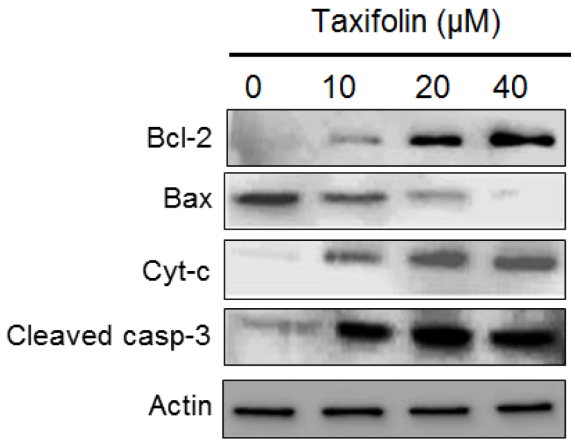

Figure 4. Effect of taxifolin evaluated by western blotting on the expression of apoptosis-associated proteins. The Figure shows that the expression of Bcl-2 is decreased, while that of Bax, Cyt-C and cleared caspase-3 increases upon taxifolin treatment. The experiments were performed in triplicate.

were performed to deduce if taxifolin inhibits the growth of the SSCC cells by prompting apoptotic cell death. The results of DAPI staining revealed that taxifolin caused remarkable changes IN the nuclear morphology of the SSCC cells, and characteristic of apoptosis (Figure 2). Next, annexin V/PI staining showed considerable increase in the apoptotic SSCC cell populations after taxifolin treatment (Figure 3). To further confirm the taxifolin-induced apoptosis, the western blot analysis was performed and it was found that taxifolin significantly enhanced the expression of Bax and cleaved caspase-3 which was concomitant with decline in the expression of Bcl-2 (Figure 4). Taken together,

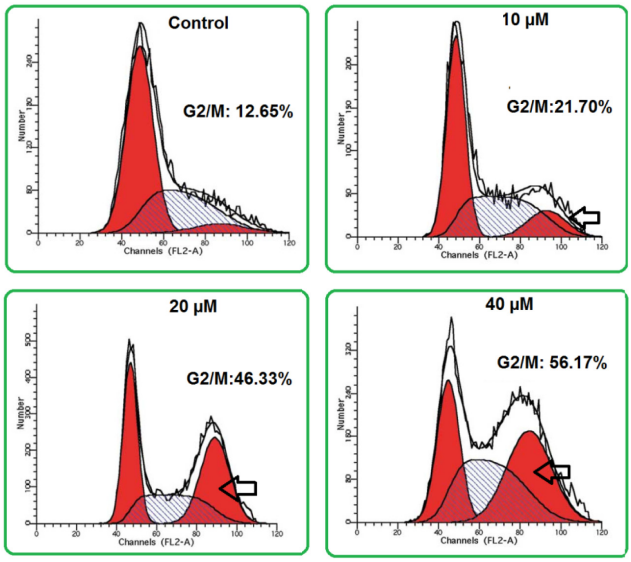

Figure 5. Taxifolin induces G2/M cell cycle arrest at varied concentrations as indicated by annexin V/PI staining. The Figure shows that G2/M phase cells increase upon taxifolin treatment in a concentration-dependent manner. Arrows depict G2/M phase cells. The experiments were performed in triplicate.

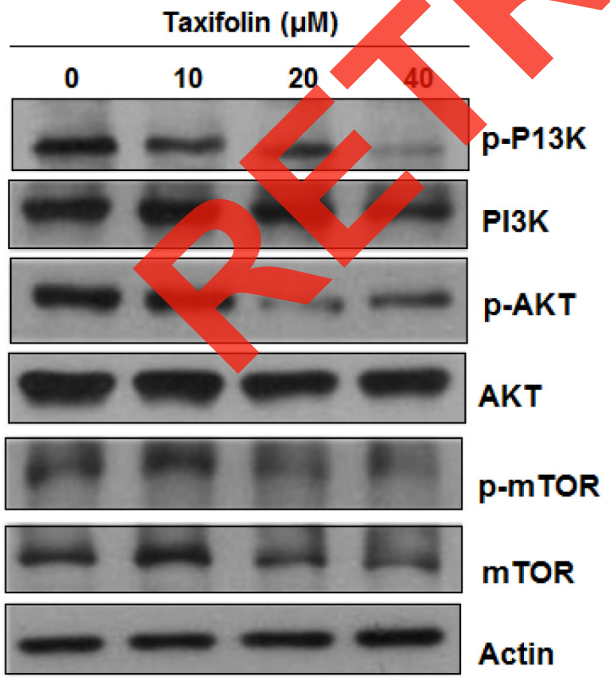

Figure 6. Effect of taxifolin on the PI3K/AKT/mTOR signalling pathway at varied concentrations as indicated by western blot analysis. The Figure shows that taxifolin blocks the PI3K/AKT/mTOR signalling pathway in a dose-dependent manner. The experiments were performed in triplicate.

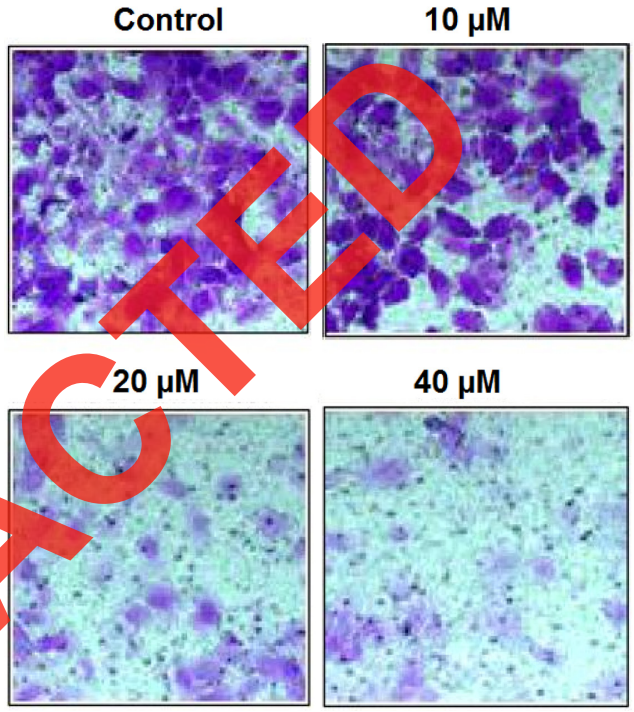

Figure 7. Effect of taxifolin on the invasion of the scar carcinoma cells at varied concentrations as depicted by transwell assay. The Figure shows that the invasion of SSCC cells is inhibited in a concentration-dependent manner by taxifolin. The experiments were repeated thrice.

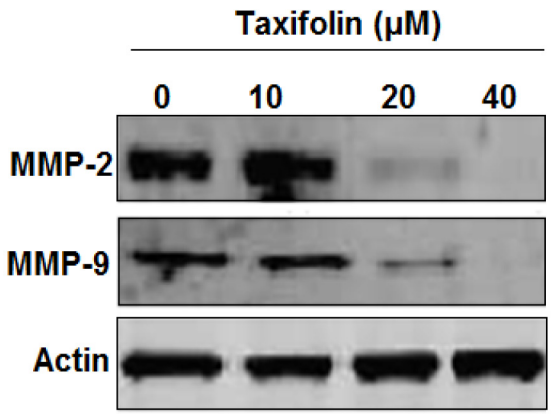

Figure 8. Effect of taxifolin on the expression of matrix metalloproteinases (MMP)-2 and 9 as depicted by western blotting. The Figure shows that taxifolin inhibits the expression of MMP-2 and MMP-9 in a concentration-dependent manner. The experiments were performed in triplicate.

these results indicate that taxifolin inhibits (at least in part) the growth of skin scar cell carcinoma cells via induction of apoptosis.

Taxifolin triggers G2/M cell cycle arrest of SSCC cells

Although taxifolin could trigger apoptosis of the SSCC cells, apoptosis alone may not be responsible for such a low IC_{50} of taxifolin. Hence, we performed the cell cycle analysis of the taxifolin treated SSCC cells. The results showed that the percentage of the G2 phase cells after treatment with taxifolin increased considerably (Figure 5) and exhibited a dose-dependent trend. The percentage of the G2/M cells increased from 12.65% to 56.17%, at 40 μM concentration of taxifolin.

Taxifolin inhibits the mTOR/PI3K/AKT signaling pathway

The mTOR/PI3K/AKT signaling pathway has been shown to play a role in cell proliferation. Therefore, we investigated that if taxifolin triggers cell cycle arrest by modulating mTOR/PI3K/AKT signaling pathway. The results showed that taxifolin could concentration-dependently inhibit the expression of phosphorylated (p)-mTOR, p-PI3K, and p-AKT, with no apparent effects on the expression on mTOR, PI3K, and AKT (Figure 6).

Taxifolin inhibits the invasion of the SSCC cells

The effects of taxifolin on the invasion of the SSCC cells were also investigated by transwell assay. It was found that taxifolin could significantly inhibit the invasion of the SSCC cells (Figure 7). Further, the inhibition of cell invasion was also linked with a decline in the expression of Metalloproteinases 2 and 9 (Figure 8).

Discussion

Scar cell carcinoma is one of the deadliest cancers and generally arising from burns or scars caused by vaccinations and several other injuries [2]. The inefficient chemotherapeutic agents and associated side effects impose barriers in the treatment of scar cell carcinoma [4]. In this study, the anticancer effects of taxifolin were investigated against SSCC and normal skin cells. Taxifolin could inhibit the growth of all SSCC; however, taxifolin could exert minimal cytotoxic effects on the normal skin cells, indicating that taxifolin selectively exerts growth-inhibitory effects on cancer cells. These observations are in good agreement with previous investigations carried out on taxifolin, where taxifolin has been shown to inhibit the growth of the cancer cells [18]. It is now well-established that many of the anticancer agents induce apoptotic cell death of the cancer cells. Taxifolin has also been reported to induce apoptosis in prostate cancer cells [12]. Apoptosis eliminates the cancer cells from the body and also prevents development of chemo-resistance [19]. In this study, it was found that taxifolin induced apoptosis in SSCC cells. In addition, taxifolin caused substantial increase in the expression of cleaved caspase-3 and Bax and decreased the expression of Bcl-2 in SSCC cells, ultimately favoring apoptosis. However, apoptosis may not be alone responsible for the low IC_{50} of 20 μM observed for taxifolin against the SSCC cells; hence, we also carried out cell cycle analysis. Interestingly, it was found that taxifolin causes arrest of the SSCC cells at the G2/M phase of the cell cycle. The arrest of cancer cells at G2/M phase prevents the cancer cells from entering mitosis, so the molecules that trigger cell cycle arrest prevent the proliferation and tumorigenesis of cancer cells [20]. Since taxifolin induced cell cycle arrest, we investigated if the taxifolin-induced cell cycle arrest happens via inhibition of mTOR/PI3K/AKT pathway [21]. It was found that taxifolin inhibits the mTOR/PI3K/AKT signaling pathway, leading to arrest of the SSCC cells at the G2/M checkpoint. The invasion of cancer cells to the neighboring organs is the initial and important step in cancer metastasis [22]. In this study, we observed that taxifolin concentration-dependently inhibits the invasion of the A549 and SSCC cells which was concomitant with the decrease in the expression of MMP-2 and 9. These results are also complemented by previous investigations wherein flavonoids (such as baicalein) inhibit the invasion of human breast cancer cells [23]. Therefore, taxifolin may prove to be an important lead molecule in the treatment of scar cell carcinoma.

In conclusion, taxifolin inhibits the development of SSCC *in vitro* by induction of apoptosis and cell cycle arrest. Hence, taxifolin may prove to be a beneficial lead molecule in the treatment of skin scar cell carcinoma, but warrants further *in vivo* investigations.

Conflict of interests

The authors declare no conflict of interests.

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