Verticillin A Overcomes Apoptosis Resistance in Human Colon Carcinoma through DNA Methylation-Dependent Upregulation of BNIP3

Feiyan Liu1,3, Qianqian Liu2, Dafeng Yang3, Wendy B. Bollag4, Keith Robertson5, Ping Wu1, and Kebin Liu3,5

Abstract

Drug resistance is a major cause of failure in cancer chemotherapy. Therefore, identification and combined use of adjuvant compounds that can overcome drug resistance may improve the efficacy of cancer therapy. We screened extracts of Verticillium species-infected mushrooms for antitumor compounds and identified the compound Verticillin A as an inducer of hepatoma cell apoptosis in vitro and an inhibitor of tumor xenograft growth in vivo. Verticillin A exhibited a potent apoptosis-sensitizing activity in human colon carcinoma cells exposed to TRAIL or Fas in vitro. Furthermore, Verticillin A effectively sensitized metastatic human colon carcinoma xenograft to TRAIL-mediated growth inhibition in vivo. At the molecular level, we observed that Verticillin A induces cell-cycle arrest in the G2 phase of the cell cycle in human colon carcinoma cells, markedly upregulating BNIP3 in both hepatoma and colon carcinoma cells. Notably, silencing BNIP3 decreased the sensitivity of tumor cells to Verticillin A–induced apoptosis in the absence or presence of TRAIL. We found that the BNIP3 promoter is methylated in both human hepatoma and colon carcinoma cells and tumor specimens. Verticillin A upregulated the expression of a panel of genes known to be regulated at the level of DNA methylation, in support of the concept that Verticillin A may act by demethylating the BNIP3 promoter to upregulate BNIP3 expression. Taken together, our findings identify Verticillin A as a potent apoptosis sensitizer with great promise for further development as an adjuvant agent to overcome drug resistance in human cancer therapy. Cancer Res; 71(21); 6807–16. ©2011 AACR.

Introduction

The ideal cancer therapy should meet 2 criteria: first, the therapeutic agent has to be effective in killing cancer cells; and second, the agent needs to exhibit low toxicity by showing selectivity for the cancer cells and avoiding systemic or off-target toxicity. Currently, cancer cell resistance to chemotherapeutic drugs and the high cytotoxicity of chemotherapeutic agents are the 2 major problems in human cancer therapy (1). Cancer cells may be intrinsically resistant to chemotherapeutic drugs, especially to cytotoxic agents, prior to treatment. Tumors can also acquire resistance during treatment. Drug resistance, whether intrinsic or acquired, is believed to account for treatment failure in more than 90% of patients with metastatic cancer (1). Therefore, finding ways to overcome drug resistance may greatly improve the survival of patients with cancer. Multiple mechanisms confer cancer cell resistance to chemotherapeutic drugs; however, when it comes to effective eradication of cancer cells by chemotherapies, all roads lead to apoptosis. Essentially, most cytotoxic anticancer drugs currently in clinical use or in clinical trials kill cancer cells through inducing apoptosis. Thus, tumor cell resistance to apoptosis, whether intrinsic or acquired, represents a major challenge in chemotherapeutic intervention of cancer, especially metastatic cancer.

TRAIL (also known as TNFSF10 or APO2L) has been under intense study for its obvious potential as a selective anticancer agent in cancer therapy because it preferentially induces apoptosis in tumor cells but not in normal cells (2, 3). TRAIL-based cancer therapies are now in multiple phase I and II clinical trials to treat human cancer including metastatic human colorectal cancer. However, although human patients exhibit excellent tolerance to humanized TRAIL receptor agonist monoclonal antibodies (mAb), the efficacy of these TRAIL receptor agonist mAbs so far is disappointing. This poor efficacy is obviously expected because most cancer cells, especially metastatic cancer cells, often exhibit a TRAIL resistance phenotype (4–8).

To increase the efficacy of TRAIL-based therapy, various therapeutic agents have been tested for their effectiveness in...
enhancing TRAIL-induced apoptosis in cancer cell lines and in human cancer patients (4, 9–17). These therapeutic agents have shown great promise in enhancing TRAIL efficacy. However, because the most attractive feature of TRAIL therapy is its tumor selectivity—conferred low toxicity, combining cytotoxic agents with TRAIL may bring back toxicity associated with the therapeutic agents. Therefore, identifying novel TRAIL sensitizers with low toxicity and high sensitization activity is urgently needed for TRAIL-based cancer therapy. We report here the identification and characterization of Verticillin A, a natural compound from pathogen-infected mushroom, as a potent TRAIL sensitizer. Our data suggest that Verticillin A holds great promise for further development as a potent sensitizer to enhance the efficacy of TRAIL-based and potentially other cytotoxic agent–based therapies against human colorectal cancer.

Materials and Methods

Purification and identification of Verticillin A

The fresh fruiting bodies of Verticillium species–infected mushroom (Amanita flavohyphasens Alk) were lyophilized and then extracted successively with light petroleum and ethyl acetate. The ethyl acetate extract was fractionated by countercurrent chromatography using a 2-phase solvent system composed of light petroleum, chloroform, and acetonitrile with a volume ratio of 6:1:3. Fractions that exhibited significant cytotoxicity were subjected to semipreparative chromatography on a reverse-phase C8 column (Hypersil ODS 20 × 250 mm²), followed by elution with a mixture of acetonitrile and water with a gradient from 10% to 100%. The structures of purified cytotoxic compounds were determined by electrospray mass spectrometry (ESI-MS) and 1- and 2-dimensional nuclear magnetic resonance (NMR) spectra.

Cell lines

All cell lines except MPNST-724 used in this study were obtained from American Type Culture Collection (ATCC). ATCC characterizes these cells by morphology, immunology, DNA fingerprinting, and cytogenetics. MPNST-724 has been previously characterized (18).

Reagents

Recombinant TRAIL protein was expressed and purified as previously described (19). TRAIL receptor DR5 agonist mAb, CD3 mAb (OKT3), and CD28 mAb (CD28.2) were obtained from BioLegend. Etoposide and cisplatin were obtained from Sigma. Mega-Fas Ligand (FaslL; kindly provided by Drs. Steven Butcher and Lars Damstrup, Topotarget A/S) is a recombinant fusion protein that consists of 3 human FasL extracellular domains linked to a protein backbone comprising the dimmer-forming collagen domain of human adiponectin. The Mega-Fas Ligand was produced as a glycoprotein in mammalian cells in Topotarget A/S.

Mice

Athymic mice were obtained from the NCI-Frederick mouse facility. Six- to eight-week-old female mice were used. Experiments and care/welfare were in agreement with federal regulations and an approved protocol by the GHSU/IACUC committee.

Cell viability and apoptosis assays

Cell viability assay was carried out using the MTT Cell Proliferation Assay Kit (ATCC). For the DNA fragmentation assay, genomic DNA was isolated from cells and analyzed by agarose gel electrophoresis. For the quantitative apoptosis assay, cells were cultured in the absence or presence of recombinant TRAIL, protein with or without Verticillin A (20), followed by staining with propidium iodide (PI; Trevigen) or PI plus Alex Fluor 647 Annexin V (BioLegend) and analyzed by flow cytometry.

Cell surface marker analysis

Tumor cells were stained with anti-TRAIL receptor DR4, DR5, T-R3, and T-R4 mAbs or an isotype-matched control IgG (Alexis Biochemicals) as previously described (20). For Fas receptor analysis, tumor cells were stained with fluorescein isothiocyanate–conjugated anti-human Fas mAb (BD Biosciences). The stained cells were analyzed by flow cytometry.

RT-PCR analysis

Total RNA was isolated from cells or tissues using TRIzol (Invitrogen) and used for semiquantitative and real-time reverse transcription PCR (RT-PCR) analysis of gene expression as described (21, 22). The PCR primer sequences are listed in Supplementary Table S1.

Western blot analysis

Western blot analysis was conducted as previously described (20). The following primary antibodies were obtained from Cell Signaling Biotech: anti-FLIP (1:250 dilution), anti-cIAP1 (1:250), anti-xIAP (1:500), anti-Bad (1:1,000), anti-Bok (1:1,000), anti-p53 (1:500), anti-PUMA (1:2,000), and anti-cleaved PARP (1:500). The following primary antibodies were obtained from Santa Cruz Biotechnology: anti-Bax (1:2,000), anti survivin (1:100), anti-Mcl-1 (1:100), and anti-BNIP3 (1:100). Anti-β-actin was obtained from Sigma and used at 1:8,000 dilution.

In vivo tumor growth inhibition

For HepG2 tumor, athymic mice were subcutaneously inoculated with the tumor cells. The control mice were given saline. The treatment group was intravenously injected with Verticillin A at doses of 1 and 2 mg/kg body weight, respectively. Seven mice were used in each group. For SW620 tumors, SW620 cells (3 × 10⁶ cells per mouse) were injected subcutaneously into athymic mice at the right flank. Three days later, the tumor-bearing mice were treated with Verticillin A (0.125 mg/kg body weight, n = 6). TRAIL (100 mg per mouse, n = 5), and Verticillin A plus TRAIL (n = 5) every 2 days for 14 days. Tumor size was measured in 2 dimensions with a digital micrometer caliper at the indicated time points. Tumor volume was calculated by the formula (tumor length × tumor width²)/2.
Cell-cycle analysis

Cell cycle was analyzed as previously described (19).

MS-PCR analysis

Genomic DNA was isolated using a DNeasy tissue kit (Qiagen). Sodium bisulfite treatment of genomic DNA was carried out using CpGenome Universal DNA modification kit (Chemicon). Methylation-sensitive (MS)-PCR was carried out as previously described (23). The PCR primers are listed in Supplementary Table S1.

Gene silencing

Scramble siRNA (Dharmacon) and human BNIP3-specific siRNA (Santa Cruz, Cat# sc-37451) were used. For HepG2 cells, tumor cells were transiently transfected with the siRNAs using Lipofectamine 2000 (Invitrogen) for approximately 24 hours. Cells were then harvested and reseeded in 24-well plates in the absence or presence of 200 nmol/L Verticillin A for approximately 24 hours before analysis for apoptosis. For SW620 cells, cells were transfected with scramble siRNA or BNIP3-specific siRNAs. Verticillin A was added to the transfection culture 6 hours later to a final concentration of 10 nmol/L and the cells were cultured overnight. Cells were then harvested and reseeded in the presence of 10 nmol/L Verticillin A with or without TRAIL (10 ng/mL) for another 24 hours and analyzed for apoptosis.

Statistical analysis

Where indicated, data were represented as the means ± SD. Statistical analysis was conducted using 2-sided t test, with values of P < 0.05 considered statistically significant.

Results

Purification and identification of Verticillin A as an antitumor cytotoxic agent

The fresh bodies of mushroom (Amanita flavorubescens Alk) infected by fungus Verticillium species were extracted, fractionated, and screened for antitumor cytotoxicity. From approximately 1,500 g fresh mushrooms, we purified a compound (~10 mg) with 99% purity and potent inhibitory activity against HepG2 cells. This compound has a formula of C₃₀H₂₈N₆O₆S₄ and a molecular weight of 696.3. Analysis with ESI-MS and NMR spectrometry, in combination with comparing the crystal structure with the database (24), identified this compound as Verticillin A (Supplementary Fig. S1).

Verticillin A inhibits the growth of hepatoma cells in vitro

Verticillin A exhibited a growth-inhibitory effect on HepG2 cells in a dose-dependent manner with an IC₅₀ value of approximately 62 nmol/L based on MTT assays (Fig. 1A). Analysis of apoptosis markers, including Annexin V plus PI,
PARP cleavage, and genomic DNA fragmentation, revealed that Verticillin A increased PI and Annexin V double-positive cells and induced PARP cleavage and genomic DNA fragmentation in HepG2 cells in a dose-dependent manner (Fig. 1B). Our data thus suggest that Verticillin A inhibits HepG2 cell growth at least partially through inducing apoptosis.

To determine whether the growth-inhibitory effect of Verticillin A can be extended to in vivo tumor growth inhibition, HepG2 cells were injected subcutaneously into athymic mice. Tumor-bearing mice were then treated with Verticillin A by intravenous injection. Verticillin A inhibited tumor growth in a dose-dependent manner, with significant inhibition of HepG2 tumor growth at a dose of 2 mg/kg body weight (Fig. 1C).

**Verticillin A is a potent suppressor of multiple types of tumor cells**

To determine whether Verticillin A inhibits the growth of other types of tumor cells, 6 types of tumor cells were cultured in the presence of different concentrations of Verticillin A and examined for their growth in vitro. Verticillin A significantly inhibited the growth of all of these tumor cells. More importantly, Verticillin A inhibited the growth of these tumor cells with concentrations in the nanomolar range with IC50 from 30 to 122 nmol/L (Supplementary Table S2). To test the toxicity of Verticillin A to normal human cells, we cultured normal human colon epithelial cell line CCD-841 in the presence of Verticillin A and determined that IC50 is 666.7 nmol/L. As expected, CCD-841 is not sensitive to TRAIL, and Verticillin A exhibited no-sensitization effect on CCD-841 cell sensitivity to TRAIL (Supplementary Fig. S2). We then obtained human white blood cells from 2 normal donors and stimulated the T cells in anti-CD3/anti-CD28–coated 96-well plates for 2 days. Verticillin A was then added to the culture for another 24 hours. The IC50 values were 65.1 and 78.8 nmol/L, respectively, for donors 1 and 2. As expected, the activated human normal T cells are not sensitive to TRAIL, and Verticillin A exhibited a small degree of effect on

**Figure 2.** Verticillin A is a potent sensitizer of TRAIL-induced cell death in human colon carcinoma cells. A, Verticillin A overcomes TRAIL resistance of metastatic human colon carcinoma cells. SW620 cells were incubated with various concentrations of Verticillin A for 24 hours and analyzed for cell death by PI staining (left). SW620 cells were also cultured in the absence or presence of Verticillin A (10 nmol/L) overnight, followed by incubation with various concentrations of TRAIL protein (middle) or DRS agonist mAb (right) for 24 hours. The tumor cells were then stained using PI and analyzed for cell death by flow cytometry. Percentage of cell death was calculated by the formula: % PI+ cells of the TRAIL- or DRS-treated cells – % PI+ cells of untreated cells. B and C, Verticillin A sensitizes human colon carcinoma (B), as well as sarcoma, lung, and mammary carcinoma (C) cells, to TRAIL-induced cell death. Tumor cells were treated with Verticillin A (20 nmol/L) overnight, followed by incubation with TRAIL protein (50 ng/mL) and then analyzed for cell death by PI staining (as in A). **P < 0.01 as compared with TRAIL or Verticillin A treatment alone. Columns, mean; bars, SD. D, Verticillin A induces tumor cell apoptosis. SW620 cells were cultured in the absence or presence of Verticillin A (10 nmol/L) overnight, followed by incubation with TRAIL protein (10 ng/mL) for another 24 hours (as in A) and stained with Annexin V and PI. The percentage of apoptotic cells (Annexin V+ and PI+ cells) is indicated in the top right. The tumor cells were also analyzed for PARP cleavage by Western blot analysis (right).
Verticillin A Overcomes Drug Resistance in Colon Cancer

activated human T-cell sensitivity to TRAIL. (Supplementary Fig. S2).

Verticillin A is a potent apoptosis sensitizer that overcomes TRAIL resistance in the metastatic human colon carcinoma cells

Combinational therapy has been shown often to be effective than single-agent therapy in suppression of tumor cell growth (25). SW620 is a metastatic human colon carcinoma cell line that is highly resistant to therapeutic agents including TRAIL (Fig. 2A; ref. 26). We observed that in addition to its ability to inhibit SW620 cell growth, Verticillin A also effectively sensitized SW620 cells to TRAIL, and TRAIL agonist mAb induced cell death at a concentration as low as 10 nmol/L (Fig. 2A). The sensitization effect of Verticillin A was also observed in 6 other human colon carcinoma cells (Fig. 2B). Next, we examined the sensitization effects of Verticillin A in other types of tumor cells. Pretreatment of sarcoma (MPNST724), lung adenocarcinoma (A549), and mammary carcinoma (MCF-7) with Verticillin A also significantly increased the tumor cells sensitivity to TRAIL-induced cell death (Fig. 2C). Analysis of tumor cell death using PI and Annexin V double staining and PARP cleavage indicated that combination treatment of Verticillin A and TRAIL induces apoptosis in SW620 cells (Fig. 2D).

Verticillin A is also a potent apoptosis sensitizer that overcomes resistance to FasL-induced cell death

Because Fas-mediated and TRAIL-induced apoptosis share similar signaling pathways, we next tested whether Verticillin A also sensitizes tumor cells to FasL-induced cell death. Verticillin A pretreatment significantly increased SW620 cells to FasL-induced cell death (Supplementary Fig. S3). We next extended our study to the chemotherapeutic drugs etoposide and cisplatin, 2 anticancer drugs that kill tumor cells by inducing apoptosis. SW620 cells were essentially resistant to both etoposide and cisplatin. Verticillin A dramatically sensitized SW620 cells to both etoposide- and cisplatin-induced cell death (Supplementary Fig. S4).

Verticillin A overcomes metastatic human colon carcinoma TRAIL resistance in vivo

To determine whether the observation that Verticillin A effectively sensitizes metastatic colon carcinoma cells to TRAIL-induced cell death in vitro can be extended to enhance TRAIL-mediated tumor suppression in vivo, SW620 cells were injected subcutaneously into athymic mice. Verticillin A and TRAIL, either used as single agents or in combination, were then injected into tumor-bearing mice. To differentiate the function of Verticillin A in TRAIL sensitization from its direct tumor growth-inhibitory activity, a low dose (0.125 mg/kg body weight) of Verticillin A was used. At this low dose, Verticillin A did not exhibit significant tumor suppression activity (Fig. 3). As expected, SW620 tumors were resistant to TRAIL (Fig. 3; ref. 26). However, combined treatment with low dose of Verticillin A and TRAIL significantly inhibited tumor cell xenograft growth (Fig. 3). Taken together, our data suggest that Verticillin A is an effective sensitizer in TRAIL-mediated suppression of metastatic colon carcinoma in vivo.

Verticillin A induces cell-cycle arrest

In the literature, enhanced cell-cycle arrest has been suggested as a possible mechanism for the synergistic effect of natural compounds combined with therapeutic agents on tumor cell apoptosis (27). To determine whether Verticillin A alters cell-cycle progression, we treated HepG2 and SW620 cells with Verticillin A and analyzed cell cycle in the treated cells. Verticillin A altered cell-cycle progression in SW620 cells (Fig. 4A) but not in HepG2 cells (Fig. 4B). It is apparent that Verticillin A induced a dramatic arrest at the G2 phase of the cell cycle in SW620 cells (Fig. 4C).

Verticillin A upregulates BNIP3 expression

The above observation that Verticillin A induces cell-cycle arrest in SW620 cells, but not in HepG2 cells (Fig. 4), suggests that enhanced cell-cycle arrest is unlikely the sole mechanism of Verticillin A function. Our data also indicate that Verticillin A does not significantly alter TRAIL or Fas receptor expression (Supplementary Fig. S5). Because Verticillin A induced tumor cell apoptosis (Figs. 1 and 2), we analyzed the protein levels of genes with known functions in the mitochondrion-dependent apoptosis pathway. As shown in Fig. 5A, Verticillin A did not alter the expression level of those antiapoptotic genes examined in SW620 cells. However, among the proapoptotic protein examined, Verticillin A increased BNIP3 protein levels in a dose-dependent manner in SW620 cells (Fig. 5B). Analysis of BNIP3 protein level revealed that Verticillin A also increased BNIP3 protein level in HepG2 cells (Fig. 5C).

We next silenced Verticillin A–induced BNIP3 in both HepG2 and SW620 cells and analyzed the effects of loss of BNIP3 on Verticillin A–enhanced apoptosis. Verticillin A upregulated BNIP3 expression and BNIP3 siRNA blocked Verticillin A–induced BNIP3 expression in HepG2 cells (Fig. 6A). Silencing BNIP3 significantly decreased Verticillin A–induced apoptosis.
Verticillin A induced BNIP3 expression in HepG2 cells (Fig. 6B). Verticillin A upregulated BNIP3 expression in SW620 cells and BNIP3 siRNA blocked BNIP3 upregulation by Verticillin A (Fig. 6C). Incubation of BNIP3-transfected and Verticillin A-treated cells with TRAIL showed that silencing BNIP3 significantly reduces Verticillin A-sensitized and TRAIL-induced apoptosis in SW620 cells (Fig. 6D).

**Figure 4.** Effects of Verticillin A on cell-cycle progression. SW620 (A) and HepG2 (B) cells were treated with Verticillin A at the indicated concentrations for 24 hours. Cells were then fixed, stained with PI, and analyzed by flow cytometry. C, quantification of percentages of cells in G1, S, and G2 phases of the cell cycle (as shown in A and B). Columns, mean; bars, SD.

**Figure 5.** Verticillin A upregulates BNIP3 expression in human colon carcinoma and hepatoma cells. Tumor cells were treated with Verticillin A at the indicated concentrations for 24 hours. Cells were then analyzed by Western blot analysis for antiapoptotic (A) and proapoptotic (B and C) proteins as indicated.
Verticillin A upregulates BNIP3 expression potentially through inducing DNA demethylation

Analysis of the human BNIP3 promoter region revealed that the BNIP3 promoter is GC rich and contains CpG islands (Fig. 7A). We then used MS-PCR to analyze the methylation status of BNIP3 in 3 human colon carcinoma cell lines, HepG2 cells, and tumor tissues dissected from 5 paraffin-embedded human colorectal carcinoma specimens (4 liver metastases and 1 primary adenocarcinoma). BNIP3 promoter is methylated in all the cell lines and the tumor specimens examined (Fig. 7B).

The above observations suggest that Verticillin A might activate BNIP3 expression in human cancer cells through inhibiting DNA methylation. To determine whether Verticillin A–inhibited DNA methylation is a general phenomenon, we tested the effects of Verticillin A on the expression of a panel of 4 genes known to be regulated by DNA methylation (23, 28, 29). As expected, azacytidine treatment increased the expression level of BNIP3 and these 4 genes. At the same time, Verticillin A treatment also increased the expression of BNIP3 and these 4 genes. Thus, our data suggest that Verticillin A might function at least partially through upregulating BNIP3 in a DNA demethylation–dependent manner.

Discussion

Verticillin A is a compound of the epidithiodioxopiprazine structural class. In a screening for antitumor cytotoxic natural compounds, we purified a compound from Verticillium-infected mushrooms Amanita flavorubescens Ak and identified this compound as Verticillin A. Neither uninfected Amanita flavorubescens Ak nor the pathogen fungus Verticillium contains Verticillin A, suggesting that Verticillin A is synthesized during the host and pathogen interaction. Here, we showed that Verticillin A is an effective tumor suppressor that induces tumor cell apoptosis at nanomolar concentrations. More importantly, Verticillin A exhibited potent activity as an apoptosis sensitizer that effectively overcame metastatic human colon carcinoma cell resistance to TRAIL-, Fas-, and other...
infected mushrooms as another natural compound that—(35)—dietary isothiocyanate found in broccoli and cauliflower (36). One example of such a compound is sulforaphane, a natural compound isolated from herb Shiraia bambusicola, has also been shown to inhibit epidermal growth factor receptor tyrosine kinase activity and to suppress tumor growth (40). Although, Verticillin A shares some structural similarity with 11,11’-dideoxy-verticillin, Verticillin A apparently possesses very different biological activity. We showed here that Verticillin A induces the expression of BNIP3 in both hepatoma and colon carcinoma cells. We also showed that this Verticillin A–elicited increase in BNIP3 expression either directly induces apoptosis in HepG2 cells or sensitizes the metastatic colon carcinoma cells to TRAIL-induced apoptosis (Fig. 6). BNIP3 is a proapoptotic member of the Bcl-2 family (42) that mediates tumor cell apoptosis (43–47). However, it has also been reported that BNIP3 upregulation is not associated with arsenic trioxide–mediated TRAIL sensitization in human glioma (48), suggesting that the function of BNIP3 in apoptosis might be tumor type or cellular context dependent. Nevertheless, we showed here that Verticillin A upregulates BNIP3 in both human hepatoma and colon carcinoma cells, and BNIP3 upregulation is at least partially responsible for the increased apoptosis in hepatoma and colon carcinoma cells. It should be pointed out that although Verticillin A induces BNIP3 in both colon carcinoma and hepatoma cells, Verticillin A sensitized the human colon carcinoma to TRAIL-induced apoptosis at a low dose (10 nmol/L). In contrast, lower dose (10 nmol/L) of Verticillin A did not overcome HepG2 cell resistance to TRAIL-induced cell death (Supplementary Fig. S6). This difference might be due, at least in part, to the different cell-cycle arrest induction in these 2 types of tumors (Fig. 4). However, whether higher doses of Verticillin A sensitize hepatoma cells to TRAIL-induced apoptosis remains to be determined.

BNIP3 expression has been shown to be regulated by DNA methylation in tumor cells (49). The promoter region of the human BNIP3 gene contains CpG islands (Fig. 7) and inhibition of DNA methylation increases BNIP3 expression in the human colon carcinoma cells (Fig. 7C). We showed here that the BNIP3 promoter is methylated in both human hepatoma and colon carcinoma cells, as well as in human colon carcinoma specimens (Fig. 7B). Furthermore, inhibition of DNA methylation with azacytidine increased BNIP3 expression in both human hepatoma and colon carcinoma cells (Fig. 7C). Thus, our data suggest that Verticillin A increases BNIP3 expression possibly by inhibiting DNA methylation or possesses biological activity as a TRAIL sensitizer. Compared with sulforaphane, which sensitizes tumor cells to TRAIL–induced apoptosis in micromolar concentrations (35–37), Verticillin A sensitizes multiple types of tumor cells to TRAIL–induced apoptosis at nanomolar concentrations, suggesting that Verticillin is potentially a more potent TRAIL sensitizer that warrants clinical testing for its effectiveness in enhancing the efficacy of TRAIL therapy in human cancer patients.

Structurally related Verticillin compounds have been shown to possess biological activities to inhibit induction of several oncopgenes (40, 41). 11,11’-Dideoxy-verticillin, a natural compound isolated from herb Shiraia bambusicola, has also been shown to inhibit epidermal growth factor receptor tyrosine kinase activity and to suppress tumor growth (40). Although, Verticillin A shares some structural similarity with 11,11’-dideoxy-verticillin, Verticillin A apparently possesses very different biological activity. We showed here that Verticillin A induces the expression of BNIP3 in both hepatoma and colon carcinoma cells. We also showed that this Verticillin A–elicited increase in BNIP3 expression either directly induces apoptosis in HepG2 cells or sensitizes the metastatic colon carcinoma cells to TRAIL-induced apoptosis (Fig. 6). BNIP3 is a proapoptotic member of the Bcl-2 family (42) that mediates tumor cell apoptosis (43–47). However, it has also been reported that BNIP3 upregulation is not associated with arsenic trioxide–mediated TRAIL sensitization in human glioma (48), suggesting that the function of BNIP3 in apoptosis might be tumor type or cellular context dependent. Nevertheless, we showed here that Verticillin A upregulates BNIP3 in both human hepatoma and colon carcinoma cells, and BNIP3 upregulation is at least partially responsible for the increased apoptosis in hepatoma and colon carcinoma cells. It should be pointed out that although Verticillin A induces BNIP3 in both colon carcinoma and hepatoma cells, Verticillin A sensitized the human colon carcinoma to TRAIL-induced apoptosis at a low dose (10 nmol/L). In contrast, lower dose (10 nmol/L) of Verticillin A did not overcome HepG2 cell resistance to TRAIL-induced cell death (Supplementary Fig. S6). This difference might be due, at least in part, to the different cell-cycle arrest induction in these 2 types of tumors (Fig. 4). However, whether higher doses of Verticillin A sensitize hepatoma cells to TRAIL-induced apoptosis remains to be determined.

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inducing DNA demethylation. However, how Verticillin A alters DNA methylation to mediate BNIP3 expression remains to be determined.

In conclusion, we have identified the natural compound Verticillin A as a potent cytotoxic agent that has the potential to be developed as a low toxicity anticancer drug. More significantly, we showed that Verticillin A is also a potent apoptosis sensitizer that has great potential to be developed as an effective, yet potentially less toxic, adjuvant agent to overcome drug resistance in cancer chemotherapy against metastatic human colorectal cancer.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

References

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Feiyan Liu, Qianqian Liu, Dafeng Yang, et al.

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