Apigenin induces autophagic cell death in human papillary thyroid carcinoma BCPAP cells

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Abstract

Apigenin, abundantly present in fruits and vegetables, is recognized as a flavonoid with anti-inflammatory, antioxidant and anticancer properties. In this study, we first investigated the anti-neoplastic effects of apigenin on papillary thyroid carcinomas (PTC) cell line BCPAP cells. Our results show that apigenin inhibited the viability of BCPAP cells in a dose-dependent manner. A large body of evidence demonstrates that autophagy contributes to cell death in certain contexts. In present study, autophagy was induced by apigenin treatment in BCPAP cells, as evidenced by Beclin-1 accumulation, conversion of LC3 protein, p62 degradation as well as the significantly increased formation of acidic vesicular organelles (AVOs) compared to the control group. 3-MA, an autophagy inhibitor, rescued the cells from apigenin-induced cell death. Notably, apigenin enhanced production of reactive oxygen species (ROS), and subsequently induction of significant DNA damage as monitored by TUNEL assay. In addition, apigenin treatment caused a significant accumulation of cells at G2/M phase via down-regulation of Cdc25C expression. Our findings reveal that apigenin inhibits papillary thyroid cancer cell viability by stimulation of reactive oxygen species (ROS) production, induction of DNA damage, leading to G2/M cell cycle arrest and following autophagic cell death. Thus, our results provide new insights into the molecular mechanisms underlying apigenin-mediated autophagic cell death and suggest apigenin as a potential chemotherapeutic agent which is able to fight against papillary thyroid cancer.

Keywords: apigenin; human papillary thyroid carcinoma; autophagy; ROS; DNA damage; cell cycle arrest.

Introduction

Apigenin (Api, 4',5,7-trihydroxyflavone, chemical structure shown in Fig. 1 A), derived from the flavanone naringenin, is abundantly present in common fruits (oranges, apples, cherries, grapes), vegetables (onions, parsley, broccoli, sweet green pepper, celery, barley, tomatoes) and beverages (tea, wine).[1] In recent years, more and more studies have reported that apigenin possesses a wide broad of biological activities, including anti-allergic, anti-microbial, anti-inflammatory, anti-viral, and anti-tumor functions.[2] Cancer chemoprevention has emerged as one of the major approaches for reducing cancer burden. Compared with other flavonoids with similar structure, apigenin has low intrinsic toxicity and differential effects in normal versus cancer cells. Therefore, it attracted particular interest as a chemopreventive and/or chemotherapeutic agent.[3, 4] Apigenin showed outstanding anticancer effect on various cancers such as breast cancer,[5] prostate cancer, [6] head and neck squamous cell carcinoma,[7] and so on. However, there is still relatively limited information about the anticancer effect of apigenin on human thyroid carcinoma cells.

Thyroid cancer is the most common form of endocrine carcinoma and its incidence is rapidly growing worldwide.[8] Papillary thyroid carcinomas (PTC), accounting for 80-85%，is the most occurring forms of human thyroid cancer.[9] Conventional therapy of thyroid cancers relied on surgery，radioiodine ablation and thyroid-stimulating hormone suppression.[10] These treatments are usually efficient for the majority of thyroid tumors. However, treating advanced thyroid cancer which is recurrent, metastatic and ^{131}I-refractory, or medullary thyroid cancer, remains a therapeutic challenge.

Recently, it has been proposed that modulation of autophagy may be a potential target for thyroid cancer therapy.[11] Autophagy is an evolutionarily conserved cellular

catabolic process by which damaged or long-lived cellular proteins and superfluous cytoplasmic materials, including organelles are degraded and recycled in lysosomes. [12, 13] Several preclinical studies have indicated that autophagy is closely correlated with ^{131}I uptake and therapeutic sensitivity. For instance, Lin et al. reported autophagy inhibition promotes PTC resistance to doxorubicin and radiation,[14] while autophagy induction with RAD001 enhances chemosensitivity and radiosensitivity in PTC.[15] These studies suggested that activation of autophagy may be a useful adjunct treatment for those PTC patients who are refractory to conventional therapy. This notion was further verified by inhibition of autophagy with ATG7 silencing, which desensitized the PTC cells to TRAIL-induced apoptosis.[16]

Both apoptosis and autophagy are genetically-regulated, evolutionarily-conserved processes that regulate cell fate. Up to now, numerous studies have indicated the apoptosis-inducing ability of apigenin in different types of cancer cells.[17] However, there are only few reports concerning the autophagy-inducing effects of apigenin as well as its underlying molecular mechanisms. It has been reported that apigenin simultaneously induced both apoptosis and autophagy in HCT116 human colon cancer cells, and autophagy plays a cytoprotective role in apigenin-induced apoptosis.[18] The induction of autophagy by apigenin treatment could also be observed in MDAMB-231 and T47D breast cancer cells.[19]

In this study, we detected the anticancer effects of apigenin on human papillary thyroid carcinoma BCPAP cells. Our data showed that apigenin can induce autophagic cell death in BCPAP cells associated with intracellular ROS accumulation, DNA damage and subsequent G2/M cell cycle arrest. These results provide evidence that apigenin is a potential chemotherapeutic agent used for treatment of thyroid cancer.

Materials and methods

Chemicals, reagents and antibodies

Apigenin (product No. A3145, purity$\geq$ 97%) was purchased from Sigma Aldrich. Apigenin was dissolved in DMSO at 27 mg/ml, and stored at -20 °C until dilution before use. Cell Counting Kit-8 was purchased from Dojindo Molecular Technologies. Trypan blue was purchased from Beyotime Biotechnology. Propidium Iodide, DAPI, Hoechst 33342, DCFH-DA, 3-MA and acridine orange were purchased from Sigma. All other chemicals were of analytical reagent grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The antibodies used were as follows: Anti-LC3B and ani-Nrf2 was purchased from Sigma Aldrich. Anti-p62, anti-Beclin 1 and anti-β-actin were purchased from Santa Cruz Biotechnology. Anti-Cdc25C was purchased from Cell Signaling Technology.

Cell line and culture conditions

Papillary thyroid cancer cell line BCPAP was obtained from the German Collection of Micro-organisms and Cell Cultures (Braunschweig, Germany) and maintained in RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin in a humid atmosphere of 5% (v/v) CO_2 and 95% (v/v) air at 37 °C. BCPAP cells in log phase were plated in cell culture plates (Corning, NY, USA). Control cells were treated with the same medium without apigenin and solvent. The solvent control contains an equivalent amount of DMSO corresponding to the highest concentration of apigenin used.

CCK-8 assay

The CCK-8 kit was used to count living cells, which are based on the conversion of a water-soluble tetrazolium salt,

2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), to a water-soluble formazan dye upon reduction by dehydrogenases in the presence of an electron carrier. 1×10^4 nonadherent BCPAP cells/well were seeded in 96-well plates with 200 μl/well of complete RPMI 1640 medium and incubated overnight, and then treated with different dosages of apigenin. 24 h later, cell proliferation was determined using cell counting kit-8. Briefly, the cultured BCPAP cells were collected and seeded in 96-well plates in 200 μl/well of complete RPMI 1640 medium, and subsequently treated with 20 μl/well of CCK-8 solution, and finally incubated for another 4 h at 37 ℃. Absorption of the wells at a wavelength of 450 nm was taken using a microplate reader (uQuant model, Bio-Tek Instruments Inc., Winooski, VT).

Trypan blue dye exclusion assay

Trypan blue dye exclusion assay was performed as previously reported.[20]

Hoechst 33342/PI staining assay

The BCPAP cells were seeded in 96-well plates overnight. Different dosages of apigenin were added to the cultures. After additional 24 h incubation, the cells were stained with 10 μg/ml of Hoechst 33342 and 15 μg/ml of PI. Images were captured under a fluorescence microscope (Olympus, X51, Japan).

Western blotting

Apigenin treated cells were collected and extracted using lysis buffer [150 mM NaCl, 1% NP-40, 0.02% sodium azide, 100 μg/ml PMSF, 50 mM Tris–HCl (pH 8.0)]. Protein concentrations in cell lysates were measured using using Bradford assay. Equal amounts of protein were separated by 10% or 15% SDS-PAGE and transferred onto NC membranes. Membranes were blocked and incubated with the antibodies as

indicated. The protein bands were visualized using secondary antibody as instructed. The intensity values from the densitometry analysis of western blots were normalized against β-actin bands using the Image Lab analysis software (BIO-RAD). Intensity values were expressed as fold change compared to control.

RT-PCR

Cells were seeded in 6-well plates and then stimulated with the indicated concentrations of apigenin for 24 h. Total RNA was extracted using Tripure reagent (Roche). cDNA was synthesized from 2 μg of RNA with oligo(dT)$_{18}$ primers using M-MLV reverse transcriptase (TaKaRa) according to the manufacturer's instructions. The specific primers were as follows: *LC3* forward: 5'-AGCAGCATCCAACCAAAATC-3' and *LC3* reverse: 5'-CTGTGTCCGTTCACCAACAG-3'; *heme oxygenase-1 (HO-1)* forward: 5'-CACGCCTACACCCGCTACCT-3' and *HO-1* reverse: 5'-TCTGTCACCCTGTGCTTGAC-3'; *β-actin* forward: 5'-GCCGGGACCT GACTGACTAC-3' and *β-actin* reverse: 5'-CGGATGTCCA CGTCACACTT-3'.

Acridine orange staining assay

Autophagy is characterized by the formation of acidic vesicular organelles (AVOs). Acridine orange is a marker that fluoresces green in the whole cell, except in acidic compartments, where it fluoresces red. Development of AVOs is a typical feature of autophagy, because only mature/late autophagosomes are acidic. To detect AVOs, vital staining with acridine orange was performed. Briefly, 3×10^5 cells/well were seeded in 6-well plates and allowed to attach. The cells were treated with apigenin for 24 h and then harvested by trypsin-EDTA, then resuspended and incubated with 1 μg/ml acridine orange in the dark for 15 min on ice. The dye was removed, and the

stained cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, USA) at 488nm.

MTT assay

MTT assay was performed as previously reported.[21]

Detection of intracellular ROS production

Levels of intracellular O_2^- and H_2O_2 were assessed by using DCFH-DA as a specific probe. Briefly, cells were seeded in 6-well tissue culture plates. After 24 h treatment with or without apigenin, cells were harvested and washed twice in cold PBS, then resuspended in DCFH-DA (2 μM) for 30 min in the dark. Detection of intracellular ROS was carried using a flow cytometer at FL1 channel.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

TUNEL was used to assess the internucleosomal DNA fragmentation. Cells were fixed with 4% paraformaldehyde at room temperature and permeabilized with 0.1% Triton X-100 in PBS on ice for 2 min. The treated cells were incubated with the one step TUNEL reaction mixture in the presence of FITC-dUTP for 60 min at 37 °C. Then the coverslips were co-stained with DAPI and observed under a fluorescence microscope.

Cell cycle analysis

BCPAP cells (3×10^5/well) were seeded in 6-well plates, followed by 24 h starvation treatment (RPMI1640 without fetal bovine serums). After that, the cells were treated with different concentrations of apigenin. For cell cycle analysis, harvested cells were fixed in 70% ethanol for 15 min at 4℃. After centrifugation cells were incubated with

PI (50 µg/ml), 0.1 % Triton X-100 and RNase A (50 µg/ml) in PBS for 15 min at room temperature in the dark. Cell cycle distribution data was subsequently analyzed using the FlowJo software (Tree Star, Ashland, USA).

Statistical analysis

All experiments were performed with at least 3 independent biological replicates. For Western blots, one representative blot is shown in figures; quantifications were performed from 3 independent experiments and expressed as mean ± SD. Statistical evaluations were performed with the Student *t* test when 2 value sets were compared. *P* < 0.05 was considered to be statistically significant.

Results

Apigenin inhibits the cell growth of BCPAP cells

As shown in Fig. 1B, treatment of BCPAP cells with apigenin resulted in a dose-dependent inhibition of cell growth as compared with the untreated controls by CCK-8 assay. Similar results were obtained in trypan blue assay (Fig. 1C). The cytotoxic effect of apigenin on tumor cells was also validated intuitively by ascertaining the cell death based on Hoechst 33342/PI staining. As illustrated in Fig. 1D, the numbers of the red-emitting fluorescent cells (PI-positive) cells were increasing after apigenin exposure. All these data indicate that apigenin showed potent inhibition effect on the growth of BCPAP cells.

Apigenin induces autophagy of BCPAP cells

Autophagy is a conserved response to stress that facilitates cell survival in some contexts and promotes cell death in others. Autophagy involves the formation of double-membrane vacuoles, termed autophagosomes, containing cytosol and

organelles. Autophagosome formation involves a complex mechanism, wherein various autophagy-related (Atg) proteins participate, including Beclin 1, Atg5 and light chain 3 (LC3).[22] As shown in Fig. 2A, apigenin treatment dose-dependently induced LC3-II accumulations which demonstrated the autophagy activation. Moreover, p62 protein level was remarkable downregulated in apigenin-treated samples compared to the control group. On the contrary, protein levels of the Beclin1 showed an up-regulated tendency compared to the control. These results indicated that apigenin induced significant autophagy in BCPAP cells. We further examined LC3 mRNA expression after apigenin treatment. As shown in Fig. 2B, higher apigenin exposure especially at 50 μM increased LC3 mRNA level, indicating that apigenin caused upregulation of LC3 expression at transcriptional level. In addition, we determined the effect of apigenin on the formation of AVOs in BCPAP cells by flow cytometer after staining with acridine orange. As shown in Fig. 2C, apigenin treatment dose-dependently increased acridine orange staining of AVOs in BCPAP cells as compared with solvent controls. At 25 and 50 μM, the percent of acridine orange-stained cells was dramatically up to 10.5% and 17.9%, respectively. To further confirm apigenin-induced autophagy in BCPAP cells, we used a well-known autophagy inhibitor, 3-MA. As a specific inhibitor of class III phosphatidylinositol-3 kinase (PI3K), 3-MA inhibits autophagy at an early stage by inhibiting autophagic sequestration. As show in Fig. 2D, apigenin induced LC3-II accumulation was suppressed by autophagy inhibitor 3-MA. Moreover, the autophagy-associated p62 degradation induced by apigenin treatment was also blocked in the presence of 3-MA, which indicate apigenin induced autophagy in BCPAP cells specifically. Furthermore, MTT assay was performed to examine the role of autophagy in the cytotoxic effect of apigenin on BCPAP cells. As illustrated in Fig. 2E, the decreased cell viability

induced by apigenin treatment at 50 μM was sufficiently blocked by 3-MA, indicating that apigenin induced cell death is associated with autophagy in BCPAP cells.

Under conditions that activate autophagy, cells induce antioxidant proteins in order to adapt to adverse conditions. The p62/Keap1/Nrf2 system is currently recognized as one of the major cellular defense mechanisms which link autophagy and oxidative stress.[23] As shown in Fig. 2F, followed by autophagic degradation of p62, there was an increase in Nrf2 protein level after apigenin treatment. As predicated, a significant induction of Nrf2 target gene *HO-1* in response to apigenin treatment was observed, indicating a complex crosstalk between autophagy and oxidative stress in apigenin challenged cells.

Apigenin induces ROS production in BCPAP cells

Recently, several studies have indicated that ROS generation is key in triggering autophagy.[24] As shown in Fig. 3A, ROS production was monitored with DCFH-DA probe by flow cytometry. When BCPAP cells were exposed to different dosages of apigenin for 24 h, the DCFH-DA fluorescence shifted to a higher intensity, indicating an increase in cellular ROS production. As shown in Fig. 3B, within the concentrations ranging from 12.5, 25 to 50 μM, there was a significant increase in the ROS accumulation values from $19.8 \pm 0.95\%$ to $25.2 \pm 0.45\%$ and $16.2 \pm 2.27\%$ respectively, compared to the solvent control group ($3.47 \pm 1.13\%$). These results show that the autophagy induction by apigenin, at least partially, is due to the ROS production.

Apigenin induces DNA damage in BCPAP cells

It is well known that ROS are quite reactive and readily damage biological molecules, including DNA. Next, the damaging effects of apigenin-induced ROS on DNA have

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been studied. DNA fragmentation was calculated by counting positive TUNEL stained cells per microscopic field. Compared with the control cells, the number of TUNEL-positive cells of 50 μM of apigenin treated groups displayed a dramatically increase (Fig. 4), suggesting apigenin induced significant DNA damage in BCPAP cells.

Apigenin induces G2/M cell cycle arrest of BCPAP cells

Accumulating evidence show that DNA damage is involved in autophagy induction.[25] In response to DNA damage, cells trigger a complex array of processes which include activation of DNA damage checkpoints to arrest cell cycle progression. Next, the effects of apigenin on cell cycle distribution in BCPAP cells were determined. After treatment with apigenin for 24 h, the cells were stained with propidium iodide to analyze their DNA content. As shown in Fig. 5A, apigenin treatment caused an appreciable G2/M phase arrest accompanied by a decrease in G0/G1 populations in BCPAP cells. Compared to solvent control group which had 13.45% cells in G2/M phase, apigenin treatment caused an arrest of 24.8% of cells in the G2/M phase of cell cycle at a concentration of 12.5 μM; percentage of cells arrested in this phase further increased to 29.5% at 25 μM, 36.8% at 50 μM (Fig. 5B). These data indicate that apigenin causes cell cycle arrest at G2/M phase in BCPAP cells. Cdc25C, which dephosphorylates and activates Cdc2/cyclin B mitotic kinase complex, plays a vital role in G2-M transition.[26] Therefore, we examined the changes of Cdc25C, which regulate the cdc2-Y15 phosphorylation level. As shown in Fig. 5C, Cdc25C were substantially decreased in a dose-dependent manner after apigenin treatment, suggesting that apigenin-induced G2-M phase arrest of the cell cycle in BCPAP cells may be mediated via down-regulation of Cdc25C protein. Taken together, these

results demonstrate that apigenin caused G2/M cell cycle arrest in BCPAP cells, which was associated with apigenin-induced autophagic cell death.

Discussion

Previous studies have indicated that apigenin, a flavonoid abundantly present in fruits and vegetables, exhibit anti-proliferative properties on certain human thyroid carcinoma cell lines, especially on human anaplastic thyroid cancer cells harboring BRAF V600E.[27-29] Moreover, apigenin in combination with Akt inhibition can significantly enhance thyrotropin-stimulated radioiodide accumulation in thyroid cells.[30] These observations strongly suggest that the effect of apigenin which decreased growth and increased retention of iodide can be of therapeutic value in the radioiodide treatment of thyroid carcinoma. Recently, it has been proposed that development of novel treatments that can modulate autophagy level in cells would be of benefit for PTC therapy. The aim of present study is to investigate the anticancer effect of apigenin on human papillary thyroid carcinoma BCPAP cells. Here, we systematically studied the molecular mechanisms underlying apigenin-induced cell death, in particular the involvement of autophagy.

A large body of evidence indicates that autophagy plays an important role in determining cell fate. When subjected to miscellaneous stress, tumor cells initiate autophagy to overcome the tough period; nevertheless, excessive activation of autophagy appeared to result in cell death.[25] In our study, we first observed that apigenin at a concentration of 12.5 to 100 μM dose-dependently inhibit the cell viability of BCPAP cells (Fig. 1). To our interest, apigenin did not cause any apparent cytotoxicity in the normal thyroid follicular epithelial cell line Nthy-ori-3.1 throughout all the concentrations tested (data not shown). It is well-known that most

chemotherapeutic agents are toxic not only to tumor cells but also to normal cells, thus these agents produce major side effects. The selective cytotoxicity of apigenin on thyroid cancer cells makes it an attractive candidate for drug development.

Autophagy is characterized by the formation of double-membraned autophagosomes that fuse with lysosomes to form autolysosomes. Lysosomes and autolysosomes are forms of acidic vesicular organelles (AVOs). Among these complex steps, Beclin 1 is involved in the initial step of autophagosome formation. Lipidated microtubule-associated protein light chain 3 (LC3) would be delivered to autophagosome. P62 is an ubiquitin- and LC3-binding protein degraded by autophagy. It has been proposed that two pathways, PI3K/Akt/mTOR and MAPK, play a major role in regulating autophagy as well as in oncogenesis. The Akt/mTOR pathway negatively regulates autophagy, whereas the MAPK pathway positively regulates autophagy.[31] Targeting these two pathways results in autophagy activation and may be beneficial in thyroid cancer treatment by enhancing the uptake and sensitivity to [131]I.[11] As mentioned above, apigenin showed potential growth inhibitory effects in human thyroid cancer cells. However, to date, few studies have evaluated the effects of apigenin on the autophagic induction. In present study, we found that apigenin effectively inhibited thyroid cancer cell growth by inducing autophagy, as reflected by the changes in the expression or degradation of autophagy-related proteins (Beclin-1, LC3 and p62), as well as the formation of acidic vesicular organelles (AVOs) by flow cytometry (Fig. 2A and C). Importantly, both apigenin-induced conversion of LC3-II and cell viability loss were abrogated by autophagy inhibitor 3-MA (Fig. 2D and E), indicating that apigenin is associated with autophagic cell death in BCPAP cells.

Recently, autophagy and oxidative stress have been shown to be interconnected.[23] The autophagy-adaptor protein p62 is assembled on autophagic

cargos and subsequently increases its affinity for Keap1 when phosphorylated at Ser351.[32] This event induces Keap1 sequestering and leaves Nrf2 free to translocate in the nucleus. Nrf2, which is no longer degraded by the proteasome system, binds to the antioxidant-responsive elements (AREs) located in the promoter of antioxidant genes such as *HO-1* (*heme oxygenase 1*), and activates their transcription. Interestingly, Johansen's group further reported that p62 is a target gene for Nrf2, implying a positive feedback loop.[33] In the present study, occurring through p62/Keap1/Nrf2 pathway, Nrf2 was concomitantly activated after apigenin treatment (Fig. 2F), confirming the interconnection between autophagy and oxidative stress. More detailed mechanisms need to be further investigated.

Autophagy can be induced and up-regulated in response to intracellular reactive oxygen species (ROS) or extracellular oxidative stress.[34] As an effective cleaner, autophagy plays a remarkable role in removing oxidative damaged organelles, so it serves as one of the first lines of defense against oxidative stress damage. Next, we investigated whether apigenin induced autophagic death is mediated by ROS generation. Indeed, apigenin treatment caused significant intracellular ROS accumulation (Fig. 3). ROS can cause severe damage to cellular molecules, such as DNA, RNA and proteins.[35] Consistent with this notion, apigenin-induced DNA damage was also detected by TUNEL assay (Fig. 4).

In response to DNA damage, cell cycle checkpoints are engaged in preventing cells with damaged DNA from either replicating (G1-S checkpoint) or dividing (G2-M checkpoint).[36] The G2-M checkpoint is identified with a series of phosphorylation/dephosphorylation events involving cyclin B: Cdc2, Cdc25, Wee1 and Myt1, which had inherent instability.[37] The Cdc25 phosphatase family comprises three members named A, B and C. During the G2-M transition, Cdc2 is rapidly

converted into the active form by Tyr15 dephosphorylation catalyzed by the Cdc25C and drives cells into mitosis.[37] Therefore, Cdc25C activation is one of the key steps for M-phase entry. Accumulating evidence indicates many important factors, such as p53, PARP-1 and FoxO3A, which participate in DNA repair, may also play an important role in the regulation of autophagy.[38] Upon DNA damage, activation of the FoxO3A transcriptional program upregulated the expression of several autophagy-related genes, including *LC3, Gabarapl1, ATG12, BNIP3 and BNIP3L*.[39]

Previous studies have shown that apigenin caused different phase cell cycle arrest in different types of cancer cells. For instance, Maggioni, D., et al. reported that apigenin treatment induced cell cycle arrest at both G0/G1 and G2/M phase in an oral squamous cell carcinoma cell line by down-regulation of cyclin D1 and E and inactivation of CDK1.[40] In another study, apigenin induced G2/M Phase cell cycle arrest and apoptosis in T24 human bladder cancer cells.[41] In our present study, we showed that apigenin induced G2/M phase arrest which was mediated by down-regulation of Cdc25C protein in BCPAP cells (Fig. 5).

Conclusions

In summary, our data showed that apigenin treatment induced ROS generation, which resulted in DNA damage and a subsequent cell cycle arrest at G2/M phase. Evident autophagy was induced and eventually triggered human papillary thyroid cancer cells death. Based on these observations, we proposed that apigenin may act as a chemotherapeutic agent and have therapeutic implications in the treatment of thyroid carcinoma. Whether apigenin treatment by targeting autophagy pathway will lead to enhance the uptake and sensitivity to ^{131}I in thyroid cancer therapy requires further investigation.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Acknowledgements

References

1   G. L. Hostetler, K. M. Riedl and S. J. Schwartz, *Food Chem.*, 2013, **141**, 1406-1411.

2   A. Crozier, I. B. Jaganath and M. N. Clifford, *Nat. Prod. Rep.*, 2009, **26**, 1001-1043.

3   S. Shukla and S. Gupta, *Pharm. Res.*, 2010, **27**, 962-978.

4   P. Batra and A. K. Sharma, *3 Biotech*, 2013, **3**, 439-459.

5   H. Bai, H. Jin, F. Yang, H. Zhu and J. Cai, *Scanning*, 2014, **36**, 622-631.

6   F. Lindenmeyer, H. Li, S. Menashi, C. Soria and H. Lu, *Nutr. Cancer*, 2001, **39**, 139-147.

7   H. I. Swanson, E. Y. Choi, W. B. Helton, C. G. Gairola and J. Valentino, *Oral Surg Oral Med Oral Pathol Oral Radiol*, 2014, **117**, 214-220.

8   Y. E. Nikiforov and M. N. Nikiforova, *Nat. Rev. Endocrinol.*, 2011, **7**, 569-580.

9   M. Xing, B. R. Haugen and M. Schlumberger, *The Lancet*, 2013, **381**, 1058-1069.

10 D. Handkiewicz-Junak, A. Czarniecka and B. Jarzab, *Molecular and cellular*

endocrinology, 2010, **322**, 8-28.

11   H. Yi, B. Long, X. Ye, L. Zhang, X. Liu and C. Zhang, *Mol Clin Oncol*, 2014, **2**, 661-665.

12   B. Levine, *Cell*, 2005, **120**, 159-162.

13   G. Jia and J. R. Sowers, *Biochim. Biophys. Acta*, 2015, **1852**, 219-224.

14   C. I. Lin, E. E. Whang, M. A. Abramson, X. Jiang, B. D. Price, D. B. Donner, F. D. Moore, Jr. and D. T. Ruan, *Surgery*, 2009, **146**, 1208-1214.

15 C. I. Lin, E. E. Whang, D. B. Donner, J. Du, J. Lorch, F. He, X. Jiang, B. D. Price, F. D. Moore, Jr. and D. T. Ruan, *Mol Cancer Res*, 2010, **8**, 1217-1226.

16   S. M. Jin, H. W. Jang, S. Y. Sohn, N. K. Kim, J. Y. Joung, Y. Y. Cho, S. W. Kim and J. H. Chung, *Endocrine*, 2014, **45**, 256-262.

17   M. Li-Weber, *Cancer Lett.*, 2013, **332**, 304-312.

18   Y. Lee, B. Sung, Y. J. Kang, D. H. Kim, J. Y. Jang, S. Y. Hwang, M. Kim, H. S. Lim, J. H. Yoon, H. Y. Chung and N. D. Kim, *Int. J. Oncol.*, 2014, **44**, 1599-1606.

19   X. Cao, B. Liu, W. Cao, W. Zhang, F. Zhang, H. Zhao, R. Meng, L. Zhang, R. Niu, X. Hao and B. Zhang, *Chin. J. Cancer Res.*, 2013, **25**, 212-222.

20   L. Zhang, H. Yu, X. Zhao, X. Lin, C. Tan, G. Cao and Z. Wang, *Neurochem. Int.*, 2010, **57**, 547-555.

21   L. Zhang, H. Yu, Y. Sun, X. Lin, B. Chen, C. Tan, G. Cao and Z. Wang, *Eur. J. Pharmacol.*, 2007, **564**, 18-25.

22   Y. Kabeya, N. Mizushima, T. Ueno, A. Yamamoto, E. Kirisako, Y. Ohsumi and T. Yoshimori, *The EMBO J*, 2000, **19**, 5720-5728.

23   G. Filomeni, D. De Zio and F. Cecconi, *Cell Death Differ.*, 2015, **22**, 377-388.

24   Y. Chen and S. B. Gibson, *Autophagy*, 2008, **4**, 246-248.

25   H. Rodriguez-Rocha, A. Garcia-Garcia, M. I. Panayiotidis and R. Franco, *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 2011, **711**, 158-166.

26   I. Nilsson and I. Hoffmann, *Prog. Cell Cycle Res.*, 2000, **4**, 107-114.

27   F. Yin, A. E. Giuliano and A. J. Van Herle, *Thyroid*, 1999, **9**, 369-376.

28   F. Yin, A. E. Giuliano and A. J. Van Herle, *Anticancer Res.*, 1999, **19**, 4297-4303.

29   S. H. Kim, J. G. Kang, C. S. Kim, S. H. Ihm, M. G. Choi, H. J. Yoo and S. J. Lee, *J. Endocrinol. Invest.*, 2013, **36**, 1099-1104.

30   A. Lakshmanan, A. I. Doseff, M. D. Ringel, M. Saji, B. Rousset, X. Zhang and S. M. Jhiang, *Thyroid*, 2014, **24**, 878-887.

31   P. Codogno and A. J. Meijer, *Cell Death Differ.*, 2005, **12 Suppl 2**, 1509-1518.

32   Y. Ichimura, S. Waguri, Y. S. Sou, S. Kageyama, J. Hasegawa, R. Ishimura, T. Saito, Y. Yang, T. Kouno, T. Fukutomi, T. Hoshii, A. Hirao, K. Takagi, T. Mizushima, H. Motohashi, M. S. Lee, T. Yoshimori, K. Tanaka, M. Yamamoto and M. Komatsu, *Mol. Cell*, 2013, **51**, 618-631.

33   A. Jain, T. Lamark, E. Sjottem, K. B. Larsen, J. A. Awuh, A. Overvatn, M. McMahon, J. D. Hayes and T. Johansen, *J. Biol. Chem.*, 2010, **285**, 22576-22591.

34   R. Scherz-Shouval, E. Shvets, E. Fass, H. Shorer, L. Gil and Z. Elazar, *EMBO J.*, 2007, **26**, 1749-1760.

35   I. Tedesco, M. Russo, S. Bilotto, C. Spagnuolo, A. Scognamiglio, R. Palumbo, A. Nappo, G. Iacomino, L. Moio and G. L. Russo, *Food Chem. Toxicol.*, 2013, **60**, 377-384.

36   X. Xu, Z. Weaver, S. P. Linke, C. Li, J. Gotay, X. W. Wang, C. C. Harris, T. Ried and C. X. Deng, *Mol. Cell*, 1999, **3**, 389-395.

37   R. G. Clyde, J. L. Bown, T. R. Hupp, N. Zhelev and J. W. Crawford, *J R Soc Interface*, 2006, **3**, 617-627.

38   P. Czarny, E. Pawlowska, J. Bialkowska-Warzecha, K. Kaarniranta and J. Blasiak, *Int. J. Mol. Sci.*, 2015, **16**, 2641-2662.

39   F. Chiacchiera and C. Simone, *Autophagy*, 2009, **5**, 1030-1033.

40 D. Maggioni, W. Garavello, R. Rigolio, L. Pignataro, R. Gaini and G. Nicolini, *Int. J. Oncol.*, 2013, **43**, 1675-1682.

41   Y. Zhu, Y. Mao, H. Chen, Y. Lin, Z. Hu, J. Wu, X. Xu, X. Xu, J. Qin and L. Xie, *Cancer Cell Int*, 2013, **13**, 54.

Figure Legends

Fig 1. Apigenin inhibits the growth of human papillary thyroid cancer cells. (A) Chemical structure of apigenin. (B and C) Effects of apigenin on the cell viability of BCPAP cells. BCPAP cells were treated with different doses (12.5 to 100 μM) of apigenin, and then cell viability was measured by CCK-8 (B) or trypan blue exclusion assay (C). All data were expressed as mean ± S.D. of three experiments and each included triplicate sets. *P < 0.05 and **P < 0.01 *vs.* SC group. (D) Effects of apigenin on BCPAP cell death rate. BCPAP cells were cultured in the absence or presence of apigenin (12.5 to 50 μM) for 24 h. After incubation, cells were stained with PI (10 μg/ml) and Hoechst 33342 (10 μg/ml) and then observed under a fluorescence microscope. SC, solvent control. Scale bar, 20 μm.

Fig 2. Apigenin induces autophagy in BCPAP cells. (A) Effects of apigenin on the levels of autophagy-related proteins. BCPAP cells were incubated with different dosages of apigenin for 24 h and total protein extracts were prepared. Representative immunoblots showing the conversion of non-lipidated LC3 (LC3-I) to its lipidated variant (LC3-II) as well as Beclin-1 and p62 protein are shown. β-actin was used as an internal loading control. (B) Effects of apigenin on *LC3* mRNA expression. BCPAP cells were exposed to different dosages of apigenin for 24 h. Total RNAs were isolated and subjected to RT-PCR. *LC3* mRNA expression was determined and normalized to that of *β-actin*. (C) Acridine orange staining of BCPAP cells treated with different concentrations of apigenin to detect the formation of acidic vesicular organelles (AVOs) in autophagic cells. (D) BCPAP cells were incubated with apigenin (50 μM) in the presence or absence of 3-MA (1 mM) for 24 h. The levels of LC3I/II were determined by Western blot. (E) BCPAP cells were incubated with apigenin (50

µM) in the presence or absence of 3-MA (1 mM) for 24 h and the cell viability was measured by MTT assay. *P* < 0.05, **P* < 0.01. (F) The effects of apigenin on Nrf2 protein level and its target gene expression. BCPAP cells were exposed to different dosages of apigenin for 24 h. Total RNA was prepared for RT-PCR analysis of *HO-1*. The *β-actin* band is shown as a loading control.

Fig 3. Apigenin induces intracellular ROS accumulation in BCPAP cells. (A) Intracellular ROS levels were determined using flow cytometry with DCFH-DA fluorescent dye. Data were representative of three independent experiments. (B) Quantitative analysis of DCF fluorescence intensity as an indication of ROS levels in apigenin-treated BCPAP cells. The data shown represent the means ± S.D. of three independent experiments. SC, solvent control. **P* < 0.01 *vs.* SC group.

Fig 4. Apigenin induces DNA damage in BCPAP cells. Representative images of TUNEL staining of BCPAP cells after treatment with the 50 µM of Apigenin for 24 h. White arrow indicates TUNEL-positive nuclei.

Fig 5. Effect of apigenin treatment on cell cycle progression of BCPAP cells. (A and B) Effects of apigenin on BCPAP cell cycle. Synchronized BCPAP cells were treated with or without different concentrations of apigenin for 24 h. Cells were stained with PI solution followed by analyses of cell cycle distribution using flow cytometry as in (A). The data was plotted in the bar chart as shown in (B). (C) Expression of cell cycle-related protein, Cdc25C, after apigenin treatment in BCPAP cells. Cells were treated with apigenin and western blotting was performed to detect Cdc25C. Bottom graph shows relative Cdc25C levels that were normalized to β-actin (n = 3). **P* < 0.01 *vs.* SC group.
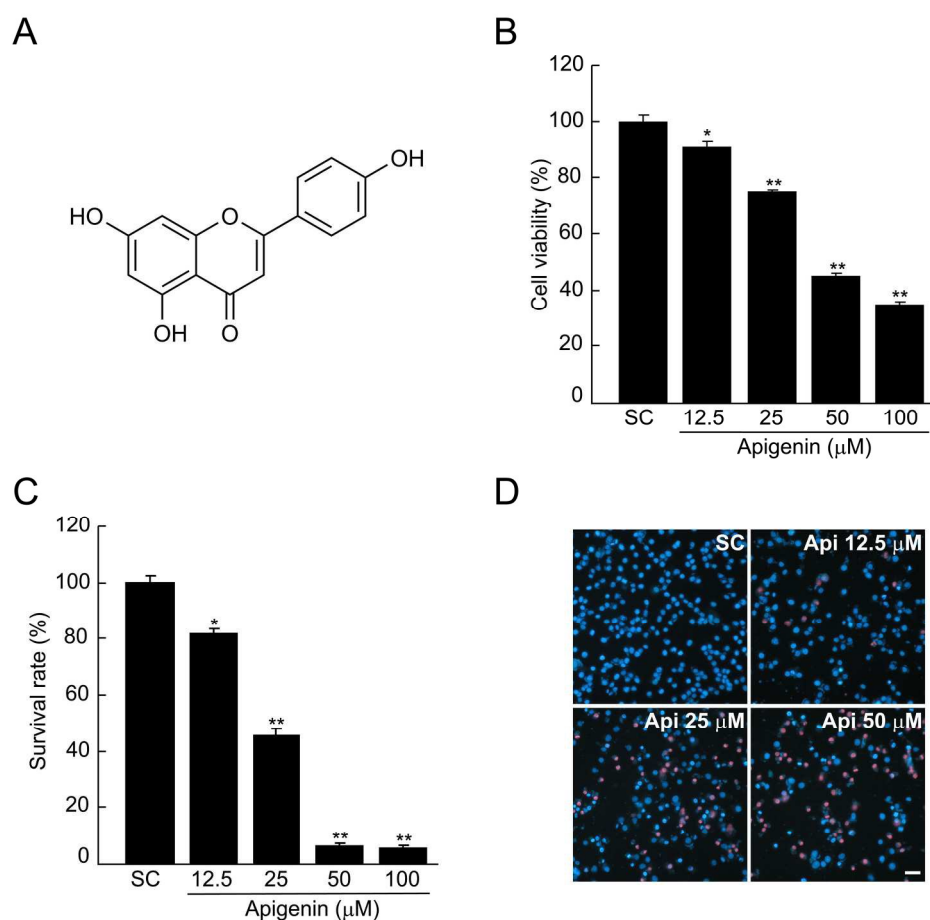
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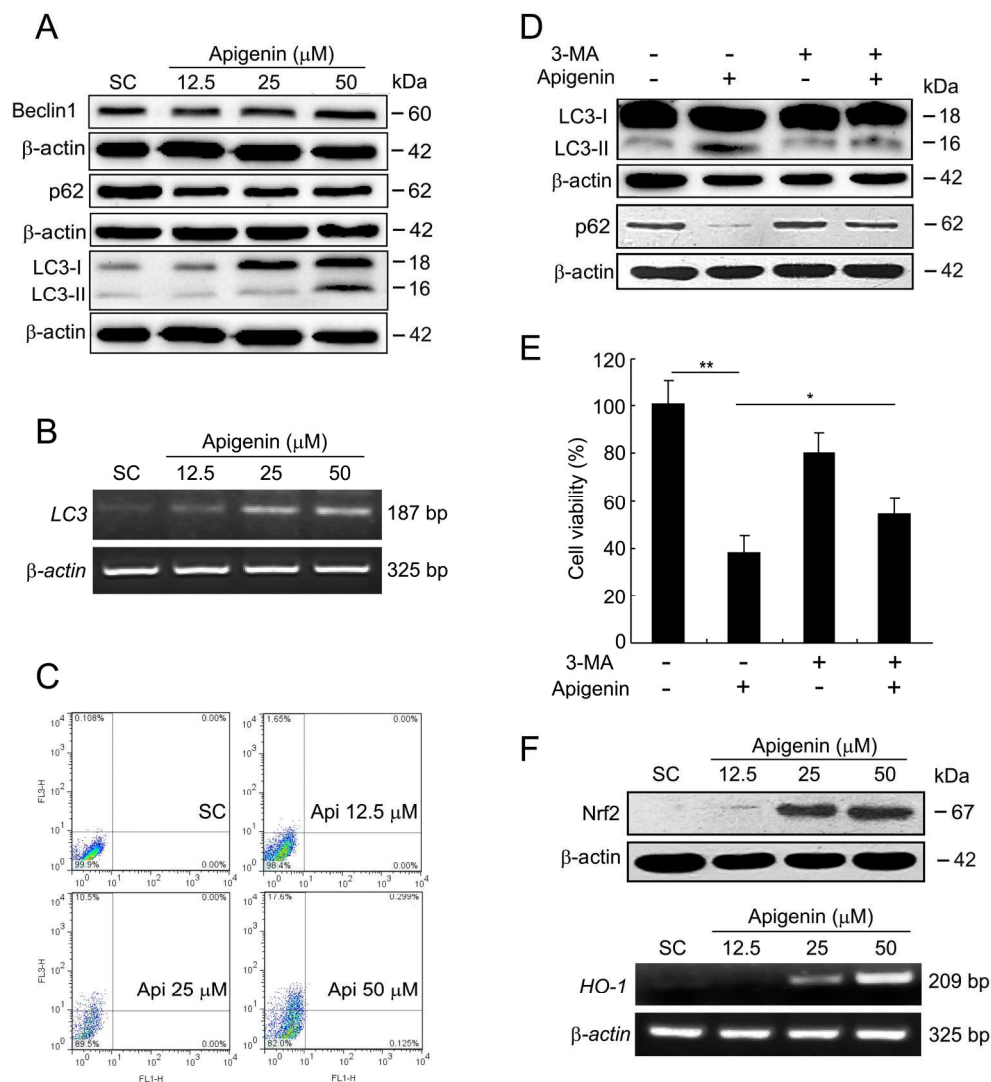198x186mm (300 x 300 DPI)

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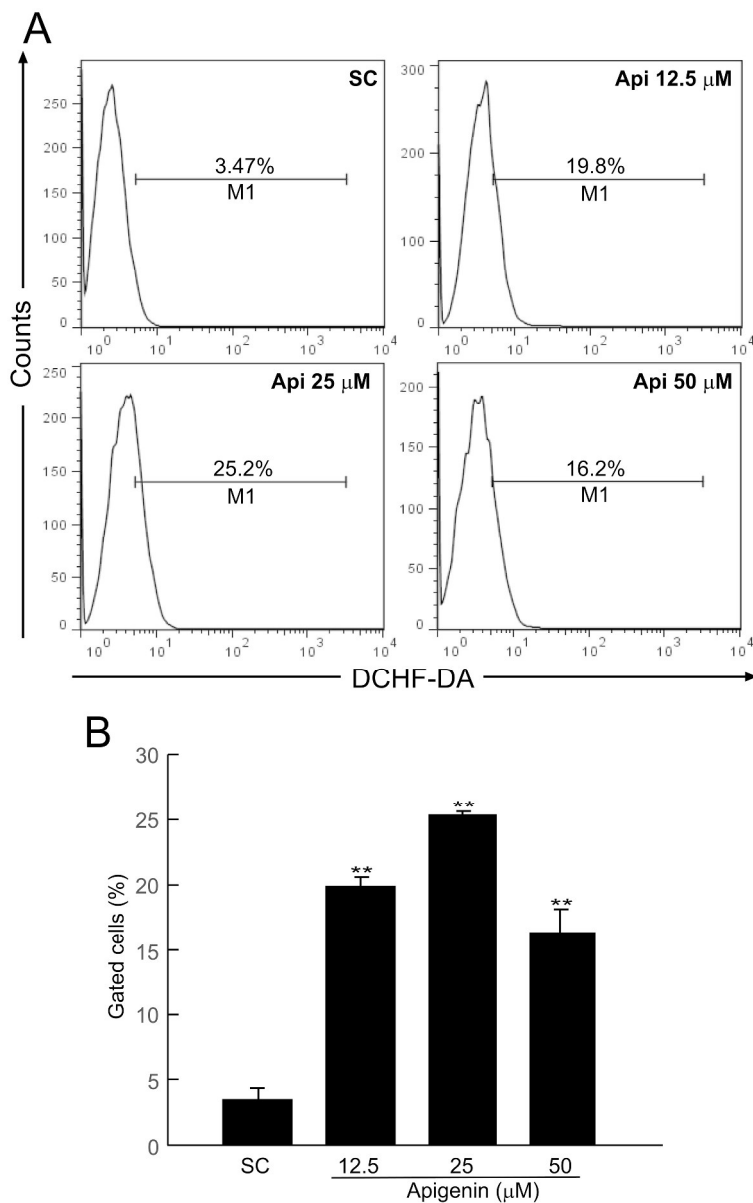185x201mm (300 x 300 DPI)

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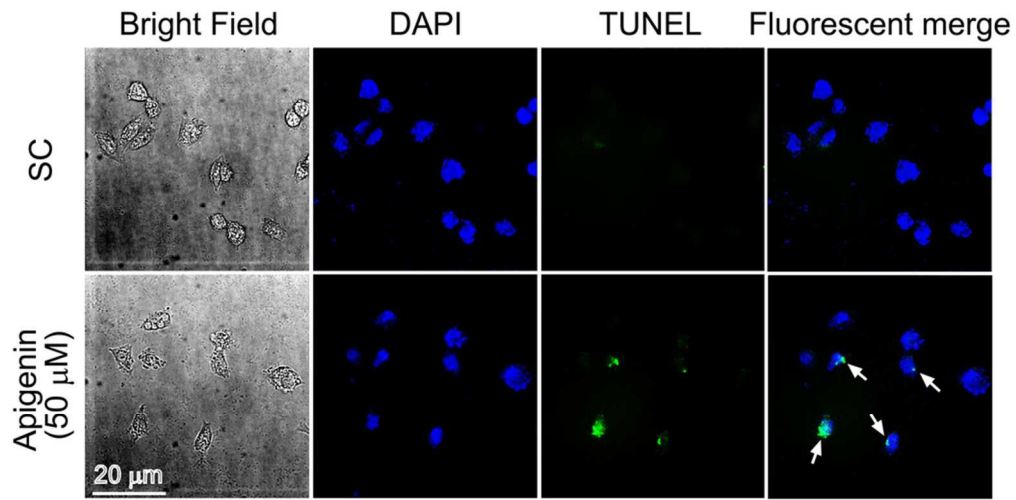127x203mm (600 x 600 DPI)

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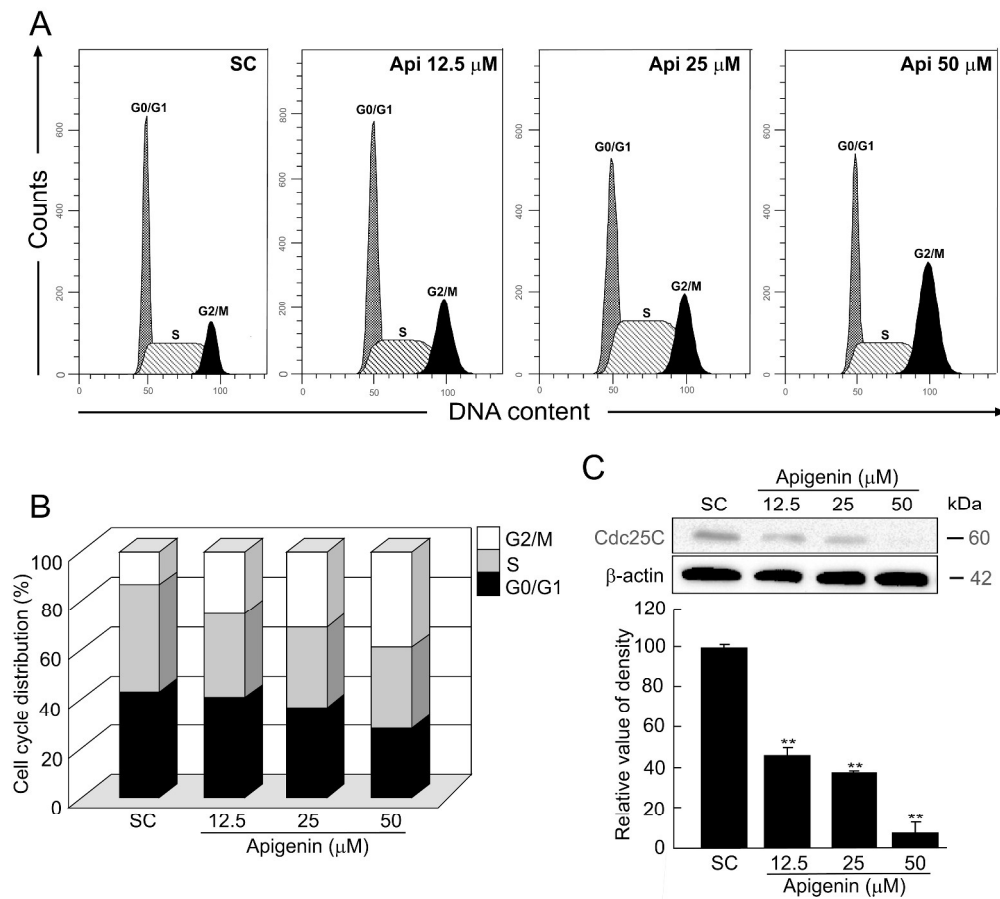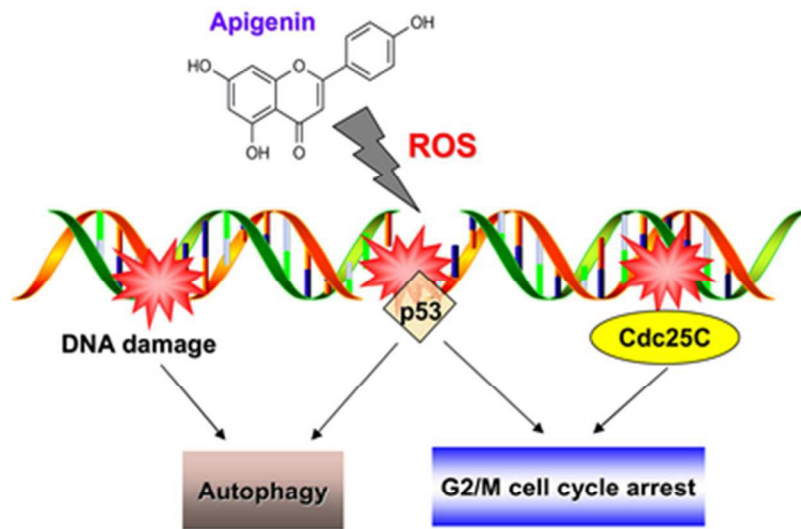88x45mm (300 x 300 DPI)

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Apigenin-induced autophagic cell death in human papillary thyroid carcinoma BCPAP cells is associated with ROS generation, DNA damage and cell cycle arrest.
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