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Molecular targets of apigenin in colorectal cancer cells: Involvement of p21, NAG-1 and p53 ☆

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ABSTRACT

Persuasive epidemiological and experimental evidence suggests that dietary flavonoids have anti-cancer activity. Since conventional therapeutic and surgical approaches have not been able to fully control the incidence and outcome of most cancer types, including colorectal neoplasia, there is an urgent need to develop alternative approaches for the management of cancer. We sought to develop the best flavonoids for the inhibition of cell growth, and apigenin (flavone) proved the most promising compound in colorectal cancer cell growth arrest. Subsequently, we found that pro-apoptotic proteins (NAG-1 and p53) and cell cycle inhibitor (p21) were induced in the presence of apigenin, and kinase pathways, including PKCδ and ataxia telangiectasia mutated (ATM), play an important role in activating these proteins. The data generated by *in vitro* experiments were confirmed in an animal study using APC^{MIN+} mice. Apigenin is able to reduce polyp numbers, accompanied by increasing p53 activation through phosphorylation in animal models. Our data suggest apparent beneficial effects of apigenin on colon cancer.

1. Introduction

In the United States, colorectal cancer (CRC) is the third most common cancer diagnosed in men and women.[1] Since conventional therapeutic and surgical approaches have not been able to fully control the incidence and outcome of most cancer types, including colorectal neoplasia, there is an urgent need to develop mechanism-based approaches for the management of cancer prevention. Many laboratories, including ours, have reported persuasive evidence of beneficial effects of plant-derived compounds in cancers of the gastrointestinal tract, lung, skin, prostate, and breast. Among these plant

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compounds, flavonoids found ubiquitously in vegetables, berries, and fruits, show anti-tumorigenic activity. Of flavonoids, apigenin has been paid much attention since apigenin is cancer suppressive by modulation of diverse kinase pathways including phosphatidylinositol 3-kinase (PI3K), protein kinase B/Akt, MAPK/ERK, casein kinase II (CKII), and other upstream kinases in the development and progression of cancer.[2,3] Apigenin arrests cell cycle at the G2/M phase accompanied by a decrease of cyclin B1 in human colorectal cancer cells.[4] Recently, animal models were used to examine if apigenin has anti-tumorigenic activity in colorectal cancer *in vivo*. Apigenin failed to inhibit adenoma formation in the APC^{MIN+} (MIN, multiple intestinal neoplasia) mice models, but it showed reduced aberrant crypt foci (ACF) formation in azoxymethane (AOM)-induced animal models.[5] In spite of the demonstrated cancer suppressing activity of apigenin in an AOM model, the molecular mechanisms to induce apoptosis and/or cell cycle arrest have not been extensively studied at the cellular level.

Many proteins related to signalling pathways play a pivotal role in colorectal tumourigenesis such as cyclin-dependent kinase inhibitor (CDKI), transforming growth factor-β (TGF-β), cyclooxygenase-2 (COX-2), β-catenin, p53, adenomatous polyposis coli (APC) and mismatch repair. Among them, the CDKI $p21^{WAF1}$ and p53 tumour suppressor are well known to function as tumour suppressors to inhibit tumour growth and induce apoptosis, respectively. The p53 tumour suppressor is a critical component of cellular mechanisms that respond to certain stresses to preserve genomic integrity by arresting cell-cycle progression or by inducing apoptosis.[6] The p53 function can potentially be affected by posttranslational modification via phosphorylation, dephosphorylation, and even acetylation, sumoylation and glycosylation. Concerning phosphorylation, the complexity of p53 biology may stem from the large number of kinases because each protein kinase phosphorylates p53 at a different site and leads to a distinct function. For example, phosphorylation of Ser-15 and Ser-37 stabilises p53 in response to DNA damage at the amino terminus of p53, which prevents Mdm2 binding[7–9]; whereas phosphorylation of Ser-392 has been linked to oncogenic properties in tumourigenesis.[10] It has been known that apigenin increases p53 expression via enhancing protein stability, thereby up-regulating p21 expression,[11] although apigenin treatment-enhanced, UVB-induced apoptosis is independent of p53 expression.[12] Thus, it is clear that apigenin affects apoptosis both in a p53-dependent and -independent manner. Since mutations commonly occur at the p53 tumour suppressor gene locus in many forms of cancer, apigenin may be a good cancer chemopreventive compound that has p53-dependent and -independent mechanisms.

Non-steroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) is a TGF-β superfamily protein involving apoptosis and anti-tumorigenesis in colorectal cancer. NAG-1 expression is positively associated with induction of apoptosis,[13] and NAG-1 transgenic mice showed much less sensitivity to carcinogens or genetic toxicity.[14]

Here, we report that apigenin enhanced apoptosis and cell growth arrest of human colon cancer cells through several kinase pathways including PKCδ and ataxia telangiectasia mutated (ATM), affecting NAG-1, p53 and p21, and the *in vivo* study was performed to support our observation from *in vitro* data.

2. Materials and methods

2.1. Materials

Human colorectal cancer cells HCT-116, SW480, HT-29 and LoVo were purchased from American Type Culture Collection (Manassas, VA). HCT-116 p53–/– cells were previously reported.[15] Actin antibody was purchased from Santa Cruz (Santa Cruz, CA). Cyclin D1 and phospho-p53 antibodies were purchased from Cell Signaling (Beverly, MA), and hemagglutinin (HA) and p21 were purchased from Covance (Berkeley, CA). NAG-1 antibody was previously described.[13] All the primary antibodies used are diluted with 5% w/v skim milk at 1:1000. Apigenin was purchased from Wako (Osaka, Japan). RO-31-8220, KU55933, Rottlerin, AG490, LY294002, SP600125, 4,5,6,7-tetrabromobenzotriazol (TBB) and SB203580 were purchased from Calbiochem (SanDiego, CA), and quercetin, phloridzin, naringenin, kaempferol, genistein, luteolin, pelargonidin, malonyl diadzin, malonyl glycitin, glycitin, daidzein, daidzin, glycitein, genistin were purchased from either MP Biomedicals or Acros organics. Protein kinase C (PKC) wild-type and dominant negative vector were provided by Dr. Jaewon Soh (Inha University, Incheon, South Korea). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

2.2. Cell culture and treatment

HCT-116 cells and HT-29 cells were maintained in McCoy's 5A medium. SW480 cells and LoVo cells were maintained in RPMI1640 and Ham's F-12, respectively. All media were supplemented with 10% v/v fetal bovine serum (FBS) and 10 μg/mL of gentamicin. Cells were grown at 37 °C under a humidified atmosphere of 5% CO_2. The cells were plated in a 12-well or 60-mm culture dish and incubated until cells were 70–80% confluent. The cells were then treated with different concentrations of apigenin at different time points as indicated in the figure legends.

2.3. Cell proliferation and apoptosis assay

Cell proliferation assay was performed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Briefly, cells were seeded at a concentration of 1000 cells/well in 96-well tissue culture plates in three replicates and maintained overnight. The cells were then treated with either DMSO or indicated compounds for 2 and/or 4 d. Twenty microliters of Cell Titer 96 Aqueous One Solution was added to each well, and the plate was incubated for 1 h at 37 °C. Absorbance at 490 nm was recorded in an ELISA plate reader (Bio-Tek Instruments Inc., Winooski, VT). For the apoptosis assay, the Annexin V-FITC apoptosis detection kit (BD Biosciences) was used. Briefly, cells were plated in 6-well tissue culture dishes and incubated with DMSO, 1 μM or 10 μM of apigenin for 72 h. The attached and floating cells were harvested together, washed with PBS, and stained with Annexin V-FITC; and early and late apoptosis was quantified according to the manufacturer's instructions. Apoptosis was analyzed using Beckman Coulter Epixs XL flow cytometer equipped with EXPO32 ADC software.

2.4. Luciferase assay

Transient transfections were performed using Lipofectamine (Invitrogen) according to the manufacturer's instruction. The cells were plated in 12-well plates at the concentration of 2×10^5 cells/well. After growth overnight, the cells were transfected with plasmid containing reporter gene (pMDM2-LUC). Then, the cells were harvested in 1× luciferase lysis buffer, and luciferase activity was determined and normalized to the pRL-null luciferase activity using a dual luciferase assay kit (Promega).

2.5. Western blotting

Cells were washed with PBS, and cell lysates were isolated in RIPA buffer (50 mM Tris–HCl, pH 7.4,150 mM NaCl, 1 mM EDTA, 1% v/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v SDS) supplemented with protease inhibitors (1 mM PMSF, 1 mg/mL aprotinin,1 mg/mL leupeptin) and phosphatase inhibitors (10 mM NaF, 0.1 mM Na_3VO_4) and centrifuged at 12,000 rpm for 5 min at 4 °C. Protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL), using bovine serum albumin as the standard. The proteins were separated on SDS–PAGE and transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN). The membranes were incubated with a specific primary antiserum in TBS containing 0.05% v/v Tween 20 (TBS-T) and 5% w/v non-fat dry milk at 4 °C overnight. After four washes with TBS-T, the blots were incubated with peroxidase-conjugated IgG for 1 h at room temperature and visualised using ECL (Amersham Biosciences, Piscataway, NJ).

2.6. Reverse transcription-polymerase chain reaction

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA), and 5 μg of total RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). One microliter of cDNA was added to a 25 μl PCR reaction mixture with human p53 gene-specific primers: F, 5′-tgcgtgtggagtatttggatg-3′ and R, 5′-tggtacagtcagagccaaccag-3′; human p21 gene-specific primers: F, 5′-agcagaggaagaccatgtg-gac-3′ and R, 5′-tttcgaccctgagagtctccag-3′; human NAG-1 gene-specific primers: F, 5′-ctccagattccgagagttgc-3′ and R, 5′-agagatacgcaggtgcaggt-3′; and human *GAPDH* gene-specific primers: F, 5′-tcaacggatttggtcgtatt-3′ and R, 5′-ctgtggtcat-gagtccttcc-3′. The thermal cycling conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 30 and 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and final extension at 72 °C for 10 min. The products were electrophoresed on 2% agarose gel and photographed under UV light.

2.7. Animal study

The $APC^{Min/+}$ mice were purchased from Jackson Laboratory (JAX Mice, Bar Harbor, ME) and bred in house. Mice were randomly divided into 3 groups of 17 mice, each to receive vehicle (0.5% w/v methylcellulose), apigenin 25 mg/kg, or apigenin 50 mg/kg by gavage. $APC^{Min/+}$ mice were treated 14 times every other day for 1 month starting at 10 weeks of age. Twenty-four hours after final treatment, the mice were sacrificed, and the intestinal tract was isolated and washed with phosphate-buffered saline (PBS). Tumor numbers and sizes in the small intestine were assessed with a stereoscopic microscope as previously described.[14] All animal research procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines.

2.8. Liquid chromatography

Apigenin standard was purchased from Fisher Scientific (Atlanta, GA, USA). Apigenin standard was made into a stock solution at a concentration of 1 mg/ml in Milli-Q water for further dilutions. Separation of apigenin from mouse serum was completed with an Agilent Technologies 1200 series HPLC equipped with an Agilent Technologies 1200 series diode array detector (DAD) (Agilent Technologies, Santa Clara, CA, USA). The HPLC was outfitted with an Agilent Zorbax XBD C18 column (4.6 mm × 50 mm, 1.8 μm) and in-line filter of 2 μm. The mobile phase consisted of (A) 0.1% v/v glacial acetic acid in Milli-Q water and (B) 0.1% v/v glacial acetic acid in acetonitrile. Gradient elution conditions consisted of 12% v/v B increasing to 90% v/v in 22 min and back to 12% v/v B in 25 min. The flow rate was 0.8 ml/min and the injection volume was 5 μl of mouse serum. The concentration of apigenin was calculated from a standard curve using three replicates of each concentration ranging from 0.1 to 10 ng/μl. The average R^2 value of 0.99 was determined at each concentration.

3. Results

3.1. Effect of apigenin on cell growth and apoptosis

We used various kinds of flavonoids such as phloridzin, quercetin, kaempferol, naringenin, apigenin, genistein, EGCG, luteolin, and pelargonidin, to examine the most effective compound on the inhibition of colon cancer cell growth. As shown in Fig. 1A, treatment of HCT-116 cells with apigenin, genistein, and luteolin resulted in a significant reduction of cell growth at 10 μM. These compounds are in the family of flavones or isoflavones; therefore, we also examined other flavones and isoflavones including glycitin, daidzein, daidzin, glycitein, genistein, genistin, and apigenin. The result showed that apigenin was the most effective compound in cell growth arrest, compared to other flavones (Fig. 1B). Based on our data, we selected apigenin for further experiments. Apoptosis analysis using annexin V showed that treatment of apigenin increased apoptosis (Fig. 1C), compared to vehicle-treated cells, indicating that apigenin increases cell growth arrest as well as apoptosis in HCT-116 cells.

3.2. Apigenin induces proteins that play a role in apoptosis and cell cycle

To investigate the effects of apigenin on the expression of proteins that are important on cell cycle (cyclin D1 and p21) and apoptosis (NAG-1 and p53), HCT-116 cells were treated with apigenin in a different dose and time, as indicated (Fig. 2A and B). Cell cycle inhibitor p21 and pro-apoptotic

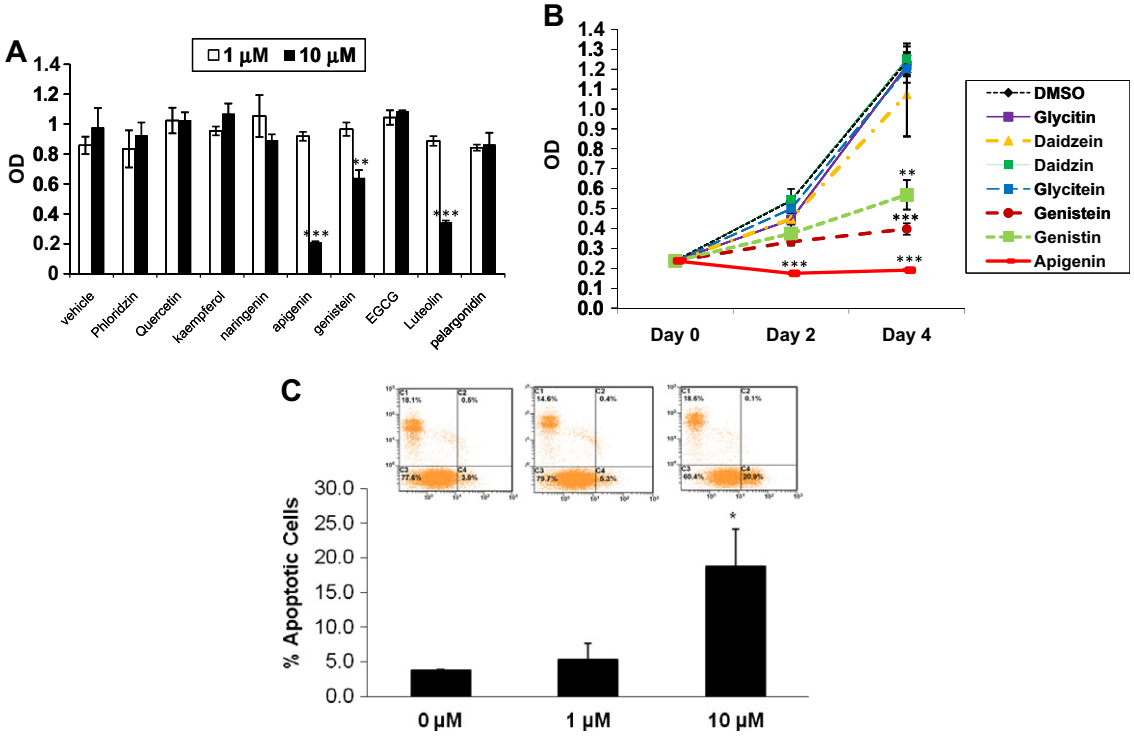

Fig. 1 – Effect of apigenin and other flavonoids on colon cancer cell growth and apoptosis. (A) HCT-116 cells were treated with DMSO or various compounds at concentrations of 1 μM and 10 μM for 4 d. Cell growth was measured using the Cell Titer96® Aqueous one solution cell proliferation assay as described in Section 2. Values are expressed as means ± SD of five replicates. **$p < 0.01$, ***$p < 0.001$ versus DMSO-treated cells at day 4. (B) HCT-116 cells were treated with DMSO or various flavones and isoflavones at 10 μM concentration for 0, 2, and 4 d. Cell growth was measured using the Cell Titer96 Aqueous one solution cell proliferation assay. **$p < 0.01$, ***$p < 0.001$ versus DMSO-treated cells at day 4. (C) HCT-116 cells were plated at 5×10^5 cells/well in six-well plates, incubated with 1 μM and 10 μM of apigenin for 72 h and analyzed for apoptosis as described in Materials and Methods. Values are expressed as means ± SD of triplicates.*$p < 0.05$ versus DMSO-treated cells.

proteins p53 and NAG-1 were induced in the presence of 10 μM apigenin in a time- and dose-dependent manner; however, cyclin D1 expression was not altered. We also performed reverse-transcription polymerase chain reaction (RT-PCR) and found that there were no changes in the expression of p21 and p53 mRNA; however, NAG-1 was induced at the transcription level in the presence of apigenin (Fig. 2C). These results indicate that apigenin affects p53 and p21 at the translational level, whereas apigenin affects NAG-1 expression at the transcriptional level. Other colorectal cancer cells, including LoVo, SW480, and HT-29 were also tested with vehicle, 1 μM or 10 μM of apigenin for 24 h. As shown in Fig. 2D, p21 was induced by apigenin in LoVo cells, whereas NAG-1 was induced in both p53 wild-type cells (LoVo) and mutant cells (SW480 and HT-29). This result indicates that p21 expression by apigenin may be affected by p53 status, whereas NAG-1 expression is independent of p53 status.

3.3. *Apigenin effects on p53 phosphorylation*

We next investigated the phosphorylation of p53 in the presence of apigenin. The p53 phosphorylation at Ser-6, Ser-9, Ser-15, Ser-37, Ser-20, Ser-392, and Ser-46 was examined after apigenin treatment. The results indicated that p53 phosphorylation at Ser-15 and Ser-37 was increased, whereas there were decreases at Ser-6 and Ser-392 (Fig. 3A). We could not detect other p53 phosphorylation sites at Ser-9, -20, and -46 in HCT-116 cells (data not shown). Time course of p53 phosphorylation was also determined. All the phosphorylation sites tested were altered at early time point (<6 h) (Fig. 3B). Thus, apigenin altered not only p53 protein level but also p53 phosphorylation. Since increasing phosphorylation of p53 at Ser-15 and -37 sites is correlated with increasing p53 activity and decreasing phosphorylation at Ser-392 is associated with de-activation of p53, we used a reporter gene containing a known p53 binding site to measure p53 activity. As shown in Fig. 3C, apigenin increased luciferase activity of the MDM2 promoter, suggesting that apigenin alters not only the p53 phosphorylation, but also the p53 activity. We also used a p21 promoter to see if apigenin increases p21 transcriptional activity; however, apigenin did not increase p21 promoter activity (data not shown), consistent with RT-PCR data indicating that apigenin increases p53 and p21 at the translational level. Finally, we measured p21 and NAG-1 expression in p53 knock-out cells. NAG-1 and p21 have been known to be induced by p53 protein at the transcriptional level.[16,17] HCT-116 p53–/– cells were treated with apigenin for 24 h at 10 μM concentration. As shown in Fig. 3D, p21 and NAG-1 were significantly increased in a dose-dependent manner in HCT-116 p53–/– cells. Although p21 induction by

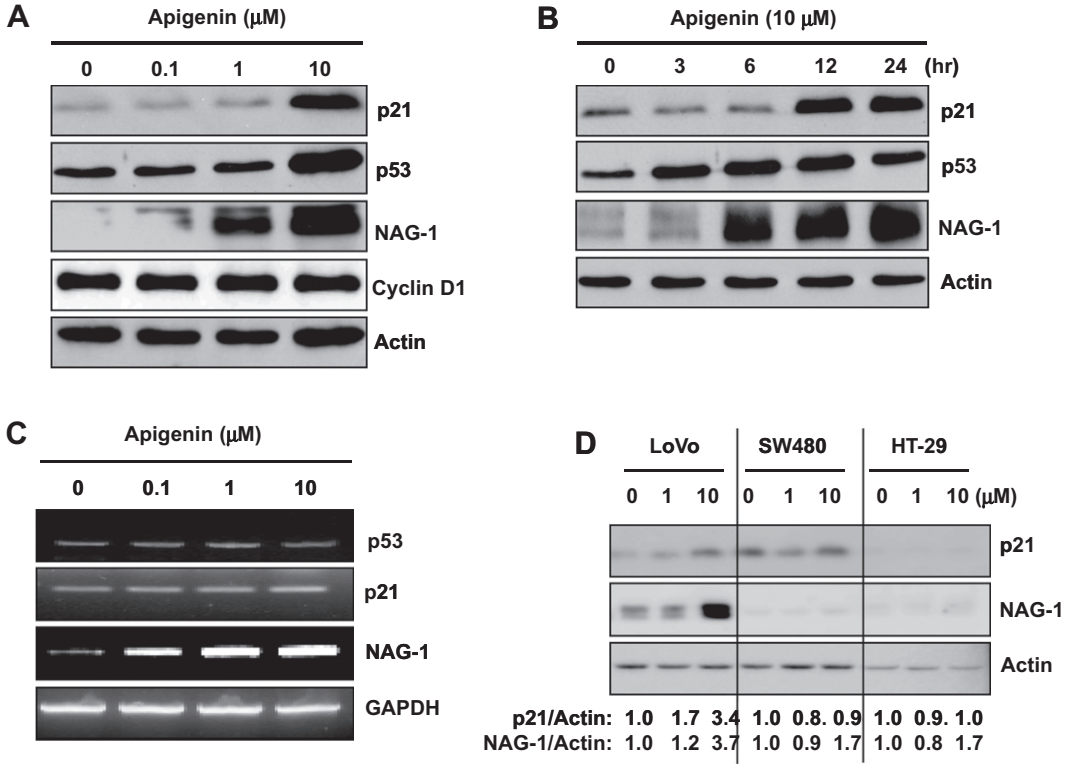

Fig. 2 – Analysis of p21, p53 and NAG-1 expressions in colorectal cancer cells treated with apigenin. (A) Western blot analysis of p21, p53, cyclin D1, NAG-1 and actin expression in HCT-116 cells treated with indicated dose of apigenin for 24 h. (B) HCT-116 cells were treated with 10 μM of apigenin, and samples were harvested at different time points. Cell lysates were then subjected to Western blot analysis for p21, p53, NAG-1 and actin. (C) HCT-116 cells were treated with 0, 0.1 μM, 1 μM, and 10 μM apigenin and then RT-PCR was performed. Number of cycles used was 29 for p53, p21 and NAG-1, and 25 cycles for GAPDH. Reaction products were analyzed on 2% agarose gel. (D) Western blot analysis of p21, NAG-1 and actin in different colon cancer cells treated with 0, 1, and 10 μM of apigenin for 24 h. Relative densitometry is shown in the bottom of the figure, as vehicle-treated sample is 1.0.

apigenin was observed only in p53 wild-type cells (Fig. 2D) and p53 phosphorylation might play a role in apigenin-induced p21 expression (Figs. 2B and 3B), these results clearly indicate that NAG-1 and p21 induction by apigenin is in a p53-independent manner in HCT-116 cells.

3.4. *Effects of kinase pathways on apigenin-induced p21 and NAG-1 expression*

To explore the molecular mechanism involved in apigenin-induced p21 and NAG-1 expression, we considered several signaling pathways that are affected by apigenin. We screened several kinase-specific inhibitors at a concentration that does not deviate from their selectivity. HCT-116 cells were pretreated with vehicle or indicated kinase inhibitor for 30 min prior to incubation with 10 μM of apigenin. As shown in Fig. 4A, apigenin-induced p21 and NAG-1 expression were strongly suppressed in the presence of staurosporin and RO-31-8220 (pan PKC inhibitor), and marginally suppressed by SP600125 (JNK inhibitor) and SB203580 (p38MAPK inhibitor). We further investigated the PKC signaling mechanisms responsible for p21 and NAG-1 induction by apigenin, since PKC has been implicated as an activator of p21 and NAG-1.[18,19] We examined the effects of apigenin on different PKC inhibitors and found that PKCδ played a role in apigenin-induced NAG-1 or p21 expression (data not shown). Pretreatment with Rottlerin (a specific inhibitor for PKCδ) showed inhibition of NAG-1 and p21 expression in a dose-dependent manner (Fig. 4B). To confirm the role of PKCδ in p21 and NAG-1 induction by apigenin, cells were transfected with empty, wild-type, or dominant negative (DN) form of HA-tagged PKCδ expression vector, then treated with apigenin. As shown in Fig. 4C, transfection of plasmid expressing DN PKCδ attenuated apigenin-induced p21 and NAG-1 expression, compared with that of wild-type transfection. Based on these data, p21 and NAG-1 expression by apigenin were, at least in part, mediated by the PKCδ signal pathway.

3.5. *Apigenin effects on intestinal tumorigenesis using APC $^{Min/+}$ mice*

Finally, we evaluated apigenin on anti-tumorigenesis using $APC^{Min/+}$ mice models. $APC^{Min/+}$ mice were separated into three groups: vehicle-treated controls (V) and mice treated with either a low dosage (L, 25 mg/kg) or high dosage (H, 50 mg/kg) of apigenin. Mice were treated with apigenin as described in Section 2, and sacrificed at 10 weeks of age. Small intestines were isolated and the polyp numbers examined.

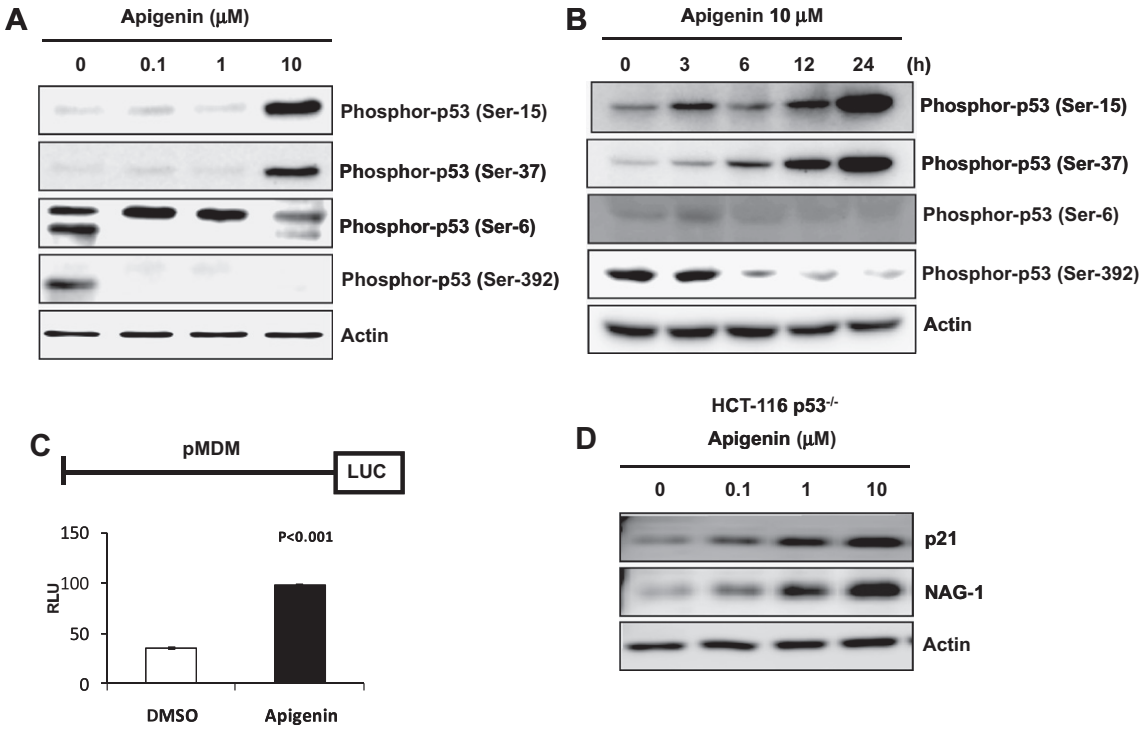

Fig. 3 – Apigenin effects on p53 expression. Western blot analysis of phosphor-p53 Ser-15, Ser-37, Ser-6, Ser-392 and actin in HCT-116 cells. (A) HCT-116 cells were treated with 0, 0.1 μM, 1 μM and 10 μM of apigenin for 24 h. (B) HCT-116 cells were treated with 10 μM apigenin with different time points. (C) Apigenin increased p53-dependent transcriptional activity. The pMdm-2 construct was transiently transfected into HCT-116 cells. After 24 h transfection, cells were treated with either DMSO or 10 μM apigenin for 24 h, and luciferase activity was measured. The value represents means ± SD from three independent experiments. (D) HCT-116 p53–/– cells were treated with 0.1 μM, 1 μM, and 10 μM apigenin for 24 h, and Western blot analysis was performed using antibodies for p21, NAG-1 and actin.

There was a statistically significant reduction in the polyp number and load in both high dose and low dose-treated groups, compared with the control group (Fig. 5A and B). However, apigenin did not affect the polyp size between vehicle and treated mice (data not shown). We measured serum concentration of apigenin in these mice and found a 13.4 ± 1.0 μM concentration in the 25 mg/kg treated group, whereas we could not detect any concentration of apigenin in vehicle-treated mice. We used the lysates of small intestinal tumors or normal tissues from vehicle and high dose apigenin-treated mice, and subjected them to Western blot. The expression of p21 is higher in tumors of apigenin-treated mice compared to those of vehicle-treated mice, whereas the expression of p53 phosphorylation at Ser-15 is much higher in apigenin-treated tumor tissues (Fig. 5C), suggesting that apigenin phosphorylates p53 protein specifically in tumor tissues.

4. Discussion

Apigenin is a non-mutagenic flavonoid found in vegetables and fruits, including parsley, onions, wheat sprouts, chamomile, seasonings, tea and oranges, and has cancer preventive activity.[20] Apigenin has been reported to act via several mechanisms, including promotion of cell cycle arrest[21] and apoptosis,[22–25] inhibition of mutagenesis,[26] and suppression of signal transduction.[21,27] It has also been reported that apigenin induces ERK and p38MAPK, but has little effect on Jun kinase activity,[28] and it arrests the cell cycle at the G2/M phase accompanied by a decrease of cyclin B1 in human colorectal cancer cells.[4] One of most obvious mechanisms of apigenin is to induce p53 tumor suppressor proteins at the translational level, followed by p21 induction. However, our data clearly indicate that p21 induction by apigenin is not dependent on p53 expression, as assessed by p53 knock-out cell lines. Although p53 phosphorylation at Ser-15 has been well known and seems to control apigenin-induced anti-tumorigenesis, other phosphorylation sites on p53 proteins may play an important role in apigenin-induced anti-tumorigenesis. As shown in Fig. 3A, phosphorylation sites at 6 and 392 showed reduction of phosphorylation in the presence of apigenin, indicating the involvement of these sites. Indeed, it has been known that phosphorylation at the Ser-392 site is important in inactivating p53 activity.[29] Thus, apigenin affects many other phosphorylation sites, including Ser-15, and controls p53 activation. Since p53 at Ser-392 is phosphorylated by casein kinase II (CKII) as well, and the treatment of CKII inhibitor did not restore p21 induction but restored NAG-1 expression (Fig. 4A) in the presence of apigenin; CKII pathway may contribute to apigenin-induced p21 expression in a p53-dependent manner, but not to apigenin-induced NAG-1 expression. Further experiments may be required to elucidate the phosphor-specific

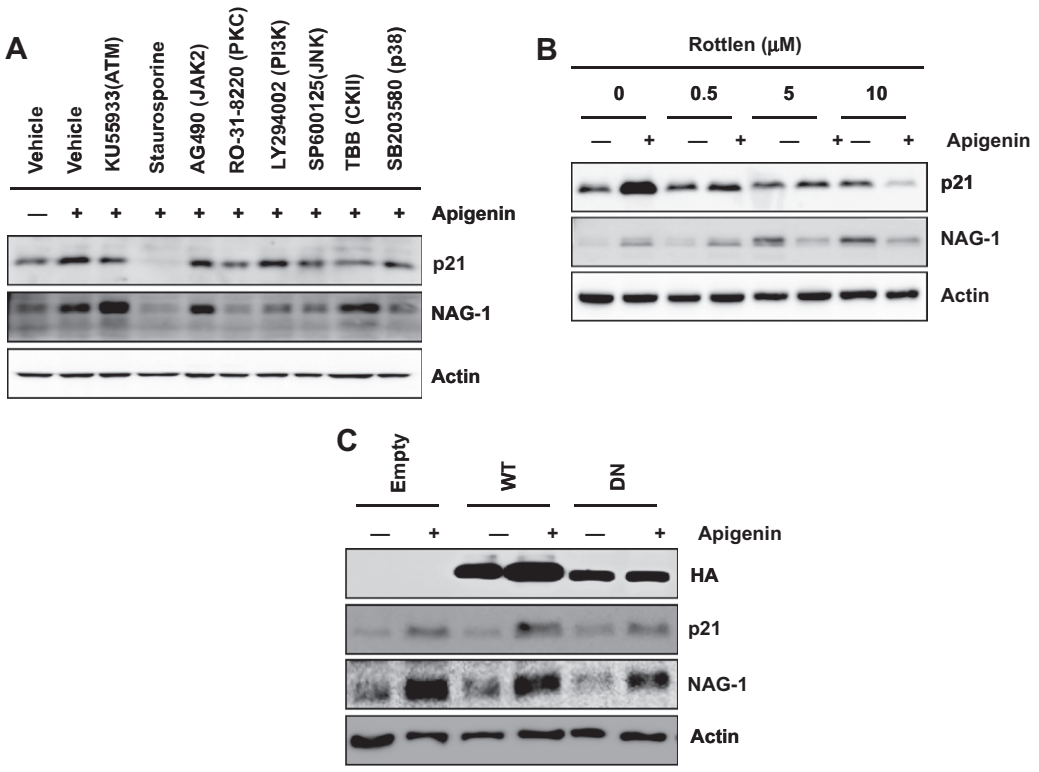

Fig. 4 – Effects of kinase inhibitors on apigenin-induced p21, and NAG-1 expression. (A) HCT-116 cells were pretreated with DMSO, ATM inhibitor (KU55933, 10 μM), p38MAPK inhibitor (SB203580, 10 μM), CKII inhibitor (TBB, 5 μM), JNK inhibitor (SP600125, 10 μM), PI3K inhibitor (LY294002, 20 μM), PKC inhibitor (RO-318220, 2.5 μM), JAK-2 inhibitor (AG490, 50 μM), and pan-kinase inhibitor (staurosporine, 5 μM), for 30 min and followed by treatment with 10 μM of apigenin for an additional 24 h. The cell lysates were harvested and subjected to Western blot analysis to examine p21, NAG-1, and actin expression. (B) HCT-116 cells were treated with DMSO, and different concentrations of Rottlerin (0.5, 5, 10 μM) in serum-free media. After 30 min pre-treatment, either DMSO or 10 μM of apigenin was added directly to the media. After 24 h incubation, cell lysates were harvested and then subjected to Western blot using antibodies for p21, NAG-1, and actin. (C) HCT-116 cells were transfected with empty, wild-type (WT), or dominant negative (DN) PKCδ expression vector as described previously. The cells were then treated with 10 μM of apigenin for 24 h. Western analysis was performed for hemagglutinin (HA), NAG-1, p21, and actin antibodies.

activity of p53 tumor suppressor protein in the presence of apigenin.

Although *in vitro* data suggests the induction of apoptosis and cell growth arrest in tumorigenesis, animal studies using apigenin are contradictory. Apigenin failed to inhibit adenoma formation in *Min* mice models, but it showed reduced ACF formation in carcinogen-induced animal models.[5] In this study, we have shown that apigenin indeed decreased tumor numbers and load in *Min* mice models. The differences between our data and the previous report result from different experimental settings, including the way of feeding apigenin (gavage versus in the diet), timing of treatment of the mice, and dose of apigenin. However, our data clearly indicate that apigenin suppressed tumor formation as well as tumor load in a dose-dependent manner. Our results are also accompanied by Western blot, showing that phosphorylated p53 at the Ser-15 is highly expressed in apigenin-treated samples, thereby controlling tumorigenesis. Many signaling pathways play a pivotal role in colorectal tumorigenesis. Specifically, the p53 pathways are important in the development of colorectal tumorigenesis. Mutations commonly occur at the p53 tumor suppressor protein locus in many forms of cancer including colorectal cancer. Considering this, there are many potential uses of apigenin in cancer prevention because the p53 mutation occurs at an early stage of tumorigenesis. Furthermore, apigenin enhances NAG-1 and p21 expression in a p53-independent manner.

Another protein we examined in this study was NAG-1. We have previously identified it as a pro-apoptotic and anti-tumorigenic protein by using PCR-based subtractive hybridization.[13] Human NAG-1 is a secreted protein with homology to members of the TGF- superfamily and has been previously identified as macrophage inhibitory cytokine-1 (*MIC-1*), placental transformation growth factor-β (*PTGFB*), prostate derived factor (*PDF*), growth differentiation factor 15 (*GDF-15*), and placental bone morphogenetic protein (*PLAB*).[30] Recently, we have generated NAG-1 transgenic mice that over-express human NAG-1 and have demonstrated that these NAG-1 transgenic mice are much less sensitive to carcinogens or genetic toxicity.[14] In addition, NAG-1 is up-regulated in a prostaglandin-independent manner in human colorectal cancer cells by several NSAIDs,[31] as well as by

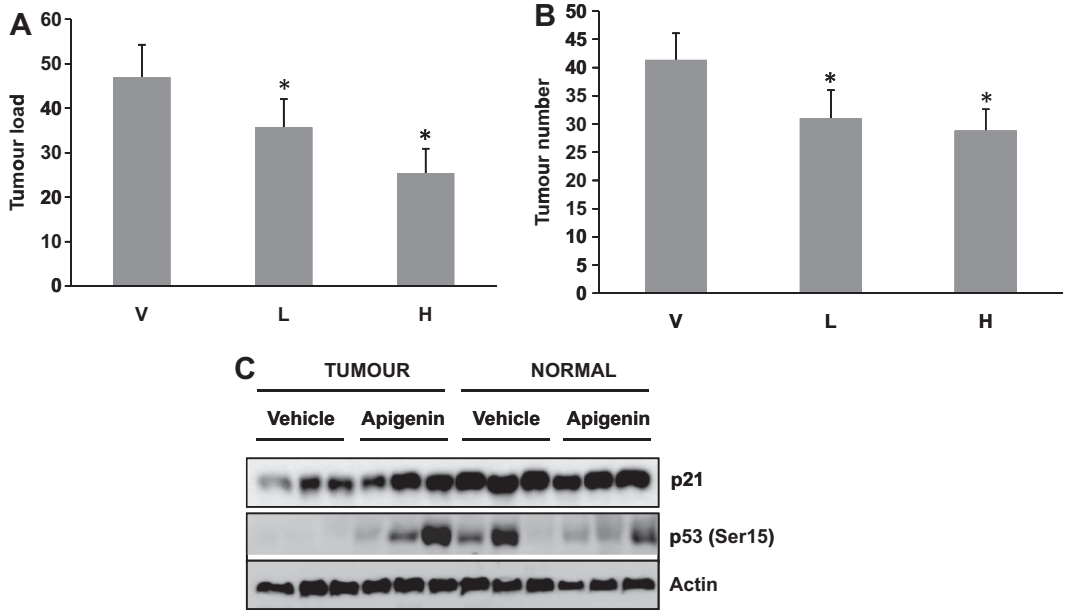

Fig. 5 – Apigenin effect on APC^{MIN+} mice. (A and B) Effects of administration of apigenin or 0.5% methylcellulose (V) on tumour number and tumour load (means ± SE) in comparison to vehicle ($n = 6$), low dosage (L) of 25 mg/kg apigenin ($n = 6$), and high dosage (H) of 50 mg/kg ($n = 5$). Tumour load was calculated by number of tumors × average diameter. Tumour load and tumour numbers were statistically analyzed using the Mixed ANOVA test with statistical significance set at $^*p < 0.05$, compared to vehicle-treated mice. (C) Cell lysates from tissue samples of three tumour and three normal tissues were isolated from vehicle-treated and apigenin-treated APC^{MIN+} mice (high dosage 50 mg/kg). Western blot analysis was performed for p21, phosphor-p53 (Ser-15), and actin.

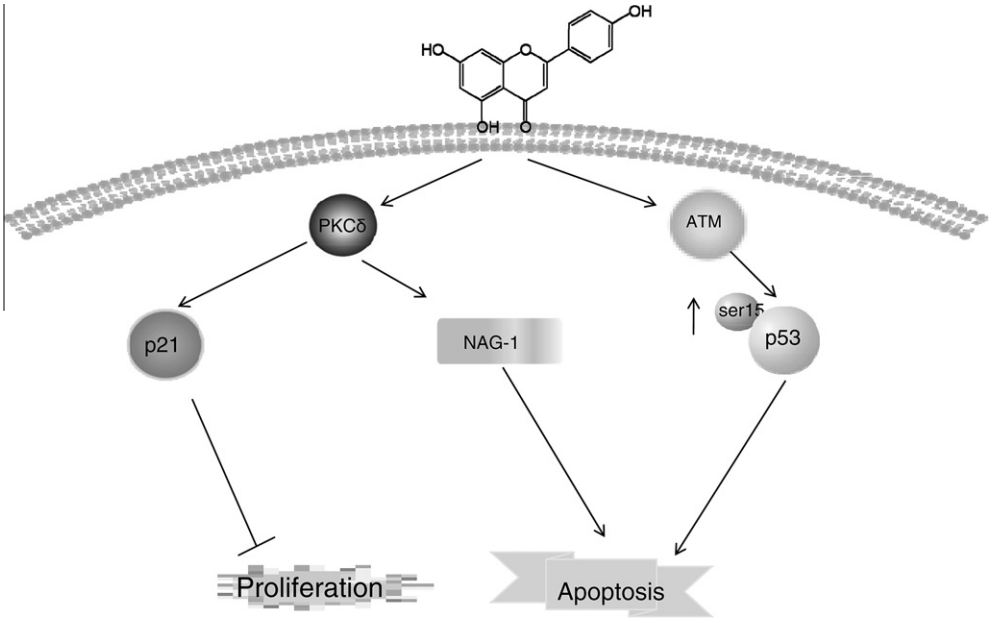

Fig. 6 – Mechanism by which apigenin induces apoptosis and suppresses cell growth in HCT-116 cells. Apigenin affects the PKCδ pathway to increase p21 and NAG-1 expressions, followed by inducing apoptosis and inhibiting cell proliferation. Phosphor-p53 at Ser-37 and Ser-15, which are active forms, were increased by apigenin through the ATM pathway. Overall, apigenin affects p21 and NAG-1 expression in a p53-independent pathway.

anti-tumorigenic phytochemicals, including resveratrol,[32] genistein,[16] green tea catechins,[33] DADS,[34] PPAR ligands,[35,36] 5F-203,[37] DIM and its derivatives)[38,39] and retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN).[40] In this study, we could add apigenin as a NAG-1 inducer mediated by a PKCδ-dependent pathway. Since NAG-1 expression is also associated with tumorigenesis in the lung,[41] it would be interesting to know if apigenin also

affects lung tumorigenesis. Indeed, one report showed that apigenin has a beneficial effect on lung cancer.[42]

Many studies have demonstrated that flavonoids provide anti-tumorigenic activity; in fact, this observation prompted extensive studies that have defined a key role for tumor suppressor proteins and identified new molecular targets for this class of compounds. In this study, we identified NAG-1 and p21 as specific targets for apigenin. Another well-accepted fact obtained during the course of these studies is that apigenin also has p53-dependent and -independent anti-tumorigenic activity (Fig. 6). The over-reaching goal of this study was to further characterize a novel molecular pathway identified by our laboratory as a target of apigenin anti-tumorigenic activity with the intention of: (1) substantiating the importance of NAG-1, p21 and p53 as apigenin target genes and (2) identifying this pathway as a target for designing pharmaceutical anti-cancer drugs in the future.

Conflict of interest statement

None declared.

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