Vitexins, Nature-Derived Lignan Compounds, Induce Apoptosis and Suppress Tumor Growth

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Abstract

Purpose: Lignans such as secoisolariciresinol diglucoside in flaxseed, are metabolizes to bioactive mammalian lignans of END and ENL. Because mammalian lignans have chemical structural similarity to the natural estrogen, they are thought to behave like selective estrogen receptor modulators and therefore have anticancer effect against hormone-related cancers. We isolated a series of lignan compounds, named as Vitexins, from the seed of Chinese herb Vitex Negundo.

Experimental Design: We purified several Vitexin lignan compounds. Cytotoxic and antitumor effects were analyzed in cancer cells and in tumor xenograft models. In vivo metabolism of Vitexins was determined in rat.

Results: Contrasts to the classic lignans, Vitexins were not metabolized to END and ENL. A mixture of Vitexins EVn-50 and purified Vitexin compound 6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaldehyde have cytotoxic effect on breast, prostate, and ovarian cancer cells and induces apoptosis with cleavage in poly ADP ribose polymerase protein, up-regulation of Bax, and down-regulation of Bcl-2. This induction of apoptosis seems to be mediated by activation of caspases because inhibition of caspases activity significantly reduced induced apoptosis. We showed a broad antitumor activity of EVn-50 on seven tumor xenograft models including breast, prostate, liver, and cervical cancers. Consistent with in vitro data, EVn-50 treatment induced apoptosis, down-regulated of Bcl-2, and up-regulated Bax in tumor xenografts.

Conclusion: Vitexin is a class of nature lignan compounds, whose action and anticancer effect is mediated by the mechanisms different from the classic lignans. Vitexin-induced antitumor effect and cytotoxic activity is exerted through proapoptotic process, which is mediated by a decreased Bcl-2/Bax ratio and activation of caspases. (Clin Cancer Res 2009;15(16):5161–9)

Lignans are a group of complex polyphenolic antioxidants found in plants. Lignans such as secoisolariciresinol (SDG) found high in flaxseed, have antitumor effect. Clinical studies suggest that lignans are one of the most promising classes of dietary agents for testing in cancer prevention (1-3). In particular, lignan may prevent hormone-dependent diseases such as breast cancer and prostate cancer. Several lignans have been extensively investigated in preclinical tumor xenograft models, prospective and case-control epidemiologic studies, and in some clinical trials (1-7). Many of the dietary plant lignans are converted by intestinal microbiota to mammalian metabolites of END and ENL (8-11). Mammalian lignans have been thought to be the major biologically active lignan, and suggested to be associated with low risk of cancer (4). Although there is no randomized clinical trial data that exist, indicating that lignan can reduce cancer growth, there are several biomarker-based neoadjuvant trials indicating that dietary intake of flaxseed lignan can inhibit tumor cell proliferation and induce apoptosis (2, 5). The ability to inhibit tumor growth kinetics in a neoadjuvant setting has previously been used as a measure of an agent's effectiveness in clinical circumstances (12-14). One neoadjuvant study, in a randomized double-blind placebo-controlled clinical trial, examined the effects of dietary flaxseed on tumor biological markers in postmenopausal patients with newly diagnosed breast cancer (5). In this study, patients were randomized to daily intake of either a 25-g flaxseed-containing muffin or...
breast cancer compared with women who had the lowest intakes. However, these inverse associations are limited to estrogen receptor (ER) and progesterone receptor (PR)-positive breast cancers, for which such inverse associations between breast cancer risk and the intakes of mammalian lignans are also observed.

We reported here an isolation of a unique class of lignan compounds named as Vitexins from the seed of Chinese herb Vitex Negundo. Contrast to the classic lignans, Vitexins did not go through metabolic activation to generate mammalian lignans. Although Vitex Negundo has been traditionally used as a folk medicine for cough, asthma, rheumatism, and arthritis and shown to have anti-inflammatory activities (23-25), we found that Vitexins activate caspases, induce apoptosis, and have broad antitumor activity in cancer xenograft models.

**Materials and Methods**

*Extraction and isolation.* Air-dried seeds of Vitex Negundo (10 kg) were extracted with 40% ethanol (2 x 10 L) and subjected to separation on polyamide chromatography column (30-60 mesh, 10 x 120 cm). After eluting with ethanol-H2O (0%, 40%, 60% and 95%, 32 liters each), the strongest cytotoxicity against MCF-7 was found to be present in the 40% ethanol. The 40% ethanol elution was partitioned between EtOAc and H2O. The EtOAc layer was concentrated to form a mixture of lignan extracts, EVn-50 (81 gms). EVn-50 was first subjected to column chromatography over Sephadex LH-20 (5 x 70 cm) and eluted by increasing concentrations of methanol (between 0 and 60%) in water to give five fractions. Fraction D was crystallized from methanol to yield 6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-7-methoxy-3, 4-dihydro-2-naphthaldehyde (VB1).

**EVn-50 and novel lignan compounds Vitexins.** We isolated a mixture of lignan compounds from Vitex Negundo seed and named this lignan extract as EVn-50. We further purified a total of 15 lignan compounds, named as Vitexins, from the extract EVn-50 and identified two metabolites namely VB-M1 and VB-M2. Among these isolated Vitexins, there are two major lignans: VB1 and VB2 (6-hydroxy-4-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-5-methoxy-3, 4-dihydro-2-naphthaldehyde). High performance chromatography/mass spectrometry analyses showed that the extract EVn-50 contains mainly VB1 and VB2 and the overall lignan compounds in the extract is ~70%. VB1 represents the most abundant lignan, accounting for 38% of total EVn-50; VB2 accounts for 17% of EVn-50 (Fig. 1A). In contrast to the classic lignans SDG or MAT (Fig. 1B and C), all these Vitexins isolated from EVn-50 have a different general structure and belong to the class of neolignan (Fig. 1D).

**Vitexin metabolism and identification of metabolites.** Male Sprague-Dawley rats (n = 4) were given a single gavage of either EVn-50 (100 mg/kg) or SDG (20 mg/kg). All agents were dissolved in 5% Tween 80, 8% DMSO in PBS. Urine and feces within 0 to 48 h were collected through metabolic cages. Urine and fecal samples were extracted with methanol and filtered. The filtrate was concentrated under reduced pressure and subjected to macroporous resin AB-8 with H2O/ethanol in gradient elution. The 10% ethanol and 20% ethanol elution were subjected to Sephadex LH-20 and high performance liquid chromatography on ODS column (C-18, 250 x 4.6 mm; Hypersil). IR spectra were determined on AVATAR 360 FT-IR spectrometer (Nicolet) in KBr pellets. UV spectra were measured on a Shimadzu UV-2450 spectrometer (Shimadzu). HRSI-MS was recorded with Finnigan LCQ-Advantage (Thermo). Nuclear magnetic resonance spectra were measured on Varian-INOVA-400FT spectrometer (Varian) with tetramethylsilane as an internal standard.

**Determination of apoptotic cells.** Apoptotic cells were determined by propidium iodide staining and flow cytometry. Following treatments, cells were fixed and stained with 50 μg/mL propidium iodide for 20 min. The propidium iodide fluorescence was measured with flow cytometer. Quantification of apoptotic cells was determined by measurement of sub-G1 DNA content.
Cytotoxicity assay. Cells were cultured in 5% FCS and incubated with EVn-50 or purified Vitexin compounds for 72 h. To quantitate cytotoxic activities, metabolically active cells were determined by quantitative colorimetric method of MTT assay.

Tumor growth in athymic nude mice. A nude mouse tumorigenic assay was done as we previously described (26, 27). We used female athymic nude mice at the age of 6 to 7 wk for MA782 murine breast cancer cells, MCF-7, T47D, MDA-MB-435s human breast cancer cell, and HeLa human cervical cancer cells. For PC-3 prostate and HepG2 liver cancer cells, male mice at the same age were used. For hormone-dependent T47D and MCF-7 tumor xenografts, 17β-estradiol pellets (0.72 mg/pellet, 60-d releasing; Innovative Research of America) were implanted s.c. 1 d before the injection of tumor cells. For all breast cancer xenografts, ~3 × 10⁶ cells were injected. Each animal received two injections, one on each side, in the mammary fat pads between the first and second nipples. For other human tumor xenografts (PC-3, HepG2, HeLa), ~2 × 10⁶ cells were s.c. injected into the right and left flanks. When tumor xenografts were established, mice bearing tumors were randomly allocated to different treatment groups. Each group has six mice. Drug treatments were either given orally by gavage or administrated by i.p. injection, 3 d/w. Tumor size was determined by three-dimensional measurements (mm) using a caliper.

Detection of tumor apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. As previously described (27), tumor samples were harvested, fixed, and embedded in paraffin for immunohistochemical analysis. The two-step TdT in situ 3,3′-diaminobenzidine apoptotic terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (R&D System) was done according to the manufacturers’ instructions. The apoptotic index was determined by the percentage of cells scored under a light microscope at 200-fold magnification. Three independent observers counted the positive cells and each observer randomly counted three fields. The numbers represent the average percentage of positive cells from nine fields.

Statistical analysis. Results were reported as the mean ± SD for typical experiments done in three replicate samples and compared by...
the Student's t test. Results were considered significantly different for a P value of <0.05. All experiments were done at least twice to ensure reproducibility of the results.

Results

Different metabolism pathway. The biological efficacy of classic lignans is primarily mediated by their metabolized bioactive mammalian lignans END and ENL, which function like phytoestrogens. Because Vitexins have different general structure (Fig. 1) and belong to the new class of neolignan, we were interested in study whether these Vitexin lignans, such as the classic plant lignans, are also metabolized to END and ENL. We fed rat with either SDG or lignan extract EVn-50 by gavage, collected urine and feces samples, and did high performance liquid chromatography/Mass spectrometry analyses on the samples. As expected, two mammalian lignans END and ENL were found in the urine samples from SDG-treated rats (Fig. 2A). However, no END and ENL were found in the urine and feces samples from EVn-50–treated rats; in contrast, among many metabolites, two major gluconate metabolites namely VB-M1 and VB-M2 were identified from EVn-50–treated rats (Fig. 2B). These data indicate that the new class of lignan compounds Vitexins undergoes a different metabolism and doesn't generate mammalian lignans.

EVn-50 has cytotoxic effect and induces apoptosis. We first determined the cytotoxic effect of EVn-50 on a variety of different tumor cells. EVn-50 has cytotoxic effects on MCF-7, ZR-75-1, SK-BR-7, MDA-MB-231, and MDA-MB-435s breast cancer cells, PC-3 and LNCaP prostate cancer cells, and COC1 ovarian cancer cells (Table 1). COC1 is most sensitive cell line to EVn-50–mediated cytotoxicity, and SK-BR-7 is the least sensitive cell line. Because the total lignan compounds in the extract EVn-50 is 70%, it is important to exclude the possibility that the EVn-50–possessed cytotoxicity is not mediated by Vitexin lignans but via other unidentified components. To this end, purified Vitexin compounds have to be investigated. To start a biological study in individual purified Vitexin lignan compound, we picked up the Vitexin compound VB1, which is the most abundant Vitexin lignan and accounts for 38% of total EVn-50. We tested the cytotoxic effect of VB1 on MCF-7, ZR-75-1, MDA-MB-231, and COC1 cells. VB1 possessed strong cytotoxic effects with IC50 ranging from 0.39 to 3.2 μmol/L. These data suggest that Vitexin lignan per se are biologically active and that EVn-50–induced cytotoxicity may be mediated by Vitexin lignans, particularly VB1. Figure 3A is representative cell morphology from VB1-treated MDA-MB-231 breast cancer cells. VB1 had a dose-dependent cytotoxic effect. At the dose range of 4 to 6 μg/mL, over 60% cells died. We also examined the effects of VB1 on induction of apoptosis. Treatment of MDA-MB-231 cells with VB1 induced a significant increase in apoptosis resulting in up to 43% of apoptotic cells (Fig. 3B).

During induction of apoptosis, caspase activates poly (ADP-ribose) polymerase (PARP) and leads to its cleavage into a COOH-terminal fragment, which is considered as an important biomarker of apoptosis. Western analysis showed a robust dose-dependent increase in cleaved PARP in cells treated with EVn-50. As shown in Fig. 3C, we found that the full-size PARP protein (116 kDa) was cleaved to yield an 85-kDa fragment after treatment of cells with EVn-50 at the doses of 4 and 6 μg/mL for 48 hours. Because Bax and Bcl-2 play crucial roles in apoptosis, we next studied the dose-dependent effects of

![Fig. 2. In vivo metabolism of Vitexins. Male Sprague-Dawley rats were given a single gavage of EVn-50 (100 mg/kg) or SDG (20 mg/kg) and the lignan metabolites were analyzed in urine and feces samples as described in Materials and Methods.](image-url)
EVn-50 induces apoptosis of caspases may be one of the underlying mechanisms by which inhibition reached to 92%, which is compatible to the levels of dependent tumor growth inhibition with the maximum tumor growth by 95% and 91%, respectively. EVn-50 had a dose–

We next extend the study on EVn-50–mediated antitumor effect to three human breast cancer xenografts: T47D, MCF-7, and MDA-MB-435s (Fig. 4C). Mice bearing established tumors were treated with Adriamycin as a positive control and with different doses of EVn-50. For hormone-dependent MCF-7 and T47D xenografts, mice were also treated with Tamoxifen. As expected, treatment of mice with Adriamycin inhibited tumor growth by 85%, 73%, and 87% in T47D, MCF-7, and MDA-MB-435s xenografts, respectively. Tamoxifen treatment also significantly suppressed T47D and MCF-7 tumor growth resulting in a 61% to 68% growth inhibition. EVn-50 had a dose-dependent tumor growth inhibition with the maximum tumor inhibition reached to 75% to 82% (by volume) at the end of dosing period of 80 mg/kg. Similar tumor suppressions were also observed when the inhibitions were calculated based on the tumor weights. Oral administration of EVn-50 was well-tolerated in mice with no overt clinical sign of toxicity.

**EVn-50 suppresses growth of liver, prostate, and cervical tumor xenografts.** Because the biological efficacy of classic flaxseed lignan SDG is primarily mediated by its metabolized mammalian metabolites END and ENL, which occurs at the gut and converted by colon microflora (4), the antitumor effect of SDG is usually observed when it is administrated orally. The antitumor effect of EVn-50 on breast cancer xenografts was shown when the drug administrated orally. We were interested in studying whether we can achieve a similar antitumor effect when EVn-50 is administrated nonorally. Here, we showed an antitumor effect EVn-50 on PC-3 prostate, HepG2 liver, and HeLa cervical tumor xenograft models when administrated nonorally but by i.p injection (Fig. 5). For positive controls, we used chemotherapy drugs Adriamycin, 5-fluorouracil, and Cisplatin for PC-3, HepG2, and HeLa xenografts, respectively. As expected, all three chemotherapy drugs induced significant tumor suppression. Repeated i.p. administration of EVn-50 (once every other day for 16 days) also produced significant antitumor activity in mice bearing established PC-3, HepG2, and HeLa xenografts. Maximal inhibition (by volume) of 62% and 71% was observed at the end of highest dosing period of 20 mg/kg for HeLa and HepG2 (Fig. 5D) xenografts, respectively. For PC-3 prostate tumor xenograft, a maximal tumor growth inhibition of 65% was observed at the end of highest dosing period of 30 mg/kg (Fig. 5A). Similar tumor suppressions were also observed when the inhibitions were calculated based on the tumor weights: 67% for HeLa, 56% for PC-3, and 69% for HepG2.

We also analyzed apoptotic index in EVn-50–treated PC-3 tumor xenografts. Immunohistochemical analysis of apoptotic cells using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling reaction showed a significant increase in tumor cell apoptosis in EVn-50–treated PC-3 xenografts, resulting in an average of 3.6-fold increase in apoptotic cells over control xenografts (Fig. 5B). Because EVn-50 treatment was observed to modulate the expression levels of Bax and Bcl-2 in vitro, we determined the effect of EVn-50 on the expression levels of Bax and Bcl-2.

### Table 1. Cytotoxicity of vitexins

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Cytotoxicity IC50 (EVn-50 μg/mL)</th>
<th>VB-1 (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>2.3 ± 0.19</td>
<td>3.2 ± 0.27</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Breast</td>
<td>0.95 ± 0.01</td>
<td>2.1 ± 0.15</td>
</tr>
<tr>
<td>SK-BR-7</td>
<td>Breast</td>
<td>4.8 ± 0.24</td>
<td>—</td>
</tr>
<tr>
<td>MDA-MB-435s</td>
<td>Breast</td>
<td>2.1 ± 0.15</td>
<td>—</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>1.5 ± 0.11</td>
<td>1.8 ± 0.12</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Prostate</td>
<td>1.3 ± 0.09</td>
<td>—</td>
</tr>
<tr>
<td>PC-3</td>
<td>Prostate</td>
<td>0.8 ± 0.06</td>
<td>—</td>
</tr>
<tr>
<td>COC1</td>
<td>Ovarian</td>
<td>0.25 ± 0.019</td>
<td>0.39 ± 0.023</td>
</tr>
</tbody>
</table>

**NOTE:** All cell lines, cultured with 10% FBS, were treated with EVn-50 (ranging from 0.05–20 μg/mL) or VB1 (50 nmol/L to 30 μmol/L) for 72 h. Cytotoxicity was measured by MTT assay. The IC50 values were determined by regression analysis. Data presented here are mean ± SEM of duplicate cultures from two separate experiments.

EVn-50 on the protein levels of Bax and Bcl-2 in the treated cells. Treatment of the cells with EVn-50 induced a significant decrease in Bcl-2 expression with a concomitant increase in the protein level of Bax (Fig. 3C). This resulted in a substantial increase in Bax/Bcl-2 ratio, which favors apoptosis. Purified Vitexin VB1 had a similar effect on induction of Bax and reduction of Bcl-2 (Fig. 3C).

**EVn-50 induces apoptosis via activation of caspases.** To test whether caspases are involved in EVn-50–induced apoptosis, we first evaluated the protein levels of caspase-3 and caspase-9 in EVn-50–treated cells. In most cancer cells, caspases are present in the proforms and require site-specific cleavage of the protein to become active in the apoptotic process. As shown in Fig. 3C, EVn-50 treatment of MDA-MB-231 cells induced a significant and progressive increase in the levels of active caspase-3 and caspase-9 protein with a concomitant decrease in the proforms of caspase-3 and caspase-9. To study whether EVn-50–induced apoptosis is mediated by activation of caspases, we used a general caspases inhibitor Z-VAD-FMK and showed that blocking caspases activity can partially inhibit EVn-50–induced apoptosis. As shown in Fig. 3D, whereas treatment of the cells with EVn-50 (5 μg/mL) resulted in a 43% apoptotic cells, blocking caspases activity significantly reduced EVn-50–mediated cytotoxic effect resulting in a 24% apoptotic cells. Western blot showed that EVn-50–induced activation of caspases-9 was significantly reduced after caspases inhibitor treatment (Fig. 3D, insert). These results suggest that activation of caspases may be one of the underlying mechanisms by which EVn-50 induces apoptosis.

**EVn-50 suppresses tumor growth of mammary xenografts.** To investigate whether EVn-50 inhibits tumor growth, we first studied the tumor-suppressing effect of EVn-50 on MA782 murine breast cancer cells using an orthotopic nude mouse model. Twelve days after implantation of MA782 cells, mice bearing MA782 tumor xenografts were treated with different doses of EVn-50 (day 0). For positive controls, we also treated the mice with either Adriamycin or Tamoxifen. As illustrated in Fig. 4A, treatment of mice with Adriamycin and Tamoxifen inhibited tumor growth by 95% and 91%, respectively. EVn-50 had a dose–dependent tumor growth inhibition with the maximum tumor inhibition reached to 92%, which is compatible to the levels of growth inhibition achieved by Adriamycin. Oral administration of mice with EVn-50 caused 56%, 86%, and 92% tumor inhibition at 20, 40, and 80 mg/kg, respectively. Similar tumor suppressions were also observed when the inhibitions were calculated based on the tumor weights. Figure 4B shows the real tumor size when mice were sacrificed at day 20 following the treatment.
Bax and Bcl-2 in tumors from control and treated xenografts. Consistent with the in vitro data on decreased Bcl-2/Bax ratio and in vivo data on increased apoptosis in tumor xenograft, EVn-50 treatment also significantly increased Bax protein expression and decreased Bcl-2 expression in PC-3 tumor xenograft (Fig. 5C).

**Discussion**

Studies suggest that lignans may be helpful for cancer prevention and treatment, particularly on the malignancy of breast (1, 7). Vitex Negundo is a native to China and its seed is used as antiasthmatics and a cough remedy. Moreover, Vitex Negundo seed is also used, in some regions of China, for the treatment of gynecologic cancers. However, few antineoplastic constituents from Vitex Negundo were ever reported up to now. In the present study, we have isolated a series of novel lignan compounds Vitexins from the seed of Vitex Negundo. We showed that a mixture of Vitexin lignans EVn-50 and the purified Vitexin compound VB1 possessed a broad cytotoxic effect against many different cancer cells. Our data showed that Vitexin treatment of breast cancer cells resulted in (a) cytotoxic effect; (b) apoptosis in a dose-dependent fashion with cleavage in PARP protein, up-regulation of Bax, and down-regulation of Bcl-2; and (c) induction of apoptosis by activation of caspases. These are important observations because regulations of Bax/Bcl-2 and caspases activity become appreciated targets for cancer intervention. Our data suggest that Vitexin-induced cytotoxic effect is through the induction of apoptosis, which is mediated by activation of caspases.
To establish the relevance of these in vitro findings to in vivo situation, we studied the antitumor effect of EVn-50 on eight tumor xenograft models, which include four breast cancer (MA782, MCF-7, T47D, MDA-MB-435s) xenografts, PC-3 prostate, HepG2 liver, and HeLa cervical tumor xenografts. Notably, we observed a significant and dose-dependent broad antitumor effect on all studied tumor xenograft models. For many of the studied tumor xenografts, the maximum tumor inhibition reached to 62% to 92%, which is compatible to the levels of growth inhibition achieved by chemotherapy drugs such as Adriamycin, 5-fluorouracil, and Cisplatin. We provided direct experimental evidence that EVn-50 has potent and broad antitumor efficacy in preclinical models of ectopic growth of breast, prostate, liver, and cervical cancer cells in nude mice (Figs. 4 and 5). Using PC3 tumor xenograft, we showed that in vivo tumor-suppressing effect of EVn-50 could be correlated well with the induction of apoptosis. A significant increase in apoptotic cells and an increase in Bax protein expression with a concomitant decrease in Bcl-2 protein expression in EVn-50–treated PC-3 tumor xenograft suggest the involvement of similar molecular events as those observed in the in vitro studies.

High dietary intakes of plant lignans and high exposure to mammalian lignans are associated with reduced risks of ER- and PR-positive breast cancer, suggesting an antitumor effect of lignans on hormone-dependent cancers (7). Although acting as phytoestrogens, mammalian lignans alters steroid metabolism and signaling, the exact mechanisms for antitumor effect are poorly understood. The antitumor effect of flaxseed lignan often depends on the actual amount of END and ENL, which can be affected by many factors such as the activity of intestinal bacteria and the use of antibiotics. Although Vitexins are structurally quite like classic lignan, e.g., SDG (Fig. 1), the mechanism of action of Vitexin is different from classic lignans. Very importantly, compared with classic plant lignans, e.g., SDG and MAT, these new lignan Vitexin compounds have...
several unique characteristics. First, as illustrated in Fig. 1, the new lignan compounds Vitexins have a unique structure, in which the position 4 and 10 are connected. Lignans belong to this general structure (Fig. 1D) are classified as neolignans. In contrast, the classic lignans have an open position at 4 and 10 (Fig. 1B and C). Second, whereas classic lignans are precursor of active mammalian lignans, which are converted by the colonic bacteria to the two mammalian lignans of END and ENL, isolated Vitexins are converted to different metabolites but not END and ENL. Furthermore, we showed that EVn-50-mediated antitumor effects on tumor xenografts can be achieved both by oral gavage and by i.p. injection, suggesting that, in contrast to classic lignans, the efficacy of Vitexins does not require in vivo metabolic activation by colonic bacteria. Third, although classic lignans act as phytoestrogens, the exact mechanisms are poorly understood. The new lignan compounds Vitexins induce apoptosis by activation of caspases-3 and -9, up-regulation of Bax, and down-regulation of Bcl-2.

Members of the Bcl-2 family proteins are critical regulators of the apoptotic pathway (28). Bcl-2 is an upstream effector molecule in the apoptotic pathway and has been identified as a potent suppressor of apoptosis (29). Bcl-2 is found at inappropriate high levels in more than half of all human tumors (28), thereby rendering tumors escaping apoptosis and undermining therapy. Bcl-2 forms a heterodimer with the apoptotic protein Bax and thereby neutralizes its apoptotic effects. Alternation of Bax/Bcl-2 ratio in favor Bax is a decisive factor to determine whether cells will undergo apoptosis (30). In our study, EVn-50 treatment of MDA-MB-231 cells induced a decrease in Bcl-2 protein expression with an increase in Bax protein expression. Such Bax/Bcl-2 ratio change in favor for apoptosis was also observed EVn-50-treated tumor xenografts (Fig. 5C). Therefore, Vitexin-possessed broad antitumor effect and cytotoxicity might be mediated by alternation of Bax/Bcl-2 ratio in favor Bax and by activation of caspases.

We are well aware that EVn-50 contains 70% of Vitexin lignan compounds, and thus may be multifunctional and affect multiple signaling pathways relevant for cancer growth and progression. Nevertheless, we showed that purified Vitexin compound VB1, the most abundant Vitexin compound in EVn-50, has potent cytotoxic effect, regulates Bax/Bcl-2, and induces apoptosis. In general, an extract from a plant contains
multiple known and unknown ingredients and it is not uncommon that the plant extract may present same or even better therapeutic efficacy and often less toxic effect than pure compound isolated from the extract. We have shown that EVn-50 has broad antitumor activity on breast and many other different tumor xenograft models. Developments of EVn-50 or individual Vitexin compound for potential cancer intervention agents warrant further investigation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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