Induction of caspase-dependent, p53-mediated apoptosis by apigenin in human neuroblastoma

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Abstract

Neuroblastoma is a pediatric tumor accounting for 15% of childhood cancer deaths and has a poor prognosis in children > 1 year of age. We investigated the ability of apigenin, a nonmutagenic dietary flavonoid that has been shown to have antitumor effects in various tumor cell lines, to inhibit growth and induce apoptosis of the human neuroblastoma cell lines NUB-7, LAN-5, and SK-N-BE(2). Apigenin inhibited colony-forming ability and survival, and induced apoptosis of NUB-7 and LAN-5 cells. The presence of the C2-C3 double bond and the 4′-OH group on the flavonoid structure correlated with the growth-inhibitory potential of apigenin. Furthermore, apigenin inhibited NUB-7 xenograft tumor growth in a nonobese diabetic/severe combined immunodeficiency mouse model, likely by inducing apoptosis. Apigenin did not inhibit survival of primary sympathetic neurons, suggesting that it is not toxic to nontransformed cells. The mechanism of action of apigenin seems to involve p53, as it increased the levels of p53 and the p53-induced gene products p21WAF1/CIP1 and Bax. Furthermore, apigenin (15–60 μmol/L) induced cell death and apoptosis of neuroblastoma cells expressing wild-type but not mutant p53. Apigenin increased caspase-3 activity and PARP cleavage, and Z-VAD-FMK, a broad-spectrum caspase-3 inhibitor, rescued NUB-7 cells from apigenin-mediated apoptosis indicating that apigenin induced apoptosis in a caspase-dependent manner. Overexpression of Bcl-XL rescued NUB-7 from apigenin-induced cell death, suggesting that Bax activity is important for the action of apigenin. Apigenin is thus a candidate therapeutic for neuroblastoma that likely acts by regulating a p53-Bax-caspase-3 apoptotic pathway. [Mol Cancer Ther 2005;4(1):1–11]

Introduction

Neuroblastoma, a neuroectodermal tumor of the peripheral nervous system, is the most common extracranial solid tumor of childhood, accounting for 15% of pediatric cancer-related deaths (1). Neuroblastoma is thought to be derived from embryonic neural crest cells (2, 3) that form the peripheral nervous system (4). Despite aggressive multimodal therapy, this devastating tumor often acquires drug resistance and metastasizes (5). Therefore, our goal is to identify agents that will promote apoptosis of neuroblastoma cells without toxic effects to nontransformed cells.

One candidate agent is apigenin and related dietary flavonoids, which have been shown to have antitumor effects in several human adult tumor cell lines, including those derived from prostate, colon, and breast cancer (6–12). Apigenin also inhibits UV-induced tumor promotion (13), and decreases melanoma lung metastasis (14) in mouse models of melanoma. In addition, apigenin mediates the stabilization and transcriptional activation of the tumor suppressor p53 in keratinocytes (15), and induces morphologic differentiation and G1 arrest in a rat neuroblastoma cell line (16). Apigenin is of particular interest as an anticancer agent because it exhibits low intrinsic toxicity and is not mutagenic compared with other structurally related flavonoids (17). The exact molecular targets of apigenin and related flavonoids are currently unknown, but they have been shown to bind ATPase domains (18) and may act as kinase inhibitors (9, 11, 12, 19, 20). Other targets may include heat shock proteins (21), telomerase (22), ornithine decarboxylase (23), and aryl hydrocarbon receptor activity (24).

The goal of this study is to determine whether apigenin is apoptotic for neuroblastoma in vitro and in vivo, and to determine whether it induces apoptosis by activating caspases and p53. Although p53 is frequently mutated in many human cancers, it is wild-type in neuroblastoma but functionally inactive due to cytoplasmic sequestration (25, 26). We, along with others, have shown that nuclear translocation and activation of p53 can be induced in neuroblastoma cells by various stimuli, including chemotherapeutic agents (27–30). Nontoxic agents that stimulate p53 activity may be ideal candidates as therapeutics for neuroblastoma.
In this study, we report that apigenin inhibits tumor growth and induces caspase-dependent apoptosis in neuroblastoma cell lines in vitro and in a nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse xenograft model with NUB-7 s.c. tumors. We show that the mechanism by which apigenin induces its anti-tumor effects is by activating caspases, concurrent with increased expression of nuclear p53 and of the p53 target gene products p21WAF1/CIP1 and Bax.

Materials and Methods

Cell Culture

NUB-7, developed in our laboratory, was established from a primary neuroblastoma tumor (31) and has been previously characterized (32). LAN-5 was kindly provided by Dr. R.C. Seeger (Department of Pediatrics, University of California at Los Angeles School of Medicine, Los Angeles, CA). Both NUB-7 and LAN-5 are MYCN-amplified and represent high-risk, metastatic tumors (32, 33). SK-N- BE(2) (ATCC 8CR-L-2271) was derived from disseminated neuroblastoma located in the bone marrow (34, 35). The cell lines were cultured in α-MEM supplemented with fetal bovine serum, and penicillin/streptomycin at 37°C, 5% CO2. Cells were plated in T25 or T75 culture flasks (Falcon, Becton Dickinson, Oakville, ON, Canada), 24- or 6-well plates (Costar, VWR CanLab, Mississauga, ON, Canada) or 96-well black walled plates (Falcon) prior to treatment. The protocol for superior cervical ganglia (SCG) culture was adapted from Ma et al. (36).

Flavonoids

Cells were treated with apigenin (Sigma, St. Louis, MO), quercetin, naringenin, diosmetin, chrysin, baicalein, and flavone (Indofine Chemical, Belle Mead, NJ) at indicated concentrations, or the equivalent volume of vehicle (DMSO).

Antibodies

Primary antibodies were obtained as follows: anti-phospho mitogen-activated protein kinase (Promega, Madison, WI), anti-Bcl-2, anti-Bcl-XL, anti-p53 (DO1), and anti-p53 (FL393; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53 (Ab-8) (Neomarkers, Montreal, PQ, Canada), anti-cleaved PARP (Asp214) and anti-cleaved caspase-3 (Cell Signaling, Beverly, MA), anti-p21WAF1/CIP1 and anti-Bax (Upstate Biotechnology, Lake Placid, NY), anti-β actin (Sigma). Secondary antibodies: goat anti-mouse horse radish peroxidase (Bio-Rad, Hercules, CA), goat anti-rabbit horseradish peroxidase (Chemicon International, Temecula, CA) and goat anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA).

Cell Viability Assays

Cell viability was determined using three methods: cell death assay, survival assay, and terminal nucleotidyl transferase (TdT)–mediated nick end labeling (TUNEL) assay.

Cell Death Assay.

Following indicated treatments, cells were incubated with trypan blue (Sigma; final volume sec20% added to media) for 10 minutes, 37°C. Trypan blue–positive and total cells were counted per microscope field, for a total of four fields per condition (a minimum of 3,000 cells were counted per condition). The proportion of cell death was calculated by dividing the number of dead cells by total cell number/field. Alternatively, viable cell number was counted. The error bars correspond to SE. Results are representative of two or three experiments.

Survival Assay. AlamarBlue (Biosource International, Camarillo, CA), which measures mitochondrial activity, was used to quantitatively measure cell survival (protocol from Biosource). Preliminary experiments determined that relative fluorescence of AlamarBlue correlated with cell number (data not shown). Fluorescence was measured with excitation at 540 nm, and emission at 590 nm, with a cutoff at 550 nm. The average percentage of survival is reported with its associated SE.

TUNEL Assay. The protocol was adapted from Li et al. (37). Cells were plated in 24-well plates and treated with varying doses of apigenin for 24 hours. Floating cells were collected and pelleted and added to the trypsinized adhered cells. Cells were washed twice in PBS and centrifuged onto glass cover slips by Cytospin (Shandon, Inc., Pittsburgh, PA). The cells were fixed in 1% paraformaldehyde for 10 minutes and stored in PBS at 4°C. Nuclear DNA was denatured with a 2:1 mixture of ethanol and acetic acid for 10 minutes. The cells were washed twice in PBS for 5 minutes and incubated for 1 hour at 37°C in 50μL of TdT reaction buffer containing: 1× concentrated buffer solution, 2.5 mmol cobalt chloride, 12.5 units of TdT (Roche, Indianapolis, IN), and 0.25 nmol of ChromaTide BODIPY FL-14-dUTP (Molecular Probes, Eugene, OR) in distilled water. For control, TdT was substituted with distilled water. The cells were washed twice with 15 mmol EDTA (pH 8.0), once with 0.1% Triton X-100 in PBS, and twice with PBS. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in Prolong Antifade (Molecular Probes) mounting media. The slides were examined under epifluorescence microscopy and photographed on Kodak EL 135-36, ASA 400. A total of six photographs were taken of each slide—three photographs were taken under the UV filter to obtain a total cell count with the DAPI counterstain, and a blue filter was then used to photograph the BODIPY-stained cells. Total cells and TUNEL-positive cells were counted in three photographed fields, and the experiment was done in duplicate.

Flavonoid Structure/Function Assay

Cells were incubated with varying concentrations (0–60 μmol/L) of flavonoid analogues for 48 hours, and cell survival was determined by AlamarBlue.

Effect of Serum Concentration

Cells were seeded in 96-well black walled plates (Falcon) at 10,000 cells/well. Apigenin (0–100 μmol/L) in combination with 2.5%, 5%, 10%, or 20% fetal bovine serum was added 24 hours after seeding. For the 6-day treatment, fresh apigenin was added every other day. Cell survival was measured by AlamarBlue.

Clonogenic Assay

The soft agarose method was used to measure colony formation by neuroblastoma cell lines (31). Briefly, a bottom
layer of agarose solution containing 1.5% agarose (FMC Colloids, Rockland, MA) in a final concentration of 1× McCoy’s medium and various concentrations of apigenin was poured into gridded 35 mm dishes (Nunclon, Fisher Scientific, Napean, ON, Canada) (1 mL/dish) and allowed to gel. The top layer contained trypsinized, counted cells, 1.2% agarose, 1× McCoy’s medium, and apigenin. After 1 to 2 weeks, the cultures were fixed with formalin and colonies were scored under phase microscopy.

**Fluorescence-Activated Cell Sorting Analysis**

Propidium iodide, staining of DNA, and fluorescence-activated cell sorting (FACS) were used to determine the effect of apigenin on DNA content. The protocol was adapted from Matteucci et al. (38). Propidium iodide fluorescence was used to determine DNA content. Cells were analyzed by flow cytometry with FACSscan (Becton Dickinson), acquisition software Cell Quest (Becton Dickinson), and the DNA curve fitting program Modfit LT (Verity Software, Becton Dickinson).

**Xenograft Mouse Model**

All animal studies were approved by our institutional animal care committee in accordance with accepted guidelines for animal care and experimentation. NUB-7 (5 × 10⁶ cells) were suspended in Matrigel (BD Biosciences, Bedford, MA) and injected s.c. in the scapular region of female NOD/SCID mice (five mice per condition). Tumors were grown for 1 week. To prepare the test concentration of 25 mg/kg, 25 mg apigenin was dissolved in 5% DMSO, 10% PEG400, and 1% Tween 80, mixing well between each addition (adapted from ref. 14). Sterile PBS was added to a final volume of 10 mL with a working concentration of 2.5 mg/mL. Apigenin (200 μL) or vehicle control was injected i.p. daily for 5 days. Mice were sacrificed and tumors excised and weighed. Tumor slices were dissociated to a single cell suspension by trypsinization, gentle vortexing and trituration, and stained with propidium iodide and analyzed by FACS.

**Caspase-3 Activity Assay**

Cells were treated with apigenin for 24 hours in 96-well plates. Caspase-3 activity assay was adapted from Carrasco et al. (39). Briefly, caspase assay buffer containing a caspase-3-specific cleavage substrate linked to a fluorophore (Ac-DEVDP-AMC; Biomol, Plymouth Meeting, PA) was added to each well. Staurosporine (Biomol; 60 μmol/L, 4 hours) was used for positive control and the broad-spectrum caspase inhibitor Z-VAD-FMK (Biomol; 100 nmol/L, 24 hours) was used for negative control. Caspase-3 activity was quantified by AMC fluorescence read every 2 minutes for 90 minutes. Activity was normalized to the untreated control.

**Bcl-X<sub>L</sub> Overexpression**

NUB-7 cells were trypsinized, counted, and plated at 200,000 cells/well in six-well plates coated with poly-d-lysine (Sigma). Sixteen hours later, cells were infected with Bcl-X<sub>L</sub> Ad5 (multiplicity of infection, 50) or GFP Ad5 (multiplicity of infection, 5) adenoviruses that express cytomegalovirus promoter–driven Bcl-X<sub>L</sub> or GFP (Aegera, Iles-Des-Soeurs, PQ, Canada). Twenty-four hours post-infection, cells were treated with 60 μmol/L apigenin for a subsequent 24 hours. Bcl-X<sub>L</sub> and GFP expression was confirmed by Western blotting and cell death was measured by trypan blue exclusion assay.

**Western Blot Analysis**

Cells were treated or infected as above, and lysed in lysis buffer (Cell Signaling) with added protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates were quantified by bicinchoninic acid protein assay kit (Pierce, Brockville, ON, Canada). Equalized lysates were denatured in sample loading buffer [1.67% SDS, 8.3% glycerol, 1.3% DTT (Sigma), trace bromophenol blue (Fishert), boiled and run on 7% to 15% gradient SDS-PAGE. Protein was transferred to nitrocellulose membrane, and the membrane was blocked in 5% bovine serum albumin (Calbiochem, VWR Can Lab)/TBS-T [TBS with 0.2% Tween-20 (Fishert)] for antibodies against phosphorylated proteins, or 5% skim milk powder (Carnation)/TBS-T for other antibodies, for 1 hour at room temperature. Primary antibodies were diluted in TBS-T and incubated with the membrane overnight at 4°C. Following washes, membranes were incubated in secondary antibodies diluted in TBS-T for 1 hour at room temperature. Antibodies were visualized with enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ). For reprobes, membranes were stripped with 2% SDS, 62 mmol Tris (pH 6.8), 0.7% β-mercaptoethanol for 15 minutes at 55°C, rinsed in TBS, blocked and reprobed. Densitometry analysis was done with Total Lab software (Amersham Biosciences).

**Immunofluorescence Staining**

Cells were plated in two-well chamber slides coated with poly-d-lysine (Sigma), treated with 60 μmol/L apigenin for indicated times and fixed for 15 minutes in ice-cold 4% paraformaldehyde. Cells were washed thrice in HBSS, permeabilized for 5 minutes with 0.5% NP40, washed four times in HBSS and blocked in 3% normal goat serum for 1 hour. Cells were incubated with rabbit anti-p53 (FL393) overnight at 4°C and washed four times. Results were confirmed with an additional p53 antibody, Ab-8 (data not shown). Primary antibodies were detected using goat anti-rabbit Cy3-coupled secondary antibody and nuclei were stained for 2 minutes with Hoechst 33258 (Sigma). Immunostained cells were photographed using constant exposure time with a 100× lens and Norton Eclipse software version 6.1.

**Statistical Analysis**

Graphing, and statistical analysis by ANOVA and t test were done with GraphPad Prism software.

**Results**

**Apigenin Inhibits the Survival of Neuroblastoma Cell Lines**

Previous reports have shown that structurally, the C2-C3 double bond and the 4'-hydroxy group on the flavone diphenyl ring of flavonoids correlate with increased growth-inhibitory effects in tumor cell line models...
We tested whether these structural characteristics of flavonoids are implicated in inhibition of growth and/or survival of human neuroblastoma. NUB-7 and LAN-5, two MYCN amplified, wild-type p53, human neuroblastomacell lines, that represent the type (intermediate) and N-type (neuronal) morphology, respectively, and the metastatic, high stage phenotypes (40), were treated for 24 hours with a series of structural analogues of apigenin including: naringenin, which differs from apigenin by the lack of the C2-C3 double bond, diosmetin which has a 4'-methoxy group, baicalein and chrysin, which lack the 4'-substitution, quercetin that has an additional 3'-OH group, and flavone (unsubstituted; Fig. 1A). The analogues were ranked as follows, from the strongest to weakest inhibitor of cell survival in NUB-7: apigenin, quercetin, diosmetin, chrysin > baicalein > flavone > naringenin (Fig. 1B). At the concentrations tested, naringenin had no effect on cell survival (Fig. 1B). Similar results were observed in LAN-5 (data not shown). Our data was in agreement with previous reports indicating that the presence of the C2-C3 double bond and (to a lesser extent in our model) the 4'-OH substitution group are important structural requirements for inhibition of melanoma cell survival (6, 14). Although quercetin, diosmetin, and chrysin had similar inhibitory potencies as apigenin, apigenin was chosen for further study as it is prevalent in the diet and has low intrinsic cytotoxicity (17).

We next tested apigenin for dose-dependent effects on cell survival. Apigenin (24 hours) inhibited cell viability in a dose-dependent manner in human neuroblastoma cell lines with an EC50 = 35 μmol/L in NUB-7, and EC50 = 22 μmol/L in LAN-5 as determined by trypan blue exclusion assay (Fig. 2A). The dose-dependent effect was also observed by AlamarBlue survival assay (Fig. 2C).

An important factor that might influence the survival response of cells to a particular drug is the method of culture growth. Cells grown in monolayer culture are often more sensitive to death-inducing agents compared with cells grown as colonies in three dimensions (41). Therefore, we asked whether apigenin could inhibit neuroblastoma colony formation in an agarose culture model. Cells were seeded in agarose that was premixed with 5 to 60 μmol/L apigenin or vehicle control, and colonies were counted after 2 weeks. Apigenin induced a dose-dependent decrease in colony number from 25% at 5 μmol/L to 100% at 60 μmol/L relative to control in NUB-7 and by 30% at 5 μmol/L and 100% at 60 μmol/L in LAN-5 (Fig. 2B). Taken together, the monolayer and colony assay results show that apigenin effectively induced dose-dependent reduction in the survival of these neuroblastoma cell lines. In addition, in monolayer culture, increasing serum concentration in the culture medium partially rescued NUB-7 cells from cell death over long-term treatment (6 days; Fig. 2C). However, the presence of high serum concentration (20%) did not completely protect these cells from apigenin-induced death (Fig. 2C). This suggests that a relatively high concentration of serum is not sufficient to block apigenin from inducing cell death, however, it does mediate moderate resistance to cell killing. We therefore chose 10% fetal bovine serum for all following experiments to facilitate the study of apoptotic mechanisms in this model.

Apigenin Reduces Tumor Mass in a Mouse Xenograft Model of Neuroblastoma

Our data indicated that apigenin inhibited in vitro survival of neuroblastoma cell lines. We therefore asked if apigenin inhibited neuroblastoma survival in a NOD/SCID xenograft model. Initial trials indicated that mice bearing scapular NUB-7 tumors treated with 25 mg/kg apigenin for 5 days did not display overt toxicity relative to untreated mice, as determined by monitoring mice for signs of morbidity, weight gain, and by histopathologic examination of major organs. Mean tumor mass in the treated group decreased by 50% (Table 1). Portions of the excised tumors were dissociated, stained with propidium iodide, and analyzed by FACS and showed a significant increase in the percentage of apoptotic cells. These results suggest that apigenin has potential as a therapeutic agent for the treatment of neuroblastoma.
in the subdiploid fraction of tumors from apigenin-treated mice (Table 1). This preliminary evaluation of apigenin in an in vivo model shows that tumors from treated mice were substantially smaller than control, and that these tumors had significantly increased apoptosis, suggesting that apigenin affected tumor cell survival at a well-tolerated dose.

Apigenin Does Not Induce the Death of Nontransformed Primary Cells

Having established that apigenin has a potent effect on neuroblastoma tumor cell growth, we sought to determine whether apigenin-induced death is specific to neuroblastoma cells. We therefore asked whether apigenin inhibits survival of nontransformed cells. Because neuroblastoma cells are derived from the sympathoadrenal precursor lineage of the embryonic neural crest, we tested the effect of apigenin on differentiated peripheral sympathetic neurons isolated from primary cultures of rat SCG cells. Treatment for 24 hours with 15 to 120 μmol/L apigenin, which inhibited the survival of neuroblastoma cells, did not affect the survival of SCG cells, as determined by AlamarBlue survival assay (Table 2). These results show that apigenin specifically induced dose-dependent death of neuroblastoma cells without toxicity to nontransformed neuronal cells. Furthermore, induction of morphologic differentiation of NUB-7 with retinoic acid (32) inhibited apigenin-induced cell death, suggesting that apigenin preferentially induces death of undifferentiated neuroblastoma cells (data not shown).

Apigenin Mediates Cell Death by Induction of Apoptosis

To determine whether the observed apigenin-induced cell death in neuroblastoma cell lines occurred via induction of apoptosis, TUNEL and FACS analysis were done. NUB-7 and LAN-5 cells were treated with 0 to 100 μmol/L apigenin for 24 hours and double-stained with TUNEL and DAPI to calculate the percentage of TUNEL-positive cells per microscopic field. Cells treated with 50 μmol/L apigenin had an 8% apoptotic fraction as compared with 14% in 100 μmol/L and 1% in untreated cells (Fig. 3A). This result is also supported by FACS analysis of NUB-7 cells which showed that compared with control, 60 μmol/L apigenin induced a 50% increase in the number of apoptotic cells in NUB-7 xenograft tumors in a NOD/SCID mouse model of neuroblastoma

<table>
<thead>
<tr>
<th>NUB-7 Xenograft mouse treatment group</th>
<th>Tumor weight (g), mean ± SE</th>
<th>% Cells in subdiploid peak, mean ± SE</th>
</tr>
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<tbody>
<tr>
<td>Vehicle control</td>
<td>1.986 ± 0.172</td>
<td>7.519 ± 1.014</td>
</tr>
<tr>
<td>Apigenin*</td>
<td>1.092 ± 0.22†</td>
<td>11.96 ± 0.98†</td>
</tr>
</tbody>
</table>

*NOD/SCID mice bearing subcutaneous NUB-7 xenograft tumors were treated with 25 mg/kg apigenin for 5 days.

†Significant difference from control, \( P < 0.05 \).
percentage of gated cells in the subdiploid peak (i.e., apoptotic cells; data not shown).

Caspase-3 activation and PARP cleavage are characteristic indicators of apoptosis. Caspase-3 is a downstream effector in the caspase cascade, activation of which represents the apoptotic “point of no return” (42). First, to determine whether apigenin activated caspase-3, cells treated with 15 to 60 μmol/L apigenin for 24 hours were lysed in the presence of Ac-DEVD-AMC that fluoresces upon specific cleavage by caspase-3. In NUB-7, caspase-3 activity increased by 5-fold at 60 μmol/L apigenin after 24 hours of treatment (Table 3). Staurosporine and Z-VAD-FMK, a broad-spectrum caspase inhibitor (43), were used as positive and negative controls, respectively, for caspase activation (Table 3). Second, we assessed PARP cleavage, a nuclear protein that is specifically cleaved by activated caspases (44), by Western blot. In NUB-7 cells, cleaved PARP increased in a dose-dependent manner with apigenin treatment (15–60 μmol/L) after 24 hours (Fig. 3B). Naringenin, the structurally related, inactive analogue (Fig. 1A and B) did not induce PARP cleavage (Fig. 3B). Furthermore, cells that were pre-treated with Z-VAD-FMK followed by treatment with apigenin had no cleaved PARP. The trypan blue cell death assay also showed that Z-VAD-FMK significantly inhibited apigenin (15–120 μmol/L)–induced cell death (Fig. 3C). Taken together, these results show that apigenin induced apoptosis in a dose-dependent manner, and that apoptosis required caspase activation.

**Apigenin Induces Apoptosis of Neuroblastoma in a p53-Mediated Manner**

p53 is most commonly wild-type in neuroblastoma tumors and cell lines, albeit inactive due to sequestration in the cytoplasm (28). It has also been shown in neuroblastoma that cytoplasmic p53 can be induced to translocate to the nucleus, where it activates gene transcription and apoptosis upstream from mitochondrial events related to caspase activation (27, 28). We therefore asked whether apigenin required wild-type p53 for induction of caspase-mediated apoptosis. NUB-7 (wild-type p53, additional cytogenetic information available in ref. 45) and SK-N-BE(2), a rare neuroblastoma cell line expressing a nonfunctional mutant p53 (6), were treated with 60 μmol/L apigenin and lysed after 4, 8, 16, and 24 hours, as this concentration of apigenin induced PARP cleavage at 24 hours post-treatment (Fig. 3B). Western blot analysis of NUB-7 lysates showed that starting at 8 hours post-treatment, the cleaved PARP product level rose and peaked at 16 hours (Fig. 4A), suggesting caspase activation at or before 8 hours post-treatment. Consistent with p53 being upstream of caspase activation, p53 levels increased at 4 hours post-treatment, followed by the increase of two p53 target genes, p21WAF1/CIP1 and Bax (Fig. 4A). Immunofluorescence staining showed that in untreated NUB-7, p53 expression is low and sparsely distributed in the

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**Table 2. Apigenin does not inhibit survival of nontransformed rat SCG**

<table>
<thead>
<tr>
<th>Apigenin (μmol/L)</th>
<th>AlamarBlue relative fluorescence*</th>
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<tbody>
<tr>
<td>15</td>
<td>102.57 ± 0.876</td>
</tr>
<tr>
<td>30</td>
<td>102.241 ± 0.998</td>
</tr>
<tr>
<td>60</td>
<td>97.62 ± 3.3209</td>
</tr>
<tr>
<td>120</td>
<td>97.884 ± 1.857</td>
</tr>
</tbody>
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NOTE: Rat SCG cells were treated with apigenin for 24 hours and cell survival was quantified by AlamarBlue.

*pNormalized to untreated control ± SE.

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5 Dr. J. Squire, personal communication.

Figure 3. A, apigenin induces apoptosis in NUB-7 and LAN-5. Cells were treated with increasing concentrations of apigenin for 24 h. TUNEL labeling was performed in conjunction with a DAPI counterstain. Total cells were counted in three fields photographed under a UV filter. Apoptotic cells were enumerated and expressed as a percentage of the total DAPI-positive cells per field. Bars, SE. B, apigenin induces PARP cleavage in a dose- and caspase-dependent manner. NUB-7 cells were treated with 15 to 60 μmol/L apigenin, 60 μmol/L naringenin or 100 nmol/L Z-VAD-FMK. Staurosporine (60 μmol/L, 4 h) served as a positive control for PARP cleavage. The cleaved form of PARP was analyzed by Western blot with an antibody against cleaved PARP. C, apigenin induces cell death in a caspase-dependent manner. NUB-7 cells were pretreated with Z-VAD-FMK (100 nmol/L) for 24 h prior to addition of apigenin for 24 h. Cell survival was quantified by trypan blue assay and expressed as dead/total cells counted per field × 100. Each condition was quantified in quadruplicate. P < 0.05. Bars, SE.
Table 3. The effects of apigenin (15–60 μmol/L) on caspase-3 activity in NUB-7 and SK-N-BE(2)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Caspase-3 activity*</th>
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<tbody>
<tr>
<td></td>
<td>NUB-7</td>
</tr>
<tr>
<td>15 μmol/L AP</td>
<td>1.02 ± 0.02</td>
</tr>
<tr>
<td>30 μmol/L AP</td>
<td>1.48 ± 0.27</td>
</tr>
<tr>
<td>60 μmol/L AP</td>
<td>4.91 ± 1.23</td>
</tr>
<tr>
<td>60 μmol/L NA</td>
<td>0.83 ± 0.11</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>9.59 ± 4.26</td>
</tr>
<tr>
<td>Z-VAD-FMK</td>
<td>0.17 ± 1.22</td>
</tr>
</tbody>
</table>

NOTE: Cells were treated with apigenin (AP), naringenin (NA), staurosporine (positive control), and Z-VAD-FMK (broad-spectrum caspase inhibitor).

*Numbers represent relative fluorescence resulting from caspase-mediated specific cleavage of the fluorogenic substrate Ac-DEVD-AMC, normalized to control ± SE.

cytoplasm and nucleus, but seems to be excluded from the nucleus (Fig. 4B). In contrast, NUB-7 cells treated for 4 hours with 60 μmol/L apigenin expressed high levels of p53 relative to control, with most of the protein located throughout the nucleus. This suggests that apigenin mediated nuclear accumulation of p53, where it activated transcription of p21WAF1/CIP1 and Bax. Results were confirmed with an additional p53 antibody, Ab-8 (data not shown). We then asked if wild-type p53 was required for apigenin-induced apoptosis. In apigenin-treated SK-N-BE(2), caspase-3 was not activated as in NUB-7 (Table 3), and there was no PARP cleavage (Fig. 4C). In addition, apigenin did not induce increased p21WAF1/CIP1 or Bax levels in SK-N-BE(2) (Fig. 4C). Apigenin (15–60 μmol/L) did not induce overt cell death of SK-N-BE(2), the mutant p53 cell line (Fig. 2D); there were small increases in death at 60 μmol/L by ~20% and at 120 μmol/L by ~25% which might indicate that at higher concentrations, apigenin induces death by a p53-independent mechanism. However, the lack of caspase activity and PARP cleavage in SK-N-BE(2) at 60 μmol/L suggests that apigenin required wild-type p53 for induction of apoptosis.

We also observed that after 4 hours, apigenin induced an increase in extracellular signal-regulated kinase (ERK) activity, as assessed by using an antibody to phosphorylated and activated ERK (Fig. 4A), suggesting that apigenin requires activated ERK for apoptosis. To test this, NUB-7 cells were pre-treated with the selective ERK inhibitor, U0126, at 20 μmol/L, a concentration that inhibited ERK phosphorylation, for 4 hours and cell viability was assessed. The presence of the ERK inhibitor did not rescue the cells from apigenin-induced cell death (data not shown), indicating that apigenin does not require ERK activity to induce apoptosis.

**Discussion**

In this study, we show that apigenin, a nonmutagenic antitumor flavonoid, exhibits tumor cell–specific effects on survival of neuroblastoma both in vitro and in vivo. We provide the first evidence that apigenin given to mice with neuroblastoma xenograft tumors reduced tumor mass possibly by induction of apoptosis. Subcutaneous xenograft tumor drug testing models are limited to measurement of growth-inhibitory effects of the drug in question, whereas orthotopic models provide information on effects of a drug on growth in a more clinically relevant environment and provide a model for assessment of metastatic effects (50). Because this apoptotic effect was limited to neoplastic cells with no evident toxicity to normal tissue, apigenin represents a potential compound for further development as a neuroblastoma therapeutic agent. In this regard, another flavonoid, flavopiridol, a semisynthetic polyhydroxylated flavone, exhibits in vitro activity and is currently in clinical trials as an antitumor agent for various cancers (51, 52). The mechanism whereby apigenin acts seems to involve p53; apigenin modulated p53 protein levels and accumulation in the nucleus, and induced the expression of the p53 target proteins p21WAF1/CIP1 and Bax. Thus, neuroblastoma tumors, which in virtually all...
cases examined express wild-type p53 that is inactive possibly due to sequestration in the cytoplasm (26, 28, 53, 54), can be chemically targeted by apigenin for activation resulting in apoptosis. It has been shown that the in vitro growth inhibitory effects of flavonoids correlate with variations in chemical structure (6, 19). Flavonoids are characterized by a benzene ring (A) fused with a pyrone ring (C), that carries a phenyl ring (B) in position 2 or 3 (see Fig. 1A). Previous studies have shown that the C ring with oxo function at position 4, and C2-C3 double bond correlate with antiproliferative activity (6, 55). The presence of the C2-C3 double bond strongly correlated with the anti-survival effect in NUB-7 and LAN-5. Naringenin, which is identical to apigenin but lacks the C2-C3 double bond, had no effects on survival or PARP cleavage, further suggesting that the presence of the C2-C3 double bond is necessary for activation of apoptosis. In addition, the presence of hydroxy or methoxy substitutions on the flavone ring structure have been shown to correlate with increased inhibitory activity (6, 7, 18, 19, 56–58), as also shown here by the lack of growth-inhibitory activity of nonhydroxylated flavone and the strong anti-survival effects of hydroxylated apigenin, quercetin, diosmetin, and chrysin. Structural characteristics of flavonoids may also contribute to their modulatory effects on P-glycoprotein (18), protein kinase C (56), phosphatidylinositol 3-kinase (19), cyclin-dependent kinases 1 and 2 (6), mitogen-activated protein kinase (20), and tyrosine kinases (e.g., EGF-R and Her2/neu; refs. 11, 19). A recent study reported that aminoflavone, a compound based on the flavone parent structure of apigenin (Fig. 1A), had potent antiproliferative effects in NUB-7, but not in SK-N-BE(2) (mutant p53). NUB-7 cells were treated with 60 μmol/L apigenin and protein was extracted at 4, 8, 16, and 24 h. Protein was separated by SDS-PAGE and analyzed by Western blot for cleaved PARP, phospho-ERK, p53, p21WAF1/CIP1, Bax, Bcl-XL, and β-actin (loading control). B, apigenin mediates nuclear accumulation of p53 in NUB-7. NUB-7 were treated with 60 μmol/L apigenin followed by immunostaining with anti-p53 and Hoechst nuclear stain. Cells were photographed under 100× magnification, constant exposure time of 1 s. C, SK-N-BE(2) cells were treated with 60 μmol/L apigenin for 4, 8, 16, and 24 h and protein extracts were analyzed by Western blot for cleaved PARP, p53, p21, Bax, and β-actin (loading control).
a breast cancer cell line by activation of aryl hydrocarbon receptor signaling (59). Thus, modifications to the chemical structure might modulate the antitumor activity and potency of apigenin.

The exact molecular targets of apigenin and related flavonoids are currently unknown. Many studies (with some exceptions refs. 11, 18, 56) showed that cells treated in culture respond by modulation of kinase activity, but did not show direct binding to suspected kinase targets via kinase assays done with lysates from untreated cells. Therefore, the observed effects on kinase activity might have been due to an alternate mechanism governing inhibition of cell growth and survival, and not to direct kinase inhibition because the protein lysates tested were from treated cells that were undergoing death. We have determined that apigenin did not directly inhibit AKT by in vitro kinase assays and by Western blot for phosphorylated, active AKT, or tyrosine kinase activity in NUB-7.6 We did observe that the presence of serum in the culture medium decreased the sensitivity of NUB-7 and LAN-5 to the anti-survival effects of apigenin, suggesting that factors in serum mediate resistance to apigenin in neuroblastoma cells, possibly by activation of survival signaling pathways. For example, growth factor signal transduction through the IGF1/IGFIR/Pi3K/AKT axis has been shown to be essential for neuroblastoma survival (60–62), but does not seem to be targeted by apigenin. An alternative mechanism besides kinase inhibition that may be targeted by apigenin is inhibition of survivin, an “inhibitor of apoptosis” protein whose expression in neuroblastoma tumor cells correlates with the clinically aggressive phenotype (63). Silibinin, a flavolignan component of milk-thistle, inhibited survivin mRNA and protein expression in a bladder cancer cell line (64). Further studies will be required to elucidate the precise targets of apigenin in neuroblastoma cells, in particular with regard to the activation of p53-mediated apoptosis.

We show that apigenin induced apoptosis via up-regulation of p53 possibly by increasing p53 protein stability and/or transcription. Apigenin had similar effects on p53 and Bax in human prostate cancer cells (65), which supports our findings. However, our study presents novel functional data (that was not presented in the study by Gupta et al. 65) that the presence of mutant p53 or overexpression of Bcl-XL rescues from apigenin-induced apoptosis. In keratinocytes, apigenin has been shown to stabilize p53 protein by disrupting its interaction with mdm2, which targets p53 for ubiquitination, without modulating p53 mRNA levels (15). In contrast, preliminary real-time PCR studies conducted on NUB-7 cells treated with apigenin showed that p53 mRNA increased at 1 to 4 hours post-treatment.7 However, the mechanism of apigenin-mediated up-regulation and activation of and/or dependence on p53 is not yet confirmed and requires further investigation. The effects of apigenin in isogenic cell line pairs, such as wild-type and mutant p53 colorectal carcinoma cell lines (66), may suggest an additional application for apigenin in other tumor types with functional p53.

In addition to the effects on p53, apigenin mediated a decrease in hyperphosphorylated pRb (Fig. 5), thereby inhibiting E2F-mediated cell cycle progression (67). The hyperphosphorylation of pRb, together with the apigenin-induced increase in the level of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 likely leads to cell cycle arrest. The unscheduled cell cycle arrest in tumor cells that are constitutively cycling could account for the apoptotic actions of apigenin.

Plant-derived anticancer agents have provided novel prototypes for drug design (68, 69). However, the concentration of apigenin (60 μmol/L) used to induce overt apoptosis in our study may not be physiologically achievable in humans. Because concentrations that induce antitumor effects in vitro and in vivo often differ, future experiments need to delineate the specific plasma and

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6 R. Torkin and H. Yeger, unpublished data.
7 R. Torkin, S. Der, and H. Yeger, unpublished data.
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intratumoral levels in order to directly correlate reduction in tumor mass with in vivo concentrations of apigenin. Lower concentrations (<20 μmol/L) also induced apoptosis albeit with slower kinetics. Nevertheless, diets high in fruits and vegetables can lead to consumption of >1 g of flavonoids daily, possibly leading to tissue accumulation (70). Our in vivo mouse study showed that apigenin decreased tumor mass and increased the apoptotic fraction in xenografted tumors, suggesting that apigenin is biologically active after absorption and distribution, possibly via blood circulation. Several studies suggest that flavonoids have limited bioavailability and are metabolized extensively upon ingestion (71). However, epidemiologic data shows that diets rich in flavonoids correlate with decreased incidence of certain cancers, suggesting that ingested flavonoids retain biological activity against tumors and are chemopreventive (69, 71, 72). A recent study showed that epigallocatechin gallate, a polyphenolic compound related to apigenin, underwent structural rearrangements (autooxidation, oxidative addition, and/or condensation) after brief incubation in human or mouse plasma (pH 6.8). The resulting new structures had dramatically increased biological activity, including inhibition of telomerase activity (22). This finding suggests that although the parent flavonoid structures might not be detectable in plasma, biologically active breakdown products might achieve high concentrations and accumulate in tissues. It also suggests that combinations of active agents, due to variable intake of plant-based flavonoids, might yield effective antitumor activity. Identification of the active products would present plant-based flavonoids, might yield effective antitumor activity (22). This finding suggests that although the parent flavonoid structures might not be detectable in plasma, biologically active breakdown products might achieve high concentrations and accumulate in tissues. It also suggests that combinations of active agents, due to variable intake of plant-based flavonoids, might yield effective antitumor activity.

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References

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