Tetrandrine Inhibits Wnt/β-Catenin Signaling and Suppresses Tumor Growth of Human Colorectal Cancer⑤

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ABSTRACT

As one of the most common malignancies, colon cancer is initiated by abnormal activation of the Wnt/β-catenin pathway. Although the treatment options have increased for some patients, overall progress has been modest. Thus, there is a great need to develop new treatments. We have found that bisbenzylisoquinoline alkaloid tetrandrine (TET) exhibits anticancer activity. TET is used as a calcium channel blocker to treat hypertensive and arrhythmic conditions in Chinese medicine. Here, we investigate the molecular basis underlying TET's anticancer activity. We compare TET with six chemotherapy drugs in eight cancer lines and find that TET exhibits comparable anticancer activities with camptothecin, vincristine, paclitaxel, and doxorubicin, and better than that of 5-fluorouracil (5-FU) and carboplatin. TET IC_{50} is ≤ 5 μM in most of the tested cancer lines. TET exhibits synergistic anticancer activity with 5-FU and reduces migration and invasion capabilities of HCT116 cells. Furthermore, TET induces apoptosis and inhibits xenograft tumor growth of colon cancer. TET treatment leads to a decrease in β-catenin protein level in xenograft tumors, which is confirmed by T-cell factor/lymphocyte enhancer factor and c-Myc reporter assays. It is noteworthy that HCT116 cells with allelic oncogenic β-catenin deleted are less sensitive to TET-mediated inhibition of proliferation, viability, and xenograft tumor growth. Thus, our findings strongly suggest that the anticancer effect of TET in colon cancer may be at least in part mediated by targeting β-catenin activity. Therefore, TET may be used alone or in combination as an effective anticancer agent.

Introduction

Colon cancer is one of the most common malignancies in the United States and is primarily initiated by abnormal activation of the Wnt/β-catenin pathway (Kinzler and Vogelstein, 1996). Despite significant developments in the treatment and substantial benefits that have been achieved for some patients, overall progress has been more modest than had been hoped (Aggarwal and Chu, 2005). Thus, there is a great clinical need to develop new treatment regimens. Herbal and natural products are valuable resources for anticancer drugs (Cragg et al., 2009). Plant-derived active principles and their semisynthetic and synthetic

ABBREVIATIONS: VP-16, etoposide; VM-26, teniposide; 5-FU, 5-fluorouracil; DMSO, dimethyl sulfoxide; EGFP, enhanced green fluorescence protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PBS, phosphate-buffered saline, TCF/LEF, T-cell factor/lymphocyte enhancer factor; TET, tetrandrine; PI, propidium iodide.

analogs have served as major sources for new anticancer drugs (Mann, 2002; Koehn and Carter, 2005). Since 1961, nine plant-derived compounds have been approved for use as anticancer drugs in the United States (Mann, 2002). These agents include vinblastine (Velban; Eli Lilly & Co., Indianapolis, IN), vincristine (Oncovin; Eli Lilly), etoposide (VP-16), teniposide (VM-26), paclitaxel (Taxol; Bristol-Myers Squibb Co., Stamford, CT), vinorelbine (Navelbine; Pierre Fabre Pharmaceuticals Inc., Parsippany, NJ), docetaxel (Taxotere; sanofi-aventis, Bridgewater, NJ), topotecan (Hycamtin; GlaxoSmithKline, Uxbridge, Middlesex, UK), and irinotecan (Camptosar; Pfizer, New York, NY). Several plant-derived anticancer agents, such as flavopiridol, acronycine, bruceantin, and thalicarpin, are currently being used in clinical trials in the United States (Mann, 2002). Thus, natural products have been the mainstay of cancer chemotherapy for the past decades (Mann, 2002).

We have found that a natural product, tetrandrine (TET), exhibits significant anticancer activity. TET (International Union of Pure and Applied Chemistry name: 6,6′,7,12-tetramethoxy-2,2′-dimethyl-1 β-berbaman; Chemical Abstracts Service number 518-34-3; $C_{38}H_{42}N_2O_6$; molecular weight, 622.74988; Supplemental Fig. S1) is a bisbenzylisoquinoline alkaloid purified from the root of *Stephania tetrandra* (or *hang fang ji*) of the Menispermaceae and has been used as an effective antihypertensive and antiarrhythmic agent in Chinese medicine (Wang et al., 2004). Although TET can block calcium channels (King et al., 1988), TET exhibits anti-inflammatory and anticancer activity. Although it has been reported that TET targets certain regulatory signals that are involved in cell cycling and cytotoxicity, the molecular mechanism that underlies its anticancer activity is not fully understood.

Here we investigate the molecular basis underlying the anticancer activity of TET. We find that TET exhibits anticancer activity comparable with camptothecin, vincristine, paclitaxel, and doxorubicin (Pharmacia, Kalamazoo, MI) and better than that of 5-FU and carboplatin. The IC_{50} for TET is ≤5 μM, which is within a range similar to that of doxorubicin, camptothecin, vincristine, and paclitaxel in most of the tested cancer lines. TET exhibits synergistic anticancer activity with 5-FU in colon cancer line HCT116. Furthermore, TET significantly reduces HCT116 cells' migration and invasion activities. TET is shown to induce apoptosis in HCT116 cells and effectively inhibit xenograft tumor growth of colon cancer. Xenograft tumors in TET treatment group exhibit a decreased level of β-catenin protein, which is confirmed by TCF/LEF and target gene c-Myc reporter assays. It is noteworthy that HCT116 cells with allelic deletion of the oncogenic β-catenin are less sensitive to TET-induced inhibition of cell proliferation, cell viability, and xenograft tumor growth. These results strongly suggest that the inhibitory effect of TET on colon cancer cells may be at least in part mediated by targeting β-catenin activity. Our results further indicate that the sensitivity of cancer cells to TET may be determined by the functional status of β-catenin, although further investigation is required. Nonetheless, TET may be used alone and in combination as an effective anticancer agent.

Materials and Methods

Cell Culture. Human cancer lines SW480 and HCT116 (colorectal cancer; also known as parental or HCT116$^{wt/mut}$), MDA-MB-231 and MDA-MB-468 (breast cancer), PC3 and DU145 (prostate cancer),

and MG63 and 143B (osteosarcoma), as well as human embryonic kidney-293 cells, were purchased from the American Type Culture Collection (Manassas, VA) and grown in McCoy's 5A medium or Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 50 U penicillin/streptomycin in 5% CO_2 at 37°C. The oncogenic β-catenin allelic deletion line HCT116$^{wt/ko}$ was derived from the parental HCT116$^{wt/mut}$ line and was kindly provided by Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD).

Chemicals and Drug Preparations. TET, doxorubicin, and vincristine were purchased from Sigma-Aldrich (St. Louis, MO). Camptothecin, carboplatin, and 5-fluorouracil were obtained from ENZO Life Sciences (Plymouth Meeting, PA); and Taxol was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These compounds were dissolved in DMSO to make stock solutions and were kept at −80°C as aliquots. Unless otherwise indicated, other chemicals were from Fisher Scientific (Waltham, MA) or Sigma-Aldrich.

Establishment of Stably Tagged HCT116-Luc Cell Lines. The parental HCT116$^{wt/mut}$ and the oncogenic β-catenin allelic deletion line HCT116$^{wt/ko}$ cells were stably transduced with firefly luciferase by using a retroviral vector expressing firefly luciferase as described previously (Luo et al., 2008a; Su et al., 2009; He et al., 2010). In brief, recombinant retrovirus was packaged in human embryonic kidney-293 cells by cotransfecting cells with pSEB-Luc and pAmpho packaging plasmid using LipofectAMINE (Invitrogen). Pooled stable cells were selected with blasticidin S (0.6 μg/ml) for 7 days. Firefly luciferase activity was confirmed by using the Luciferase Assay kit (Promega, Madison, WI).

MTT Proliferation Assay. A modified MTT assay was used to examine the cell proliferation as described previously (Luu et al., 2005b; Luo et al., 2008a,b; Su et al., 2009; He et al., 2010). In brief, cells were seeded in 96-well plates (10^4 cells/well, 50–70% density). Drugs were added to the cells at variable concentrations or solvent control. At 48 h after treatment, 15 μl of MTT dye solution was added to each well and incubated for an additional 4 h. Thereafter, 100 μl/well Solubilization/Stop Solution was added to terminate the reactions and to dissolve formazan crystals in a humidified atmosphere overnight. Absorbance at 570 nm was measured using a 96-well microplate reader (He et al., 2010).

Boyden Chamber Trans-Well Cell Invasion Assay. The experiments were carried out as described previously (Luo et al., 2004, 2008a,b; Luu et al., 2005a; Si et al., 2006; Su et al., 2009; He et al., 2010). Subconfluent HCT116 cells were trypsinized and washed in Dulbecco's modified Eagle's medium/0.1% bovine serum albumin medium twice. Pre-equilibrated media containing 0.5% fetal bovine serum as a chemoattractant was placed into the bottom chamber of six-well transwell unit (Corning Life Sciences, Lowell, MA). Cells (5×10^4) were placed onto each upper chamber of the transwell unit, in which the polycarbonate 8-μm pore membrane was precoated with 100 μg/ml rat tail type I collagen or matrigel mix (BD Biosciences, San Jose, CA) for 2 h and washed in PBS. Cells were allowed to migrate at 37°C and 5% CO_2 for 3 h. The unattached cells were rinsed with PBS, and the membrane containing attached cells was fixed in 10% formalin and washed with PBS. The cells were stained with hematoxylin and rinsed with water. Cells on the unmigrated side were gently wiped off with a wet cotton-tip applicator, and the membrane was rinsed with water. The membranes containing the migrated cells were dried and mounted onto slides with Permount (Thermo Fisher Scientific, Waltham, MA). The number of migrate cells per high-power fields was determined by averaging 20 randomly counted high-power fields. The assays were performed in duplicate and were reproducible in at least two batches of independent experiments.

Crystal Violet Viability Assay. Crystal violet assay was conducted as described previously (Haydon et al., 2002b; Luo et al., 2004, 2008a,b; Luu et al., 2005a,b; Si et al., 2006; Su et al., 2009; He et al., 2010). HCT116 cells were treated with drugs. At 48 or 72 h after treatment, cells were carefully washed with PBS and stained

with 0.5% crystal violet formalin solution at room temperature for 20 to 30 min. The stained cells were washed with tap water and air-dried for taking macrographic images (Haydon et al., 2002b; Luo et al., 2004, 2008a,b; Luu et al., 2005a,b; Si et al., 2006; Su et al., 2009; He et al., 2010). For quantitative measurement, the stained cells were dissolved in 10% acetic acid (1 ml per well for 12-well plate) at room temperature for 20 min with shaking. A 500-μl portion was taken and added to 2 ml of double-distilled H_2O. Absorbance was measured at 570 to 590 nm (Ishiyama et al., 1996).

Annexin V Staining. HCT116 cells were seeded in 12-well plates and treated with TET at various concentrations for 12 h. Cells were washed with PBS twice and incubated with 500 μl of binding buffer and 2 μl of Annexin V-EGFP fusion protein (GenScript USA Inc., Piscataway, NJ) each well for 5 min, followed by washing with PBS twice. Green fluorescent protein signal was detected under a fluorescence microscope.

Fluorescence-Activated Cell Sorting Analysis. Subconfluent HCT116 cells were seeded in six-well plates and treated with TET at various concentrations for 12 h. The treated cells were harvested, washed twice with PBS, and stained with YO-PRO-1/propidium iodide for 20 min on ice. The stained cells were subjected to flow cytometry. Each assay condition was performed in triplicate.

Vybrant Apoptosis Assay. Subconfluent HCT116 cells were seeded in six-well plates and treated with TET at various concentrations for 12 h. The treated cells were harvested and washed twice with PBS. The resuspended cells (in 1.0 ml) were incubated with 1 μl of Hoechst 33342 (8.1 mM) solution, 1 μl of YO-PRO-1 (100 μM) solution, and 1 μl of propidium iodide (1.5 mM) solution, provided with the Vybrant Apoptosis Assay kit (Invitrogen) for 20 min on ice. The stained cells were collected by a brief centrifugation. The cell pellets were transferred to slides with coverslips and examined under a fluorescence microscope. Live cells were shown in blue fluorescence; apoptotic cells were shown in bright green/blue fluorescence; and necrotic cells were shown in bright red fluorescence. Each assay condition was performed in triplicate.

Transfection and Luciferase Reporter Assay. Firefly luciferase reporter assay was carried out as described previously (Zhou et al., 2002, 2003; Luo et al., 2004, 2008a, 2010; Si et al., 2006; Sharff et al., 2009; Tang et al., 2009). In brief, HCT116 cells were seeded in 25-cm^2 culture flasks and transfected with 3.0 μg per flask of pTOP-Luc or Myc/Max-Luc luciferase reporter (Zhou et al., 2002, 2003) using LipofectAMINE (Invitrogen). At 16 h after transfection, cells were replated in 12-well plates and treated with various concentrations of TET or solvent control. At 36 h, cells were lysed and subjected to luciferase activity assays using Luciferase Assay kit (Promega). Each assay condition was done in triplicate. Luciferase activity was normalized by total cellular protein concentrations among the samples. Reporter activity was expressed as mean ± S.D.

Xenograft Tumor Model of Human Colon Cancer. The use and care of animals was carried out by following the guidelines approved by the Institutional Animal Care and Use Committee. Female athymic nude mice (4–6 weeks old, five mice per group; Harlan, Indianapolis, IN) were used. Subconfluent HCT116-Luc cells were harvested and resuspended in PBS to a final density of 2×10^7 cells/ml. Before injection, cells were resuspended in PBS and analyzed by 0.4% trypan blue exclusion assay (viable cells, >90%). For subcutaneous injection, approximately 10^6 HCT116-Luc cells in 50 μl of PBS were injected into the flanks of each mouse using 27-gauge needles. At 1 week after tumor cell injection, TET was administered at 60 mg/kg body weight to mice once every 2 days via intraperitoneal injection.

Xenogen Bioluminescence Imaging. Small animal whole-body optical imaging was carried out as described previously (Luo et al., 2008a; Su et al., 2009; He et al., 2010). In brief, mice were anesthetized with isoflurane attached to a nose-cone mask equipped with Xenogen IVIS 200 imaging system (Caliper Life Sciences, Hopkinton, MA) and subjected to imaging weekly after subcutaneous injection. For imaging, mice were injected intraperitoneally with D-lucife-

rin sodium salt (Gold Biotechnology, St. Louis, MO) at 100 mg/kg body weight in 0.1 ml of sterile PBS. Acquired pseudo images were obtained by superimposing the emitted light over the grayscale photographs of the animal. Quantitative analysis was done with Xenogen's Living Image V2.50.1 software as described previously (Luo et al., 2008a; Su et al., 2009; He et al., 2010). Animals were sacrificed after 3 weeks, and tumor samples were retrieved for histological examination.

Histological Evaluation and Immunohistochemical Staining. Retrieved tumor tissues were fixed in 10% formalin and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin. For immunohistochemical staining, slides were deparaffinized and then rehydrated in a graduated fashion (Haydon et al., 2002a; Luu et al., 2005a,b; Luo et al., 2008a; Su et al., 2009; He et al., 2010). The deparaffinized slides were subjected to antigen retrieval and probed with an anti-β-catenin antibody (Santa Cruz Biotechnology) or isotype IgG control, followed by incubation with biotin secondary antibodies and streptavidin-horseradish peroxidase. The β-catenin protein was visualized by 3,3′-diaminobenzidine staining (Haydon et al., 2002a; Luu et al., 2005a,b; Luo et al., 2008a; Su et al., 2009; He et al., 2010).

Statistical Analysis. All quantitative experiments were performed in triplicate and/or repeated three times. Data were expressed as mean ± S.D. Statistical significances between vehicle treatment versus drug-treatment were determined by one-way analysis of variance and the Student's t test. A value of $p < 0.05$ was considered statistically significant.

Results

TET Exhibits an Antiproliferative Activity that Is Comparable with Several Commonly Used Chemotherapy Drugs in Human Cancer Cells. Although there have been several reports about the cytotoxicity of TET, it remains unclear how TET's anticancer activity is compared with six commonly used chemotherapy drugs. We sought to compare the antiproliferative activity between TET and six commonly used chemotherapy drugs in human colon cancer (HCT116 and SW480), breast cancer (MDA-MB-468 and MDA-MB-231), prostate cancer (PC3 and DU145), and osteosarcoma lines (MG63 and 143B). In human colon cancer line HCT116 cells, TET was shown to inhibit cell proliferation as effectively as four of the six chemotherapy drugs, including camptothecin, vincristine, paclitaxel, and doxorubicin, whereas TET was more effective in inhibiting cell proliferation than 5-FU and carboplatin (Fig. 1A).

The calculated IC_{50} for TET in the eight tested cancer lines was approximately 5 μM or less, which is lower than that for 5-FU and carboplatin, and within a similar range, if not better, for doxorubicin, camptothecin, vincristine, and paclitaxel in many of the tested cancer lines (Table 1). It is noteworthy that TET seemingly exhibits similar cytotoxicities (IC_{50} range, 1.25–5.7 μM) in the eight tested lines, whereas other drugs exert huge IC_{50} ranges (e.g., doxorubicin, 0.15–7.06 μM; camptothecin, 0.025–5.57 μM; paclitaxel, 0.01 nM to 41.1 μM; and vincristine, 0.013 nM to 100 μM) (Table 1). These results strongly suggest that TET may exhibit strong cytotoxicities and anticancer activities across a board range of cancer lines. Consistent with this possibility was that TET was shown to inhibit cell proliferation of colon cancer and breast cancer lines and an osteosarcoma line with a similar efficacy (Fig. 1B).

TET Synergizes with 5-FU in Antiproliferation Effect on Human Colon Cancer Cells. Most chemotherapies

involves in using a combination of several drugs. It is conceivable that TET may act synergistically with other commonly used anticancer drugs. We examined the synergistic effect between TET and 5-FU, because 5-FU is one of the most commonly used chemotherapy drugs despite its relative high IC_{50} (Table 1). When HCT116 cells were treated with various concentrations of TET and/or 5-FU, we found that in the presence of TET (between 2.5 and 10 μM), 5-FU exhibited much stronger inhibitory effect on cell survival in crystal violet cell viability assay (Fig. 2A). For example, in the presence of 5 μM TET 5-FU significantly inhibited cell proliferation and survival at as low as 10 μM, which was significantly lower than the IC_{50} for 5-FU in HCT116 cells (Fig. 2A and Table 1). Similar results were obtained from MTT proliferation assay, in which HCT116 cell proliferation was significantly inhibited by 5-FU at as low as 2.5 μM when TET

was at or below 5 μM (Fig. 2B). These results suggest that TET may act synergistically with other chemotherapy drugs and be used as an adjuvant agent to reduce the adverse effects associated with these drugs.

TET Inhibits Colony Formation and Cell Migration of Cancer Cells. We sought to examine the possible mechanism behind TET's anticancer activity. When HCT116 cells were treated with TET for 24 h and then replated, the numbers of cell colonies formed in subsequent culture significantly decreased (Fig. 3A). Similar results were obtained from other cancer lines, including SW480 and MG63 cells (data not shown).

We further tested whether TET could affect cancer cell invasion. Using the Boyden chamber trans-well assay, we found that when HCT116 cells were treated with 5 μM TET, the numbers of migrated cells across the extracellular matrix protein-coated membranes significantly decreased (Fig. 3B). TET was shown to inhibit the numbers of migrated HCT116 cells by approximately 75% over that of the control treatment (Fig. 3C). These results have demonstrated that TET can effectively inhibit cancer cell colony formation and cancer cell invasiveness phenotype in vitro.

TET Induces Apoptosis in HCT116 Cells. Because many anticancer agents can induce cell apoptosis, we tested whether TET could induce apoptosis in cancer cells. We used three complementary apoptosis assays. When HCT116 cells were treated with 0, 5, 10, and 20 μM TET for 12 h and stained with Annex V-EGFP fusion protein, we found that TET induced EGFP staining in a dose-dependent fashion (Fig. 4A), indicating that TET can effectively induce the translocation of phosphatidylserines in cell membrane phospholipids from the inner surface to the outer surface during the early stages of apoptosis.

We conducted further analyses to distinguish TET-induced apoptosis from necrosis in cancer cells using the Vybrant Apoptosis Assay kit. During apoptosis, the cytoplasmic membrane becomes slightly permeant. Certain dyes, such as the green fluorescent YO-PROR-1 dye, can enter apoptotic cells, whereas other dyes, such as the red fluorescent dye, propidium iodide (PI), cannot. Thus, the use of YO-PROR-1 dye and PI together provide a sensitive indicator for apoptosis. We treated HCT116 cells with 0, 5, and 10 μM TET for 12 h, and the cells were stained with Hoechst 33342, YO-PRO-1, and PI. We found that both YO-PROR-1 and PI stained significantly increased in a dose-dependent manner after TET treatment (Fig. 4B). Under the above staining conditions, apoptotic cells show green fluorescence, and necrotic/dead cells show red and green fluorescence, whereas viable/live cells show blue fluorescence (Fig. 4B).

The YO-PROR-1 and PI stained cells were also ready for flow cytometry analysis. The percentage of apoptotic cells
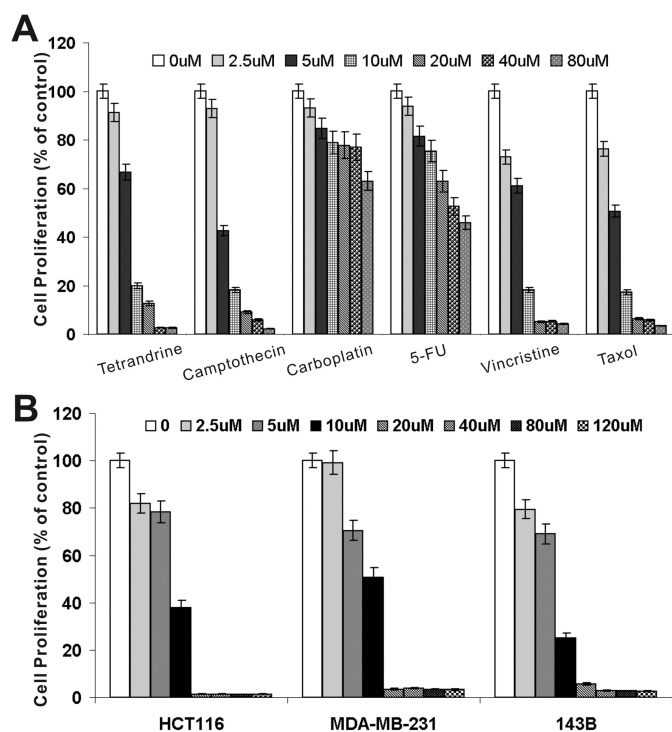
Fig. 1. Antiproliferative activity of TET in human cancer cells. A, antiproliferative activity comparison between TET and other chemotherapy drugs. Subconfluent HCT116 cells were treated with indicated concentrations of TET, camptothecin, carboplatin, 5-FU, vincristine, and paclitaxel for 48 h. The cells were then subjected to MTT assay. Each assay condition was done in triplicate. B, antiproliferative activities of TET in different types of human cancers. Subconfluent human colon cancer line HCT116, breast cancer line MDA-MB-231, and osteosarcoma line 143B were treated with TET at the indicated concentrations for 48 h. The cells were subjected to MTT assay as described under *Materials and Methods*. Each assay condition was done in triplicate.

TABLE 1

IC_{50} comparison of TET and chemotherapy drugs in various types of human cancer lines

Values shown are micromolar unless indicated otherwise.

| Compound	MDA-MB-468	MDA-MB-231	HCT116	SW480	PC3	DU145	MG63	143B
Tetrandrine	1.250	1.890	1.530	3.900	4.339	3.168	5.707	2.169
Doxorubicin	2.323	0.294	0.702	5.782	0.416	0.150	7.061	0.435
5-FU	>500	>500	>500	>500	>500	163.686	>500	>500
Carboplatin	>100	>100	>100	>100	>100	>100	>100	37.629
Camptothecin	0.027	1.226	0.147	5.569	0.025	0.030	0.265	0.022
Paclitaxel	2.381	12.809	0.400 nM	38.682	>100	<0.010 nM	41.107	0.400 nM
Vincristine	<0.100 nM	8.718	0.134 nM	>100	>100	0.0627 nM	29.669	0.013 nM

increased after TET treatment (e.g., from 23.9 to 67.3%), whereas there was a noticeable increase in necrotic cells (e.g., from 4.71 to 11.6%) after TET treatment (Fig. 4C). It is noteworthy that there was a slightly higher background of apoptotic cells (23.9%) in the control group (i.e., 0 μM TET), which may be caused by the presence of the solvent DMSO, and/or by the fact that the cells were grown overnight in low fetal bovine serum medium. Nonetheless, the results from these assays strongly suggest that TET may achieve its anticancer activity at least in part by effectively inducing apoptosis in cancer cells.

TET Inhibits In Vivo Tumor Growth in a Xenograft Tumor Model of Human Colon Cancer Cells. We next investigated the in vivo anticancer activity of TET using a xenograft model of human colon cancer cells. In brief, exponentially growing firefly luciferase-tagged HCT116 cells were injected into the flanks of athymic nude mice. At 1 week after cancer cell injection, TET was intraperitoneally administered (60 mg/kg body weight, once every 2 days). Mice were subjected to Xenogen bioluminescence imaging on a weekly base for additional 3 weeks. As shown in Fig. 5A, the TET treatment group exhibited significantly decreased Xenogen imaging signal, compared with the control group. In fact,

quantitative analysis revealed that TET-mediated inhibition of xenograft tumor growth was statistically significant ($p < 0.01$) at three weeks after treatment, even though the tumors were not completely eliminated (Fig. 5B).

Histological analysis (H and E staining) indicated that TET treatment group exhibited a decreased cellularity in the tumor mass (Fig. 5C). Moreover, we examined the β-catenin expression. As mentioned above, abnormal activation of Wnt/β-catenin signaling pathway is a critical step of colon cancer development (Kinzler and Vogelstein, 1996; Luo et al., 2007). Thus, it is of significance to analyze whether TET would inhibit β-catenin level in tumor cells. As shown in Fig. 5D, the whole-cell and nuclear staining intensities of β-catenin protein was markedly reduced in TET-treated tumors compared with that of the tumors from the control group. Taken together, these in vivo results strongly suggest that TET may inhibit the xenograft tumor growth of colon cancer, possibly by reducing proliferative activity and β-catenin protein level of colon cancer cells.

Colon Cancer Cells with Oncogenic β-Catenin Are More Sensitive to TET-Induced Antiproliferative Activity. We sought to further investigate the mechanism behind the TET-mediated inhibition of Wnt/β-catenin activity. As an initial assessment, we determined the effect of TET on
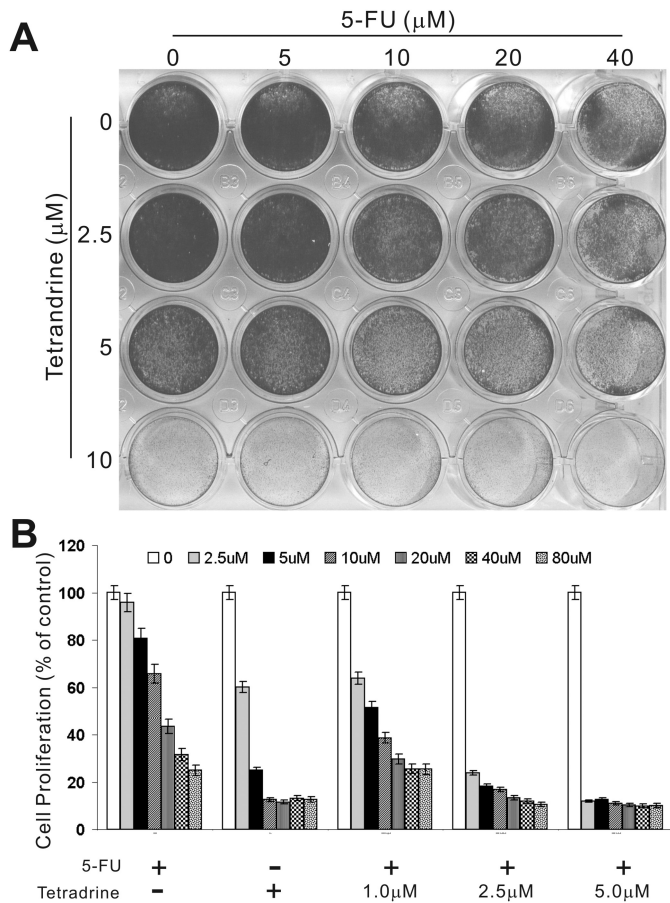
Fig. 2. Synergistic antiproliferation effect between TET and 5-FU. A, TET synergized with 5-FU in inhibiting cancer cell viability. Subconfluent HCT116 cells were treated with TET and 5-FU at the indicated concentrations for 3 days. Viable cells were subjected to crystal violet staining as described under *Materials and Methods*. B, synergistic antiproliferative effect between TET and 5-FU in cancer cells. Subconfluent HCT116 cells were treated with various amounts of 5-FU at a relatively fixed TET concentrations for 48 h and subjected to MTT assays. Each assay condition was done in triplicate.
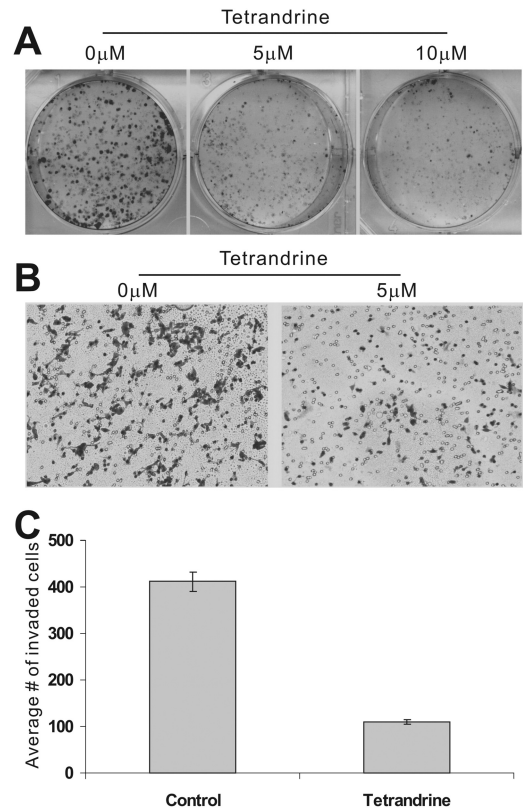

Fig. 3. TET inhibits colony formation and cell migration of cancer cells. A, TET inhibits colony formation. Subconfluent HCT116 cells were treated with TET at the indicated concentrations for 24 h. The cells were replated at a low density in duplicate and maintained in culture for 5 days. The formed colonies were subjected to crystal violet staining. Each assay condition was done in duplicate. Representative results are shown. B and C, TET inhibits cell migration in trans-well assay. Subconfluent HCT116 cells were treated with DMSO (0 μM) or 5 μM TET and used for transwell migration assay as described under *Materials and Methods*. Migrated cells were fixed, stained, and microphotographed (B) and counted as the average number of migrated cells per 5 to 10 high power fields (C). Representative images are shown in B.

the TCF/LEF-responsive reporter, TOP-Luc (He et al., 1998; Zhou et al., 2002, 2003; Tang et al., 2009) and the reporter Myc/Max-Luc of a well characterized downstream target gene c-Myc (He et al., 1998). The parental HCT116 cells were transfected with TOP-Luc and Myc/Max-Luc reporters and treated with 0, 5, or 10 μM TET. We found that TET was shown to effectively inhibit both reporter activities ($p < 0.05$ for TOP-Luc and $p < 0.001$ for Myc/Max-Luc, respectively) (Fig. 6A).

To investigate the role of β-catenin in TET-mediated anticancer activity, we took advantage of the availability of an isogenic allelic deleted HCT116 line of oncogenic β-catenin (Chan et al., 2002). The proliferative activity of the parental HCT116 (i.e., HCT116$^{wt/mut}$) and its allelic deletion of oncogenic β-catenin derivative line (i.e., HCT116$^{wt/ko}$) were first analyzed in the presence of various concentrations of TET.

We found that the parental HCT116 cells were more sensitive to TET treatment than the oncogenic β-catenin-deleted derivative cells ($p < 0.05$), at least at up to the concentration of 5 μM (Fig. 6B). However, the differential response was less pronounced but still significant at 10 μM ($p < 0.05$). Similar results were obtained in the crystal violet cell viability assay. As shown in Fig. 6C, the cell viability in parental HCT116 cells decreased more substantially than that in the oncogenic β-catenin-deleted HCT116 cells.

Finally, we conducted an in vivo experiment to test whether the loss of oncogenic β-catenin rendered HCT116 cells insensitive to TET treatment. We injected the firefly luciferase-tagged HCT116$^{wt/mut}$ and HCT116$^{wt/ko}$ cells into the flanks of athymic nude mice subcutaneously. At 1 week after injection, TET was intraperitoneally administered (60 mg/kg body weight, once every 2 days). Mice were subjected
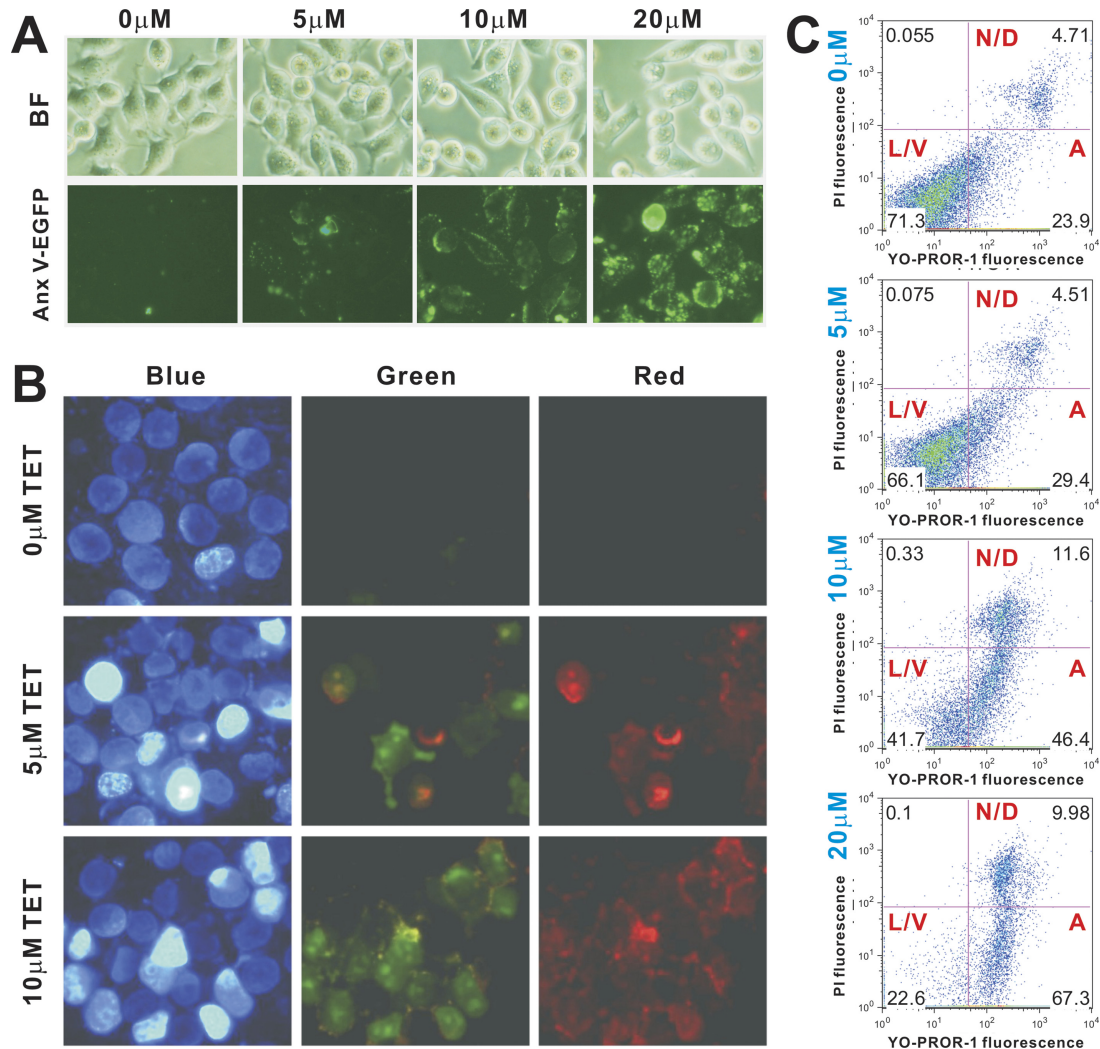
Fig. 4. TET induces apoptosis in HCT116 cells. A, Annexin V-EGFP apoptosis detection. HCT116 cells were seeded in 12-well plates and treated with TET at the indicated concentrations for 12 h. Cells were washed with PBS twice, add incubated with 500 μl of binding buffer and 2 μl of Annexin V-EGFP in each well for 5 min, followed by washing with PBS twice. Green fluorescent protein signal was detected under a fluorescence microscope. B, Vybrant Apoptosis Assay. Subconfluent HCT116 cells were seeded in six-well plates and treated with TET at the indicated concentrations for 12 h. The treated cells were harvested and washed twice with PBS. The resuspended cells (in 1 ml) were incubated with 1 μl of Hoechst 33342 (8.1 mM) solution, 1 μl of YO-PRO-1 (100 μM), solution and 1 μl of propidium iodide (1.5 mM) solution (Vybrant Apoptosis Assay Kit) for 20 min on ice. The stained cells were collected by a brief centrifugation. The cell pellets were transferred to slides with coverslips and examined under a fluorescence microscope. Live cells are shown in blue fluorescence; apoptotic cells are shown in bright green/blue fluorescence; and necrotic cells are shown bright red fluorescence. C, flow cytometery analysis of apoptotic cells. Subconfluent HCT116 cells were seeded in six-well plates and treated with TET at the indicated concentrations for 12 h. The treated cells were harvested, washed twice with PBS, and stained with YO-PRO-1/propidium iodide for 20 min on ice. The stained cells were subjected to flow cytometry. Each assay condition was done in triplicate. Representative results are shown.

to Xenogen bioluminescence imaging on a weekly base. The tumor growth (as judged by the Xenogen signal) was significantly inhibited in the parental HCT116 group after 3-week treatment, whereas the HCT116$^{wt/ko}$ group had no significant reduction in tumor growth (Fig. 6D). Taken together, these above in vitro and in vivo results strongly suggest that
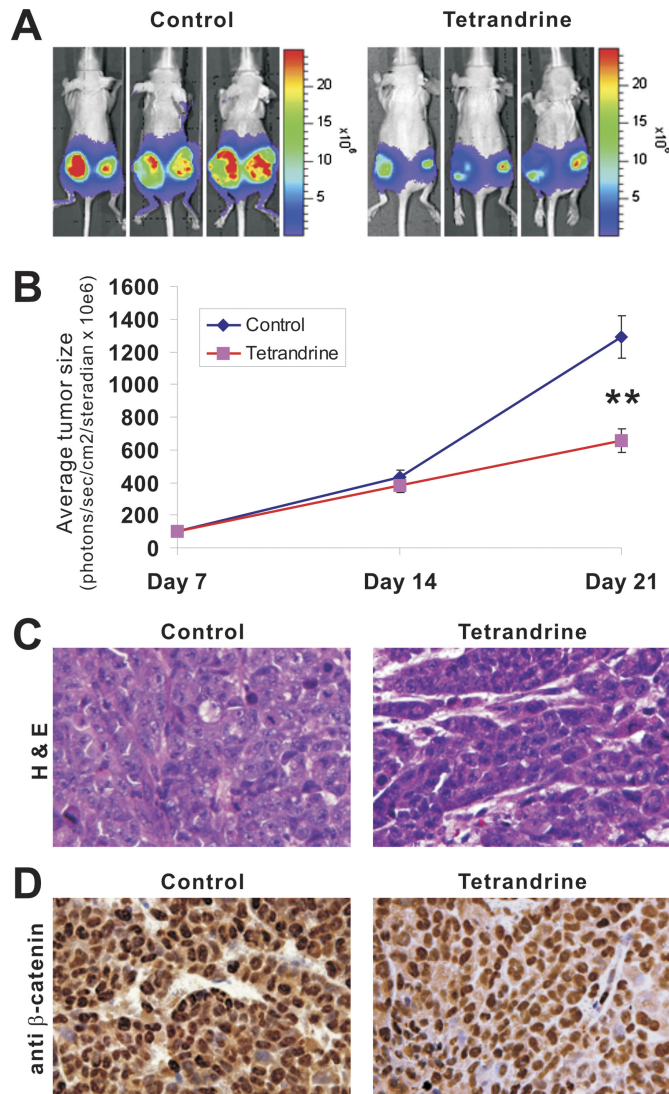

Fig. 5. TET inhibits in vivo tumor growth in a xenograft tumor model of human cancer cells. A, monitoring tumor growth by using Xenogen bioluminescence imaging. Exponentially growing firefly luciferase-tagged HCT116 cells were injected into the flanks of athymic nude mice (five mice/group; 10^6 cells/injection site). At 1 week after cancer cell injection, TET was intraperitoneally administered (60 mg/kg body weight, once every 2 days). Mice were subjected to Xenogen bioluminescence imaging on a weekly base. Representative Xenogen imaging results at week 3 are shown. B, quantitative analysis of Xenogen bioluminescence imaging data. Acquired weekly imaging data were analyzed as described under *Materials and Methods*. Average tumor size was represented by imaging signal intensities (in photons per second per steradian). ∗∗, $p < 0.01$. C, histological examination of xenograft tumor samples. Retrieved tumor samples were fixed, embedded, and subjected to hematoxylin and eosin staining. Representative images are shown (original magnification, 300×). D, expression of β-catenin in xenograft tumors. Retrieved tumor samples were prepared as described in C. Tumor sections were blocked and probed with an anti-β-catenin antibody, followed by incubating with a horseradish peroxidase-conjugated secondary antibody. The presence of β-catenin protein was visualized by developing the slides with a 3, 3'-diaminobenzidine staining kit. Representative staining results are shown (original magnification, 300×).

the inhibitory effect of TET on colon cancer cells may be at least in part mediated by β-catenin activity. Our results also indicate that the sensitivity of cancer cells to TET may be determined by the functional status of β-catenin, although further investigation is required.

Discussion

We investigated the molecular mechanism underlying the anticancer activity of a natural product, TET, in human cancer, particular in colon cancer. Although cancer treatment options have substantially increased and substantial benefits have been achieved for some patients, overall progress has been more modest than had been hoped (Aggarwal and Chu, 2005). Thus, there is a great clinical need to develop new treatment regimens. We have found that a bisbenzylisoquinoline alkaloid, TET, exhibits significant anticancer activity. However, the molecular mechanism that underlies its anticancer activity is not fully understood (Wang et al., 2004).

Here, we compared the antiproliferative activity of TET with other six commonly used chemotherapy drugs in a panel of eight lines of different cancers. We found that TET exhibits comparable anticancer activity with camptothecin, vincristine, paclitaxel, and doxorubicin. The IC$_{50}$ for TET was ≤5 μM in most of the tested cancer lines. TET exhibited synergistic anticancer activity with 5-FU in colon cancer line HCT116. Furthermore, TET significantly reduced HCT116 cells migration and invasiveness. TET was shown to induce apoptosis in HCT116 cells and effectively inhibited xenograft tumor growth of colon cancer. The xenograft tumors derived from TET treatment group exhibited a decreased level of β-catenin protein, which was further confirmed by TCF/LEF and c-Myc reporter assays. It is noteworthy that HCT116 cells with allelic deletion of the oncogenic β-catenin were insensitive to TET in cell proliferation, cell viability, and xenograft tumor growth. These in vitro and in vivo results strongly suggest that the inhibitory effect of TET on colon cancer cells may be at least in part mediated by β-catenin activity. Our results also indicate that the sensitivity of cancer cells to TET may be affected by the functional status of β-catenin, although further investigation is required.

TET was originally identified as a calcium channel antagonist (Wang et al., 2004). As a Ca^{2+} antagonist, TET can inhibit extracellular Ca^{2+} entry, intervene in the distribution of intracellular Ca^{2+}, maintain intracellular Ca^{2+} homeostasis, and then disrupt the pathological processes (Wang et al., 2004). As shown in whole-cell patch-clamp recordings, TET blocked bovine chromaffin cells' voltage-operated Ca^{2+} channel current (Wang et al., 2004). The antihypertensive effects of TET have been demonstrated in experimental hypertensive animals and in hypertensive patients (Wang et al., 2004). Recent studies showed that modulation by M receptor is one of the pharmacological mechanisms of cardiovascular effects of TET (Wang et al., 2004). TET has been shown to inhibit proliferation of vascular smooth muscle cells, induce and sensitize vascular smooth muscle cells to proapoptosis stimulation, improve the endothelial function, and increase NO production (Wang et al., 2004). These results suggest that TET was not only an antihypertensive drug but also an excellent drug to reverse cardiac and vascular remodeling.

Recent studies indicate that TET exhibits anti-inflammatory and anticancer activity (Xu et al., 2006; Liu et al., 2008; Chen et al., 2009; Wu et al., 2010). It has been reported that TET may target regulatory signaling pathways that are involved in cell cycling and cytotoxicity (Lee et al., 2002; Meng et al., 2004; Ng et al., 2006). In this study, we have demonstrated that TET effectively targets Wnt/β-catenin signaling pathway in human colon cancer cells, suggesting that inhibition of Wnt/β-catenin signaling pathway may at least in part account for TET's anticancer activity in colon cancer. Our findings are supported by a recent report in which the activation of glycogen synthase kinase 3β via AKT inhibition induced by TET resulted in enhanced phosphorylation and proteolysis of cyclin D, activation of caspase 3, and subsequent cleavage of poly(ADP-ribose) polymerase (Chen et al., 2008).

Nonetheless, it is conceivable that other signaling pathways may also participate in TET's anticancer activity, particularly in noncolon cancers in which the possible pathogenic role of TET may be less characterized. For example, TET-loaded nanoparticles were shown to activate reactive oxygen species-dependent c-Jun NH2-terminal kinase and caspase 3 in Lovo cells (Li et al., 2010). It was reported that TET-induced apoptosis might be at least partially related to

activation of the mitogen-activated protein kinase signaling pathway in human and mouse cancer cells (Nomura et al., 2007; Cho et al., 2009; Wu et al., 2010). Treatment of HepG2 cells with TET caused the up-regulation of p53, down-regulation of Bcl-X(L), cleavage of Bid and Bax, and release of cytochrome c, which were accompanied by activation of caspases 9, 3, and 8 (Oh and Lee, 2003). Furthermore, TET was shown to inhibit the expression of vascular endothelial growth factor in glioma cells, induce cytotoxicity effect on the ECV304 cells, and suppress angiogenesis (Chen et al., 2009).

As for many small molecules, it is likely that TET has multiple cellular targets. Future studies should be directed to the identification of TET target proteins, which would aid us to elucidate the molecular mechanism underlying TET anticancer activity. On the other hand, the possible in vivo toxicities of TET should be thoroughly evaluated. It is conceivable that more effective and safer TET derivatives can be generated as a new generation of anticancer agents. Taking together, we have demonstrated that TET exhibits effective anticancer activity, which may be at least in part mediated by targeting β-catenin activity. These findings suggest that TET may be used alone or in combination as a potential anticancer agent.
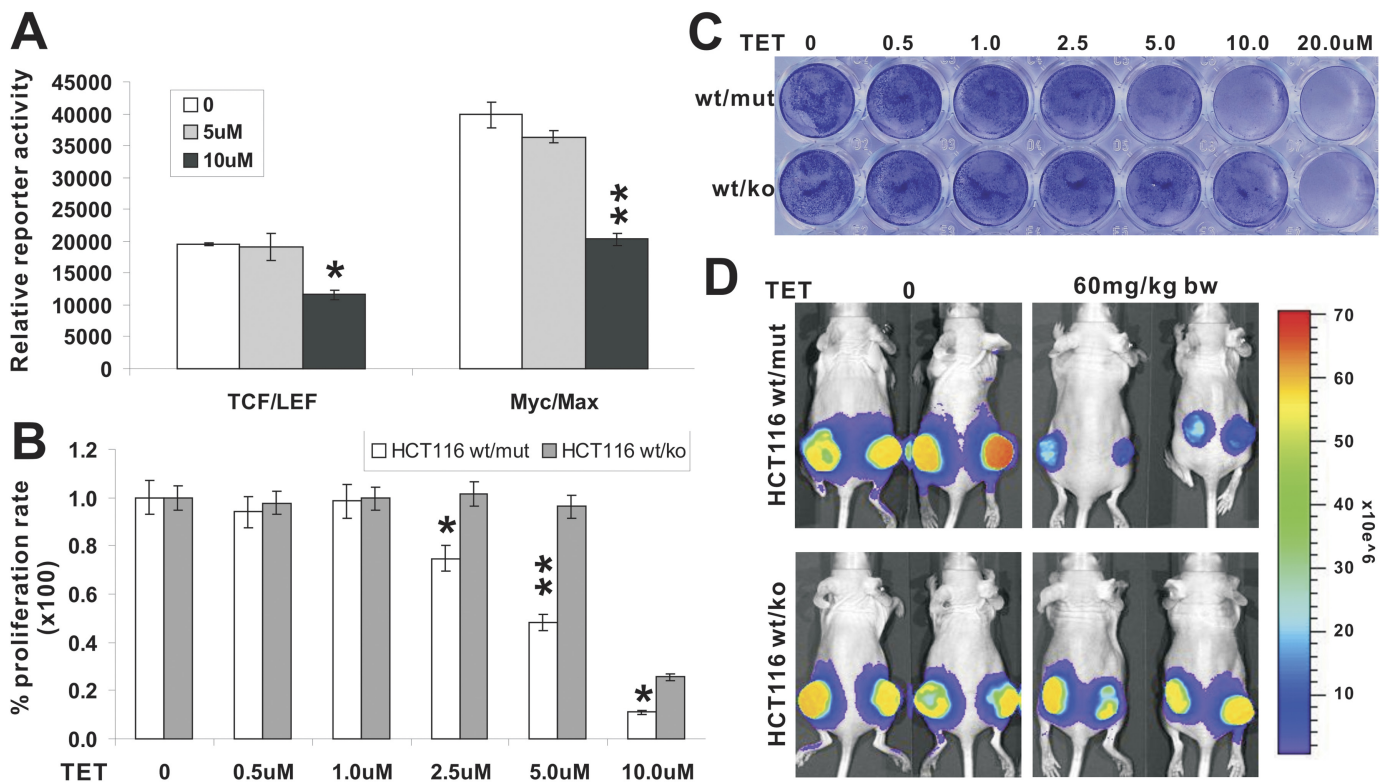
Fig. 6. Colon cancer cells with oncogenic β-catenin are more sensitive to TET-induced antiproliferative activity. A, TET inhibits β-cattenin/Tcf transcriptional activity and the Wnt target c-Myc. The HCT116 (i.e., parental line or HCT116$^{wt/mut}$) cells were plated in 25-cm^2 flasks and transfected with the β-catenin/Tcf reporter pTOP-Luc or Myc/Max reporter. At 15 h after transfection, cells were replated in 24-well plates and treated with 0, 5, or 10 μM TET. Firefly luciferase activities were measured 15 h after treatment. Each assay condition was done in triplicate. *, $p < 0.05$; **, $p < 0.001$. B, effect on oncogenic β-catenin on TET-induced antiproliferative activity. The parental HCT116 (i.e., HCT116$^{wt/mut}$) and its allelic deletion of oncogenic β-catenin derivative line (i.e., HCT116$^{wt/ko}$) were plated in 24-well plates. MTT assay was carried out as described in Fig. 1 and under Materials and Methods. *, $p < 0.05$; **, $p < 0.01$. C, TET inhibits cancer cell viability more effectively in the presence of oncogenic β-catenin. Subconfluent parental HCT116$^{wt/mut}$ and oncogenic β-catenin deleted HCT116$^{wt/ko}$ cells were treated with TET at the indicated concentrations for 3 days. Viable cells were subjected to crystal violet staining as described under Materials and Methods. D, colon cancer cells with oncogenic β-catenin are more sensitive to TET-mediated inhibition of xenograft tumor growth. Exponentially growing firefly luciferase-tagged HCT116$^{wt/mut}$ and HCT116$^{wt/ko}$ cells were collected and injected into the flanks of athymic nude mice (10^6 cells/injection site) as described in Fig. 5 and under Materials and Methods. At 1 week after cancer cell injection, TET was intraperitoneally administered (60 mg/kg body weight, once every 2 days). Mice were subjected to Xenogen bioluminescence imaging on a weekly base. Representative Xenogen imaging results at week 3 are shown.

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