ORIGINAL ARTICLE

Baicalein suppresses the growth of the human thyroid cancer cells by inducing mitotic catastrophe, apoptosis and autophagy via NF-kB signalling pathway

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Summary

Purpose: The current study aimed at investigating the anticancer effects of plant-based flavone Baicalein against the thyroid cancer.

Methods: Cell viability was assessed using MTT assay. Flow cytometric-based estimation of cell cycle analysis was done for determining the cancer cell phase distribution. DAPI staining method followed by fluorescent microscope examination was used for inferring the cancer cell apoptosis. Transmission electron microscopy (TEM) was used for detection of autophagosomes. Western blotting was done for protein concentration estimation.

Results: Baicalein induced dose-dependent decline in proliferation of MDA-T68 thyroid cancer cells, while the reduction of cell proliferation was surprisingly lower for normal thyrocytes. IC_{50} of 10µM was estimated against cancer cells. Bai

calein induced cell apoptosis in a concentration-dependent manner. Induction of apoptosis was attributed to increase in apoptotic protein concentration and signal was mediated through change Bax/Bcl-2 ratio. The autophagic cell death occurred in cancer cells when treated with Baicalein. The mechanism of cell death was inferred as modulation of NFkB signaling pathway. Baicalein was also seen to induce mitotic cell cycle arrest in thyroid cancer cells by reducing the concentration of Cyclin B1 mitotic protein.

Conclusion: The results of current study suggest Baicalein as an important anticancer agent against thyroid cancer. Future research to further investigate and enhance the effects of Baicalein against thyroid cancer is needed.

Key words: flavanone, apoptosis, autophagosomes, cell cycle arrest

Introduction

During a long evolutionary history, plants have evolved to produce a vast array of secondary metabolites [1]. Plant secondary metabolites function to enable the host plants to better survive in native habitat conditions [2]. Besides their primary role, these compounds have been found economically important and have a number of health beneficial effects [3]. Humans have learnt to make use of plants for isolation and use of such compounds

for a number of purposes. Flavonoids are an important class of polyphenolic secondary metabolites abundantly present in the plant kingdom [4]. The antioxidant potential of flavonoids is well documented [5-7] and a number of flavonoids have been reported to act as anticancer agents [8,9]. There is high interest to evaluate flavonoids for anticancer drug discovery [10]. Baicalein (5,6,7-trihydroxyflavone) is a flavonone type of flavonoid extracted

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most commonly from dried roots of Scutellaria baicalensis and Scutellaria lateriflora (Figure 1A). Baicalein has been already shown to have radical scavenging properties [11]. The anticancer potential of Baicalein has been investigated in a number of studies [12-15]. It inhibits the proliferation of human cervical cancer cells by inducing apoptosis [16]. A same type of study on anticancer activity Baicalein has shown that this compound inhibits breast cancer cell adhesion and metastasis [17]. Baicalein is also active in inhibiting the human prostate cancer cells [18]. Similarly, Baicalein was proved to inhibit the growth of ovarian cancer cells [19]. The current study focused on the evaluation of anticancer potential of this important flavonoid in thyroid cancer. Thyroid cancer is a most common malignant type of endocrine cancer. More than 90% of thyroid cancers are believed to result from follicular cells of the thyroid gland [20]. According to WHO's report, about 230,000 and 70,000 new thyroid cancer cases were recorded in 2012 among women and men worldwide, respectively, with an age-standardized rate of 6.10/100,000 women and 1.90/100,000 men [21]. It is of concern that an increase in the incidence has been noticed in recent years. Therefore, there is a need to explore anticancer measures against thyroid cancer. In the present study the anticancer effects of Baicalein were examined on human thyroid cancer cells and the underlying mechanisms were explored.

Methods

Culture of cell lines

MDA-T68 thyroid cancer cells and normal thyroid follicular cells were obtained from American Type Culture Collection (Manassas, USA). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), L-glutamine (2mM) in incubator at 37°C with 95% O_2 and 5% CO_2 , as previously reported [22].

Cell viability assay

Cell viability was determined using MTT assay. MDA-T68 thryoid cancer and normal thyrocytes were grown in a 96-well plate up to a density of 1×10^6 cells/ well. The cells were then treated with 0, 1.25, 2.5, 5, 10, 20, 40, 80, 160 and 320µM concentrations of Baicalein for 24h. MTT 0.5% (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide) was added to each well followed by incubation at 37°C for 4h. Dimethyl sulfoxide (DMSO) 150µl was added to each well to solubilize the MTT-formazan crystals formed from MTT. Optical density (OD)₅₇₀ was taken to analyze the cell viability and was presented in percentages.

Cell cycle analysis

MDA-T68 thyroid cancer cells treated with 0, 5, 10 and 20µM Baicalein were harvested and fixed using

ethanol (70%). The cells were again collected and resuspended in phosphate buffered saline (PBS) supplemented with 200µg/ml RNase A, 0.1% Triton X-100, and 50µg/ml propidium iodide (PI). Then, the cells were incubated at 37°C in the dark for 30 min. The phase distribution was examined using flow cytometry.

Assessment of cell apoptosis

Fluorescent microscopic analysis of cell morphology of MDA-T68 cells, treated with 0, 5, 10 and 20μ M concentration of Baicalein, was done to assess the induction of cellular apoptosis. Before that the cells were DAPI-stained in 6-well plates at a density of 0.6×10^6 .

Analysis of autophagy of MDA-T68 cancer cells

TEM was used to examine the autophagy in MDA-T68 cells treated with Baicalein. Briefly, the cancer cells were treated with 0, 10, 20 and 40µM Baicalein for 24h in 6-well plates. The cells were harvested by centrifugation and washed with PBS. Then, the cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer and were again fixed using 1% osmium tetroxide. Finally, the cells were ethanol-treated and resin embedded. The cells were then cut into thin sections with an ultramicrotome and studied under electron microscope.

Analysis of protein expression through western blotting

MDA-T68 cells treated with 0, 10, 20 and 40µM Baicalein were harvested and lysed with RIPA buffer. The estimation of protein concentration was done using Bradford method for the lysates. Cell lysates with about 40µg protein concentration were loaded on SDS-PAGE followed by blotting of PAGE gel to PVDF membrane which was exposed to primary antibodies. The final protein detection was done using chemilumines-



Figure 1. (A): Chemical structure of Baicalein. **(B):** MTT assay showing the effect of Baicalein on the viability of Thyrocytes and MDA-T68 thyroid cancer cells. The experiments were performed in triplicate and expressed as mean \pm SD (*p<0.05).

cence after using secondary antibodies. Actin protein was used as control for performing protein expression studies.

Statistics

The experiments were performed in triplicate and the values were presented as mean±SD. Student's t-test (for comparison between two samples) and one way analysis of variance (ANOVA) followed by Tukey's test (for comparison between more than two samples) were used for statistical analysis using GraphPad Prism software (version 7; GraphPad Software Inc, La Jolla, (USA). P<0.05 was considered to indicate statistically significant difference.

Results

Baicalein decreased thyroid cancer cell viability

A decline in the proliferation of MDA-T68 cancer cells was seen after their treatment with Baicalein. Furthermore, the decline was more prominent at higher treatment concentrations, i.e., the inhibition rate was proportional to concentration of the Baicalein used (Figure 1B). The percentage of decrease in cell viability reached near about 50% at 10μ M which was taken as IC₅₀ for Baicalein.

Baicalein caused mitotic cell arrest in MDA-T68 cells

The examination of the cell cycle was performed using flow cytometry which showed that the G0/G1 phase cells increased with lower doses of Baicalein. However, at 20µM there was a greater



Figure 2. (A): Distribution of MDA- T69 cells in different phases of the cell cycle. (B): Western blot showing that Baicalein inhibits the expression of cyclin B1 concentration-dependently. The experiments were performed in triplicate and expressed as mean \pm SD (p<0.05).

fraction of cells in the G2/M phase (Figure 2A). Baicalein prevented the mitotic entry of cancer cells and stopped their proliferation either at G0/G1 or G2/M stage of the cell cycle. The inhibition of mitotic entry was also confirmed from western blot analysis of Cyclin B1 protein the concentration of which decreased in a dose-dependent manner (Figure 2B).

Cancer cell apoptosis and cell death induction by Baicalein

Fluorescent microscopy was used to study the effects of Baicalein treatment in initiating apoptosis of MDA-T68 cancer cells. The results showed that Baicalein has a potential to induce apoptosis of cancer cells. The effects were more prominent at higher concentrations (Figure 3). To infer the mechanism of apoptosis induction, western blot-



Figure 3. DAPI staining showing the effects of Baicalein on the induction of apoptosis in MDA-T6 thyroid cancer cells. The Figure shows that Baicalein increases the number of apoptotic cells dose-dependently. The experiments were performed in triplicate.



Figure 4. Western blot analysis showing that Baicalein increases the expression of Bax and decreases the expression of Bcl-2 in MDA-T6 thyroid cancer cells dose-dependently. The experiments were performed in triplicate.



Figure 5. Electron microscopic images showing induction of autophagy by Baicalein in MDA-T68 thyroid cancer cells in a dose-dependent manner (rectangular boxes indicate the autophagosomes). The experiments were performed in triplicate.

ting of Bax and Bcl-2 apoptotic pathway proteins was done. The concentration of Bax increased in a concentration-dependent manner. The same was true for Bcl-2 protein but the trend was reverse, i.e., its concentration decreased with increase in Baicalein treatment employed ((Figure 4). To assess the induction of autophagy in thyroid cancer cells, MDA-T68 cells treated with 0, 10, 20 and 40 µM concentrations of Baicalein were processed for electron microscopic study. The electron microscope imaging showed the presence of prominent vesicular structures in the treated cells (Figure 5). These vesicles (autophagosomes) were indicative of cell death prompted by Baicalein. The apoptotic signal was also mediated through other apoptosisrelated proteins like LC3B II, whose concentration increased with increase in Baicalein concentration.

However, there was no effect of Baicalein on LC3B I protein expression (Figure 6).

Baicalein induced NF-kB signaling pathway mediated cell death of thyroid cancer cells

To look whether the induction of cell autophagy was mediated through NF-kB apoptotic signal, western blotting study was performed which showed that there was a remarkable increase in NF-kB autophagy factor. The concentration of NF-kB showed dose-dependent increase (Figure 7).

Discussion

Plants produce a diverse array of secondary manner. Induction of apoptosis was via the activametabolites whose synthesis is mainly meant for tion of Bax/Bcl-2 signaling pathway wherein Bax



Figure 6. Western blot analysis showing that Baicalein increases on the expression of LC3B I, II and Beclin 1 in MDA-T6 thyroid cancer cells dose-dependently. The experiments were performed in triplicate.



Figure 7. Western blot analysis showing that Baicalein inhibits the expression of NF-kB in MDA-T6 thyroid cancer cells concentration-dependently. The experiments were performed in triplicate.

better survival and self-defense. Flavonoids, a class of plant secondary metabolites, are almost universally present in the plant kingdom. The flavonoids, besides being beneficial to the plant itself, are having diverse positive effects on human health. Flavonoids possess anticancer potential and can serve as source of anticancer drugs. Baicalein belongs to the flavonoid group of secondary metabolites. This molecule has been investigated against a number of human cancers but there is no study of its anticancer investigation against human thyroid cancer. Human thyroid cancer is the most common malignant type of the endocrine glands [23]. The matter to worry about is that the rate of incidence has increased in recent years. The results of the current study suggest that Baicalein is having a potential to limit the growth of MDA-T68 thyroid cancer cells but the inhibitory effects were comparatively lower on normal thyrocytes. Such selective inhibition has been found for other anticancer compounds as well [24-26]. The rate of inhibition of thyroid cancer cell proliferation increased with increasing Baicalein concentrations. The anticancer effects of Baicalein were also evident as induction of apoptosis in cancer cells. Also, the apoptosis of MDA-T68 cells increased in a dose-dependent manner. Induction of apoptosis was via the activa-

concentration increased under Baicalein treatment but Bcl-2 protein was seen to reduce. Western blotting results revealed that induction of apoptosis was further enhanced by increase in protein concentration of LC3B II. To check whether Baicalein has any effect on MDA-T68 cell cycle, cells treated with Baicalein were investigated for cell phase distribution analysis using flow cytometry and it was observed that the number of cells increased at G0/ G1 phase up to IC₅₀ concentration. At concentrations higher than IC_{50} the cell cycle was arrested at G2/M phase. Whether the cell cycle is halted at G0/G1 or G2/M phase, the mitotic cell division got hindered in both cases. Western blotting of Cyclin B1, whose synthesis is important for a cell to enter mitosis, revealed that the protein decreased in a concentration-dependent manner. This indicates that Baicalein is able to cause cell cycle arrest in thyroid cancer cells by preventing their mitotic entry. This result is in accordance with a previous report [27]. Furthermore, Baicalein induced autophagy in thyroid cancer cells and the mechanism of cell death by Baicalein treatment was attributed to modulation of NF-kB signalling pathway. There was a significant decline in NF-kB protein which is important for increasing the rate of cell death. From the western blotting studies, we found that Baicalein increased the concentration of Beclin 1 and LC3B II autophagy-related proteins in a dosedependent manner. The increase in concentration of Beclin 1 and LC3B II increased the rate of cell autophagy. Altogether, the results of the current

study suggest that Baicalein has a potential to act as an anticancer agent against human thyroid cancer cells. Moreover, the compound can be chemically modified to increase its anticancer activity and as such this study has a scope to provide a basis for future research studies on this issue.

Conclusion

To conclude, Baicalein inhibited the proliferation of the thyroid cancer cells in a dose-dependent manner and induced autophagy in cancer cells. The signal for cell autophagy was prompted with increase in NF-kB protein concentration. Anticancer effects also included halting to mitotic entry. The anticancer potential of Baicalein against human thyroid cancer suggests that flavonoids like Baicalein are potential candidates for anticancer drug discovery. Semisynthetic chemical approaches must be employed to make anticancer molecules like Baicalein more active and broaden their targets.

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Conflict of interests

The authors declare no conflict of interests.

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