

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

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Running Title: Tetrandrine Targets Wnt Pathway in Human Cancer

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Text Statistics

of text pages: 32
of tables: 1
of figures: 6
of references: 38
of words in the Abstract: 234
of words in Introduction: 513
of words in Discussion: 846

List of Non-Standard Abbreviations

5-FU, 5-fluorouracil
ANOVA, analysis of variance
BSA, bovine serum albumin
DMSO, dimethyl sulfoxide
DMEM, Dulbecco's modified Eagle's medium
EGFP, enhanced green fluorescence protein
HRP, horseradish peroxidase
IC₅₀, half maximal inhibitory concentration
i.p., intraperitoneal (injection)
MTT, dimethyl thiazolyl diphenyl tetrazolium assay
PBS, phosphate buffered saline
TCF/LEF, T-cell factor/lymphocyte enhancer factor
TET, tetrandrine

ABSTRACT

As one of the most common malignancies, colon cancer is initiated by abnormal activation of 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, taxol and adriamycin, and better than that of 5-FU and carboplatin. TET IC_{50} is $\leq 5\mu M$ 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 TCF/LEF and c-Myc reporter assays. Interestingly, 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 US, and is primarily initiated by abnormal activation of Wnt/ β -catenin pathway (Kinzler and Vogelstein, 1996). Despite significant developments in the treatment 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. Herbal and natural products are valuable resources for anticancer drugs (Cragg et al., 2009). Plant-derived active principles and their semi-synthetic and synthetic analogs have served as one of the major sources for new anti-cancer drugs (Koehn and Carter, 2005; Mann, 2002). Since 1961, nine plant-derived compounds have been approved for use as anticancer drugs in the US (Mann, 2002). These agents include vinblastine (Velba), vincristine (Oncovin), etoposide (VP-16), teniposide (VM-26), taxol (Paclitaxel), navelbine (Vinorelbine), taxotere (Docetaxel), topotecan (Hycamtin), and irinotecan (Camptosar). Several plant-derived anticancer agents, such as Flavopiridol, Acronyciline, Bruceantin, and Thalicarpin, are currently being used in clinical trials in the US (Mann, 2002). Thus, natural products have been the mainstay of cancer chemotherapy for the past decades (Mann, 2002).

Recently, we have found that a natural product tetrandrine (TET) exhibits significant anticancer activity. TET is a bisbenzylisoquinoline alkaloid purified from the root of *Stephania tetrandra* (or *hang fang ji*) of the Menispermaceae (IUPAC name, 6,6',7,12-tetramethoxy-2,2'-dimethyl-1 beta-berbaman; CAS #518-34-3; C₃₈H₄₂N₂O₆; MW, 622.74988; Supplemental Fig. 1), and has been used as an effective antihypertensive and anti-arrhythmic 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. While it has been reported that TET targets certain regulatory signals that are involved in cell cycling and cytotoxicity, 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, taxol and adriamycin, and better than that of 5-FU and carboplatin. The IC_{50} for TET is $\leq 5\mu M$, which is within a similar range to that of adriamycin, camptothecin, vincristine and taxol 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. Mechanistically, 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. Interestingly, 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; aka, 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 HEK-293 cells, were purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in the McCoy's 5A medium or DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 50U penicillin

/streptomycin in 5% CO₂ 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 of Johns Hopkins Oncology Center, Baltimore, MD.

Chemicals and Drug Preparations. TET, adriamycin and vincristine were purchased from Sigma-Aldrich (St. Louis, MO). Camptothecin, carboplatin, 5-fluorouracil were obtained from ENZO Life Sciences /BIOMOL (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 (Pittsburgh, PA) 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 (He et al., 2010; Luo et al., 2008a; Su et al., 2009). Briefly, recombinant retrovirus was packaged in HEK-293 cells by co-transfecting cells with pSEB-Luc and pAmpho packaging plasmid using LipofectAMINE (Invitrogen). Pooled stable cells were selected with blasticidin S (0.6 μ g/ml) for 7 days. Firefly luciferase activity was confirmed by using Promega's Luciferase Assay kit (Promega, Madison, WI).

MTT Proliferation Assay. A modified MTT assay was used to examine the cell proliferation as described (He et al., 2010; Luo et al., 2008a; Luo et al., 2008b; Luu et al., 2005b; Su et al., 2009). Briefly, cells were seeded in 96-well plates (10⁴ cells/well, 50-70% density). Drugs were added to the cells at variable concentrations or solvent control. At 48h after treatment, 15 μ l MTT dye solution were added to each well and incubated for additional 4h. Subsequently, 100 μ l/well Solubilization/Stop Solution were 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 micro-plate

reader (He et al., 2010).

Boyden Chamber Trans-Well Cell Invasion Assay. The experiments were carried out as previously described (He et al., 2010; Luo et al., 2004; Luo et al., 2008a; Luo et al., 2008b; Luu et al., 2005a; Si et al., 2006; Su et al., 2009). Subconfluent HCT116 cells were trypsinized and washed in DMEM/0.1% BSA medium twice. Pre-equilibrated media containing 0.5% FBS as a chemoattractant was placed into the bottom chamber of 6-well transwell unit (Corning Costar, Corning, NY). 5×10^4 cells were placed onto each upper chamber of the transwell unit, in which the polycarbonate 8 μm pore membrane was pre-coated with 100 $\mu\text{g/ml}$ of rat tail type I collagen or matrigel mix (BD Biosciences) for 2h and washed in PBS. Cells were allowed to migrate at 37°C and 5% CO_2 for 3h. The unattached cells were rinsed off with PBS and the membrane containing attached cells were 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. The number of migrated cells per high power fields (hpf) was determined by averaging twenty randomly counted hpfs. 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 (Haydon et al., 2002b; He et al., 2010; Luo et al., 2004; Luo et al., 2008a; Luo et al., 2008b; Luu et al., 2005a; Luu et al., 2005b; Si et al., 2006; Su et al., 2009). Experimentally, HCT116 cells were treated with drugs. At 48h or 72h after treatment, cells were carefully washed with PBS and stained with 0.5% crystal violet formalin solution at room temperature for 20-30 min. The stained cells were washed with tap water and air dried for taking macrographic images (Haydon et al., 2002b; He et al., 2010; Luo et al., 2004; Luo et al., 2008a; Luo et al., 2008b; Luu et al., 2005a; Luu et al., 2005b; Si et al., 2006; Su et al., 2009). 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. 500 μ l were taken and added to 2 ml ddH₂O. Absorbance at 570-590 nm was measured (Ishiyama et al., 1996).

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

Fluorescence-Activated Cell Sorting (FACS) Analysis. Subconfluent HCT116 cells were seeded in 6-well plates and treated with TET at various concentrations for 12h. 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.

Vybrant Apoptosis Assay. Subconfluent HCT116 cells were seeded in 6-well plates and treated with TET at various concentrations for 12h. The treated cells were harvested and washed twice with PBS. The resuspended cells (in 1.0 ml) were incubated with 1 μ l of Hoechst 33342 (8.1mM) solution, 1 μ l of YO-PRO-1 (100 μ M) solution and 1 μ l of propidium iodide (1.5 mM) solution, provided with the Vybrant Apoptosis Assay kit (Invitrogen/Molecular Probes, Eugene, OR) for 20min 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 bright red fluorescence. Each assay condition was done in triplicate.

Transfection and Luciferase Reporter Assay. Firefly luciferase reporter assay was carried out as described previously (Luo et al., 2010; Luo et al., 2004; Luo et al., 2008a; Sharff et al., 2009; Si et al., 2006; Tang et al., 2009; Zhou et al., 2003; Zhou et al.,

2002). Briefly, HCT116 cells were seeded in 25 cm² culture flasks and transfected with 3.0µg per flask of pTOP-Luc or Myc/Max-Luc luciferase reporter (Zhou et al., 2003; Zhou et al., 2002) using LipofectAMINE (Invitrogen). At 16h post transfection, cells were replated in 12-well plates and treated with various concentrations of TET or solvent control. At 36h, 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 week old, 5 mice per group, Harlan Sprague-Dawley, Indianapolis, IN) were used. Subconfluent HCT116-Luc cells were harvested and resuspended in PBS to a final density of 2×10^7 cells/ml. Prior to injection, cells were resuspended in PBS and analyzed by 0.4% trypan blue exclusion assay (viable cells > 90%). For subcutaneous injection, approximately 1×10^6 HCT116-Luc cells in 50 µl PBS were injected into the flanks of each mouse using 27G needles. At 1 week after tumor cell injection, TET was administered at 60 mg/kg body weight to mice once every two days via *i.p.* injection.

Xenogen Bioluminescence Imaging. Small animal whole body optical imaging was carried out as described (He et al., 2010; Luo et al., 2008a; Su et al., 2009). Briefly, 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 (*i.p.*) with D-Luciferin sodium salt (Gold Biotechnology, St. Louis, MO) at 100 mg/kg body weight in 0.1 ml 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 (He et al., 2010; Luo et

al., 2008a; Su et al., 2009). Animals were sacrificed after 3 weeks, and tumor samples were retrieved for histologic examination.

Histologic 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 (H & E). For immunohistochemical staining, slides were deparaffinized and then rehydrated in a graduated fashion (Haydon et al., 2002a; He et al., 2010; Luo et al., 2008a; Luu et al., 2005a; Luu et al., 2005b; Su et al., 2009). 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-HRP. The β -catenin protein was visualized by 3,3'-diaminobenzidine staining (Haydon et al., 2002a; He et al., 2010; Luo et al., 2008a; Luu et al., 2005a; Luu et al., 2005b; Su et al., 2009).

Statistical Analysis. All quantitative experiments were performed in triplicate and/or repeated 3 times. Data were expressed as mean \pm standard deviation (S.D.). Statistical significances between vehicle treatment vs. drug-treatment were determined by one-way ANOVA and the Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

TET Exhibits an Anti-Proliferative Activity That Is Comparable to Several Commonly-Used Chemotherapy Drugs in Human Cancer Cells. While 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 anti-proliferative 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, taxol and adriamycin, while TET was more effective in inhibiting cell proliferation than 5-FU and carboplatin (**Fig. 1A**).

The calculated IC_{50} for TET in the tested 8 cancer lines was approximately $5\mu\text{M}$ or less, which is lower than that for 5-FU and carboplatin, and within a similar range, if not better, for adriamycin, camptothecin, vincristine and taxol in many of the tested cancer lines (**Table 1**). It is noteworthy that TET seemingly exhibits similar cytotoxicities (IC_{50} range: $1.25\mu\text{M}$ to $5.7\mu\text{M}$) in the tested 8 lines, whereas other drugs exert huge IC_{50} ranges, e.g., adriamycin $0.15\mu\text{M}$ to $7.06\mu\text{M}$; camptothecin $0.025\mu\text{M}$ to $5.57\mu\text{M}$; taxol 0.01nM to $41.1\mu\text{M}$; and vincristine 0.013nM to $100\mu\text{M}$ (**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 Anti-Proliferation 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, as 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 $2.5\mu\text{M}$ to $10\mu\text{M}$) 5-FU exhibited much stronger inhibitory effect on cell survival in crystal violet cell viability assay (**Fig. 2A**). For example, in the presence of $5\mu\text{M}$ TET 5-FU significantly inhibited cell proliferation and survival at as low as $10\mu\text{M}$, 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 μ M when TET was at or below 5 μ M (**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 24h 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 μ M TET the numbers of migrated cells across the extracellular matrix protein-coated membranes significantly decreased (**Fig. 3B**). Quantitatively, 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. As many anticancer agents can induce cell apoptosis, we tested whether TET could induce apoptosis in cancer cells. We employed three complementary apoptosis assays. When HCT116 cells were treated with 0, 5, 10, and 20 μ M TET for 12h 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, 10 μ M TET for 12h, 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, while 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 increased after TET treatment (e.g., from 23.9% to 67.3%), while 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 μ M 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-FBS 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. Briefly, exponentially growing firefly luciferase-tagged HCT116 cells were injected into the flanks of athymic nude mice. At one week after cancer cell injection, TET was intraperitoneally administered (60mg/kg body weight, once every two days). Mice were subjected to Xenogen bioluminescence imaging on a weekly base for additional three weeks. As shown in **Fig. 5A**, the TET treatment group exhibited significantly decreased Xenogen imaging signal, when 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**).

Histologic analysis (H & 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 if 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 Anti-Proliferative 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 the TCF/LEF-responsive reporter, TOP-Luc (He et al., 1998; Tang et al., 2009; Zhou et al., 2003; Zhou et al., 2002) 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 μ M 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/mt}) 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 $5\mu\text{M}$ (**Fig. 6B**). However, the differential response was less pronounced but still significant at $10\mu\text{M}$ ($p < 0.05$). 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.

Lastly, we conducted in vivo experiment to test if the loss of oncogenic β -catenin rendered HCT116 cells insensitive to TET treatment. We injected the firefly luciferase-tagged HCT116^{wt/mt} and HCT116^{wt/ko} cells into the flanks of athymic nude mice subcutaneously. At one week after injection, TET was i.p. administered (60mg/kg body weight, once every two days). Mice were subjected 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, while 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 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. Recently, we have found that a bisbenzylisoquinoline alkaloid, TET,

exhibits significant anticancer activity. But, the molecular mechanism that underlies its anticancer activity is not fully understood.

Here, we compared the anti-proliferative activity of TET with other six commonly-used chemotherapy drugs in a panel of 8 lines of different cancers. We found that TET exhibits comparable anticancer activity with camptothecin, vincristine, taxol and adriamycin. The IC_{50} for TET was $\leq 5\mu M$ 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. Mechanistically, 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. Interestingly, 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 VSMCs to pro-apoptosis stimulation, improve the endothelial function, and increase NO production (Wang et al., 2004). These results suggest that TET was not only an anti-hypertensive drug but also an excellent drug to reverse cardiac and vascular remodeling.

Recent studies indicate that TET exhibits anti-inflammatory and anticancer activity (Chen et al., 2009; Liu et al., 2008; Wu et al., ; Xu et al., 2006). 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 GSK3 β via AKT inhibition induced by TET resulted in enhanced phosphorylation and proteolysis of cyclin D, activation of caspase 3 and subsequent cleavage of PARP (Chen et al., 2008).

Nonetheless, it is conceivable that other signaling pathways may also participate in TET's anticancer activity, particularly in non-colon cancers in which the possible pathogenic role of TET may be less characterized. For example, TET-loaded nanoparticles were shown to activate ROS-dependent c-JNK 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 MAPK signaling pathway in human and mouse cancer cells (Cho et al., 2009; Nomura et al., 2007; Wu et al., 2010). Treatment of HepG2 cells with TET caused the upregulation of p53, downregulation 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 VEGF 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. Conversely, the possible in vivo toxicities of TET ought to 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.

Acknowledgements

The authors wish to thank Bert Vogelstein of Johns Hopkins Oncology Center, Baltimore, MD for kind provision of the oncogenic β -catenin allelic deletion line HCT116^{wt/ko} cells. The authors thank Dr. Sergey Kozmin of The University of Chicago for his critical reading of the manuscript. The reported work was supported in part by research grants CA106569, AT004418, AR50142, and AR054381 (TCH, RCH, and HHL) from the National Institutes of Health.

Authorship Contribution

T.-C. H., Y. Z., H.H.L., R.C. H., B.-C.H., and J.-L. G. designed the experiments. B.-C. H., J.L.G., B.Q. Z., Q.L., Q. S., S.H.K. E.H., Y.H., and K. Y. performed in vitro experiments. B.-C.H., J.-L.G., E.R.W., L.W., N. T., J.L., X.L., M.L., Y.B., Q.Z. performed animal experiments. B.-C.H., J.S., N.H., G.L., performed histologic analysis and immunostaining. B.-C.H., J.-L.G., Y.Z., Q.Z. analyzed the data and performed statistical analysis. T.-C.H., Y.Z., R.C.H., B.-C.H., and H.H.L. wrote the manuscript.

References

- Aggarwal S and Chu E (2005) Current therapies for advanced colorectal cancer. *Oncology (Williston Park)* **19**(5):589-595.
- Chan TA, Wang Z, Dang LH, Vogelstein B and Kinzler KW (2002) Targeted inactivation of CTNNB1 reveals unexpected effects of beta-catenin mutation. *Proc Natl Acad Sci U S A* **99**(12):8265-8270.
- Chen XL, Ren KH, He HW and Shao RG (2008) Involvement of PI3K/AKT/GSK3beta pathway in tetrandrine-induced G1 arrest and apoptosis. *Cancer Biol Ther* **7**(7):1073-1078.
- Chen Y, Chen JC and Tseng SH (2009) Tetrandrine suppresses tumor growth and angiogenesis of gliomas in rats. *International journal of cancer* **124**(10):2260-2269.
- Cho HS, Chang SH, Chung YS, Shin JY, Park SJ, Lee ES, Hwang SK, Kwon JT, Tehrani AM, Woo M, Noh MS, Hanifah H, Jin H, Xu CX and Cho MH (2009) Synergistic effect of ERK inhibition on tetrandrine-induced apoptosis in A549 human lung carcinoma cells. *Journal of veterinary science* **10**(1):23-28.
- Cragg GM, Grothaus PG and Newman DJ (2009) Impact of natural products on developing new anti-cancer agents. *Chem Rev* **109**(7):3012-3043.
- Haydon RC, Deyrup A, Ishikawa A, Heck R, Jiang W, Zhou L, Feng T, King D, Cheng H, Breyer B, Peabody T, Simon MA, Montag AG and He TC (2002a) Cytoplasmic and/or nuclear accumulation of the beta-catenin protein is a frequent event in human osteosarcoma. *International journal of cancer* **102**(4):338-342.
- Haydon RC, Zhou L, Feng T, Breyer B, Cheng H, Jiang W, Ishikawa A, Peabody T, Montag A, Simon MA and He TC (2002b) Nuclear receptor agonists as potential differentiation therapy agents for human osteosarcoma. *Clin Cancer Res* **8**(5):1288-1294.
- He BC, Chen L, Zuo GW, Zhang W, Bi Y, Huang J, Wang Y, Jiang W, Luo Q, Shi Q,

- Zhang BQ, Liu B, Lei X, Luo J, Luo X, Wagner ER, Kim SH, He CJ, Hu Y, Shen J, Zhou Q, Rastegar F, Deng ZL, Lu HH, He TC and Haydon RC (2010) Synergistic antitumor effect of the activated PPARgamma and retinoid receptors on human osteosarcoma. *Clin Cancer Res* **16**(8):2235-2245.
- He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B and Kinzler KW (1998) Identification of c-MYC as a target of the APC pathway. *Science* **281**(5382):1509-1512.
- Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y and Ueno K (1996) A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biological & pharmaceutical bulletin* **19**(11):1518-1520.
- King VF, Garcia ML, Himmel D, Reuben JP, Lam YK, Pan JX, Han GQ and Kaczorowski GJ (1988) Interaction of tetrandrine with slowly inactivating calcium channels. Characterization of calcium channel modulation by an alkaloid of Chinese medicinal herb origin. *The Journal of biological chemistry* **263**(5):2238-2244.
- Kinzler KW and Vogelstein B (1996) Lessons from hereditary colorectal cancer. *Cell* **87**(2):159-170.
- Koehn FE and Carter GT (2005) The evolving role of natural products in drug discovery. *Nat Rev Drug Discov* **4**(3):206-220.
- Lee JH, Kang GH, Kim KC, Kim KM, Park DI, Choi BT, Kang HS, Lee YT and Choi YH (2002) Tetrandrine-induced cell cycle arrest and apoptosis in A549 human lung carcinoma cells. *Int J Oncol* **21**(6):1239-1244.
- Li X, Zhen D, Lu X, Xu H, Shao Y, Xue Q, Hu Y, Liu B and Sun W (2010) Enhanced cytotoxicity and activation of ROS-dependent c-Jun NH2-terminal kinase and caspase-3 by low doses of tetrandrine-loaded nanoparticles in Lovo cells--a possible Trojan strategy against cancer. *Eur J Pharm Biopharm* **75**(3):334-340.
- Liu B, Wang T, Qian X, Liu G, Yu L and Ding Y (2008) Anticancer effect of tetrandrine on

primary cancer cells isolated from ascites and pleural fluids. *Cancer Lett* **268**(1):166-175.

Luo J, Chen J, Deng ZL, Luo X, Song WX, Sharff KA, Tang N, Haydon RC, Luu HH and He TC (2007) Wnt signaling and human diseases: what are the therapeutic implications? *Laboratory investigation; a journal of technical methods and pathology* **87**(2):97-103.

Luo J, Tang M, Huang J, He BC, Gao JL, Chen L, Zuo GW, Zhang W, Luo Q, Shi Q, Zhang BQ, Bi Y, Luo X, Jiang W, Su Y, Shen J, Kim SH, Huang E, Gao Y, Zhou JZ, Yang K, Luu HH, Pan X, Haydon RC, Deng ZL and He TC (2010) TGF β /BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells. *The Journal of biological chemistry*.

Luo Q, Kang Q, Si W, Jiang W, Park JK, Peng Y, Li X, Luu HH, Luo J, Montag AG, Haydon RC and He TC (2004) Connective tissue growth factor (CTGF) is regulated by Wnt and bone morphogenetic proteins signaling in osteoblast differentiation of mesenchymal stem cells. *The Journal of biological chemistry* **279**(53):55958-55968.

Luo X, Chen J, Song WX, Tang N, Luo J, Deng ZL, Sharff KA, He G, Bi Y, He BC, Bennett E, Huang J, Kang Q, Jiang W, Su Y, Zhu GH, Yin H, He Y, Wang Y, Souris JS, Chen L, Zuo GW, Montag AG, Reid RR, Haydon RC, Luu HH and He TC (2008a) Osteogenic BMPs promote tumor growth of human osteosarcomas that harbor differentiation defects. *Laboratory investigation; a journal of technical methods and pathology* **88**(12):1264-1277.

Luo X, Sharff KA, Chen J, He TC and Luu HH (2008b) S100A6 expression and function in human osteosarcoma. *Clinical orthopaedics and related research* **466**(9):2060-2070.

Luu HH, Kang Q, Park JK, Si W, Luo Q, Jiang W, Yin H, Montag AG, Simon MA, Peabody TD, Haydon RC, Rinker-Schaeffer CW and He TC (2005a) An orthotopic

model of human osteosarcoma growth and spontaneous pulmonary metastasis.

Clinical & experimental metastasis **22**(4):319-329.

Luu HH, Zhou L, Haydon RC, Deyrup AT, Montag AG, Huo D, Heck R, Heizmann CW, Peabody TD, Simon MA and He TC (2005b) Increased expression of S100A6 is associated with decreased metastasis and inhibition of cell migration and anchorage independent growth in human osteosarcoma. *Cancer Lett* **229**(1):135-148.

Mann J (2002) Natural products in cancer chemotherapy: past, present and future. *Nat Rev Cancer* **2**(2):143-148.

Meng LH, Zhang H, Hayward L, Takemura H, Shao RG and Pommier Y (2004) Tetrandrine induces early G1 arrest in human colon carcinoma cells by down-regulating the activity and inducing the degradation of G1-S-specific cyclin-dependent kinases and by inducing p53 and p21Cip1. *Cancer research* **64**(24):9086-9092.

Ng LT, Chiang LC, Lin YT and Lin CC (2006) Antiproliferative and apoptotic effects of tetrandrine on different human hepatoma cell lines. *Am J Chin Med* **34**(1):125-135.

Nomura M, Yamazaki R, Takaya M, Kikuchi M, Takahashi-Nishioka T, Akiyama K, Akiyama N, Moritani S and Kobayashi S (2007) Inhibition of tetrandrine on epidermal growth factor-induced cell transformation and its signal transduction. *Anticancer research* **27**(5A):3187-3193.

Oh SH and Lee BH (2003) Induction of apoptosis in human hepatoblastoma cells by tetrandrine via caspase-dependent Bid cleavage and cytochrome c release. *Biochem Pharmacol* **66**(5):725-731.

Sharff KA, Song WX, Luo X, Tang N, Luo J, Chen J, Bi Y, He BC, Huang J, Li X, Jiang W, Zhu GH, Su Y, He Y, Shen J, Wang Y, Chen L, Zuo GW, Liu B, Pan X, Reid RR, Luu HH, Haydon RC and He TC (2009) Hey1 basic helix-loop-helix protein plays an important role in mediating BMP9-induced osteogenic differentiation of

- mesenchymal progenitor cells. *The Journal of biological chemistry* **284**(1):649-659.
- Si W, Kang Q, Luu HH, Park JK, Luo Q, Song WX, Jiang W, Luo X, Li X, Yin H, Montag AG, Haydon RC and He TC (2006) CCN1/Cyr61 is regulated by the canonical Wnt signal and plays an important role in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells. *Molecular and cellular biology* **26**(8):2955-2964.
- Su Y, Luo X, He BC, Wang Y, Chen L, Zuo GW, Liu B, Bi Y, Huang J, Zhu GH, He Y, Kang Q, Luo J, Shen J, Chen J, Jin X, Haydon RC, He TC and Luu HH (2009) Establishment and characterization of a new highly metastatic human osteosarcoma cell line. *Clinical & experimental metastasis* **26**(7):599-610.
- Tang N, Song WX, Luo J, Luo X, Chen J, Sharff KA, Bi Y, He BC, Huang JY, Zhu GH, Su YX, Jiang W, Tang M, He Y, Wang Y, Chen L, Zuo GW, Shen J, Pan X, Reid RR, Luu HH, Haydon RC and He TC (2009) BMP-9-induced osteogenic differentiation of mesenchymal progenitors requires functional canonical Wnt/beta-catenin signalling. *Journal of cellular and molecular medicine* **13**(8B):2448-2464.
- Wang G, Lemos JR and Iadecola C (2004) Herbal alkaloid tetrandrine: from an ion channel blocker to inhibitor of tumor proliferation. *Trends Pharmacol Sci* **25**(3):120-123.
- Wu JM, Chen Y, Chen JC, Lin TY and Tseng SH (2010) Tetrandrine induces apoptosis and growth suppression of colon cancer cells in mice. *Cancer Lett* **287**(2):187-195.
- Xu WL, Shen HL, Ao ZF, Chen BA, Xia W, Gao F and Zhang YN (2006) Combination of tetrandrine as a potential-reversing agent with daunorubicin, etoposide and cytarabine for the treatment of refractory and relapsed acute myelogenous leukemia. *Leuk Res* **30**(4):407-413.
- Zhou L, An N, Haydon RC, Zhou Q, Cheng H, Peng Y, Jiang W, Luu HH, Vanichakarn P, Szatkowski JP, Park JY, Breyer B and He TC (2003) Tyrosine kinase inhibitor STI-571/Gleevec down-regulates the beta-catenin signaling activity. *Cancer Lett* **193**(2):161-170.

Zhou L, An N, Jiang W, Haydon R, Cheng H, Zhou Q, Breyer B, Feng T and He TC

(2002) Fluorescence-based functional assay for Wnt/beta-catenin signaling activity.

Biotechniques **33**(5):1126-1128, 1130, 1132 passim.

FOOTNOTES

B.-C.H. and J.-L.G. contributed equally to the work.

Figure legends

Figure 1. Anti-proliferative activity of TET in human cancer cells. **A.** Anti-proliferative activity comparison between TET and other chemotherapy drugs. Subconfluent HCT116 cells were treated with indicated concentrations of TET, camptothecin, carboplatin, 5-FU, vincristine, and taxol for 48h. The cells were then subjected to MTT assay. Each assay condition was done in triplicate. **B.** Anti-proliferative 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 48h. The cells were subjected to MTT assay as described in Methods. Each assay condition was done in triplicate.

Figure 2. Synergistic anti-proliferation 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 in Methods. **B.** Synergistic anti-proliferative effect between TET and 5-FU in cancer cells. Subconfluent HCT116 cells were treated with various 5-FU at a relatively fixed TET concentrations for 48h and subjected to MTT assays. Each assay condition was done in triplicate.

Figure 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 24h. 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 μ M) or 5 μ M TET and used for transwell migration assay as

described in Methods. Migrated cells were fixed, stained, and microphotographed (**B**) and counted as average # migrated cells per 5-10 high power fields (**C**). Representative images are shown in **B**.

Figure 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 12h. Cells were washed with PBS twice, add incubated with 500 μ l binding buffer and 2 μ l Annexin V-EGFP (GenScript USA Inc. Piscataway, NJ) each well for 5 min, followed by washing with PBS twice. GFP signal was detected under a fluorescence microscope. **B.** Vybrant Apoptosis Assay. Subconfluent HCT116 cells were seeded in 6 well plates and treated with TET at the indicated concentrations for 12h. The treated cells were harvested and washed twice with PBS. The resuspended cells (in 1ml) were incubated with 1 μ l of Hoechst 33342 (8.1mM) solution, 1 μ l of YO-PRO-1 (100 μ M) solution and 1 μ l of propidium iodide (1.5 mM) solution (Vybrant Apoptosis Assay kit, Molecular Probes, Eugene, OR) 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 bright red fluorescence. **C.** Flow cytometry analysis of apoptotic cells. Subconfluent HCT116 cells were seeded in 6 well plates and treated with TET at the indicated concentrations for 12h. The treated cells were harvested, washed twice with PBS, and stained with YO-PRO-1/propidium iodide for 20min on ice. The stained cells were subjected to flow cytometry. Each assay condition was done in triplicate. Representative results are shown.

Figure 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 (5 mice/group; 1×10^6 cells/injection site). At one week after cancer cell injection, TET was i.p. administered (60mg/kg body weight, once every two 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 in Methods. Average tumor size was represented by imaging signal intensities (in photons/second/steradian). “***”, $p < 0.01$. **C.** Histologic examination of xenograft tumor samples. Retrieved tumor samples were fixed, embedded, and subjected to H & E staining. Representative images are shown (magnification, x300). **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 an HRP-conjugated secondary antibody. The presence of β -catenin protein was visualized by developing the slides with DAB staining kit. Representative staining results are shown (magnification, x300).

Figure 6. Colon cancer cells with oncogenic β -catenin are more sensitive to TET-induced anti-proliferative 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 cm² flasks and transfected with the β -catenin/Tcf reporter pTOP-Luc or Myc/Max reporter. At 15h post transfection, cells were replated in 24-well plates and treated with 0, 5, or 10 μ M of TET. Firefly luciferase activities were measured at 15h after treatment. Each assay condition was done in triplicate. “*” $p < 0.05$; “***” $p < 0.001$. **B.** Effect on oncogenic β -catenin on TET-induced anti-proliferative 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 Figure 1 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/mt} 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 in 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/mt} and HCT116^{wt/ko} cells were collected and injected into the flanks of athymic nude mice (1×10^6 cells/injection site) as described in Figure 5 and Methods. At one week after cancer cell injection, TET was i.p. administered (60mg/kg body weight, once every two days). Mice were subjected to Xenogen bioluminescence imaging on a weekly base. Representative Xenogen imaging results at week 3 are shown.

Table 1. IC₅₀ Comparison between TET and Chemotherapy Drugs in Various Types of Human Cancer Lines

Table 1. IC ₅₀ Comparison between Tetrandrine and Chemotherapy Drugs in Human Cancer C uM unless indicated otherwise)							
Compound	MDA-MB-468	MDA-MB-231	HCT116	SW480	PC3	DU145	MG63
Tetrandrine	1.250	1.890	1.530	3.900	4.339	3.168	5.707
Adriamycin	2.323	0.294	0.702	5.782	0.416	0.150	7.061
5-FU	>500	>500	>500	>500	>500	163.686	>500
Carboplatin	>100	>100	>100	>100	>100	>100	>100
Camptothecin	0.027	1.226	0.147	5.569	0.025	0.030	0.265
Taxol	2.381	12.809	0.400nM	38.682	>100	<0.010nM	41.107
Vincristine	<0.100nM	8.718	0.134nM	>100	>100	0.0627nM	29.669

Figure 1

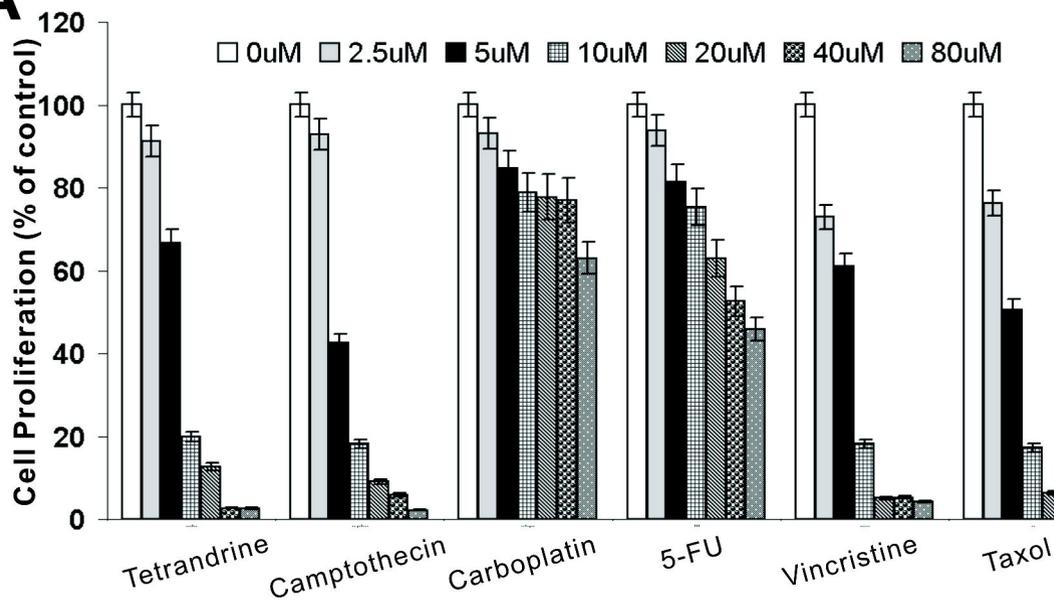
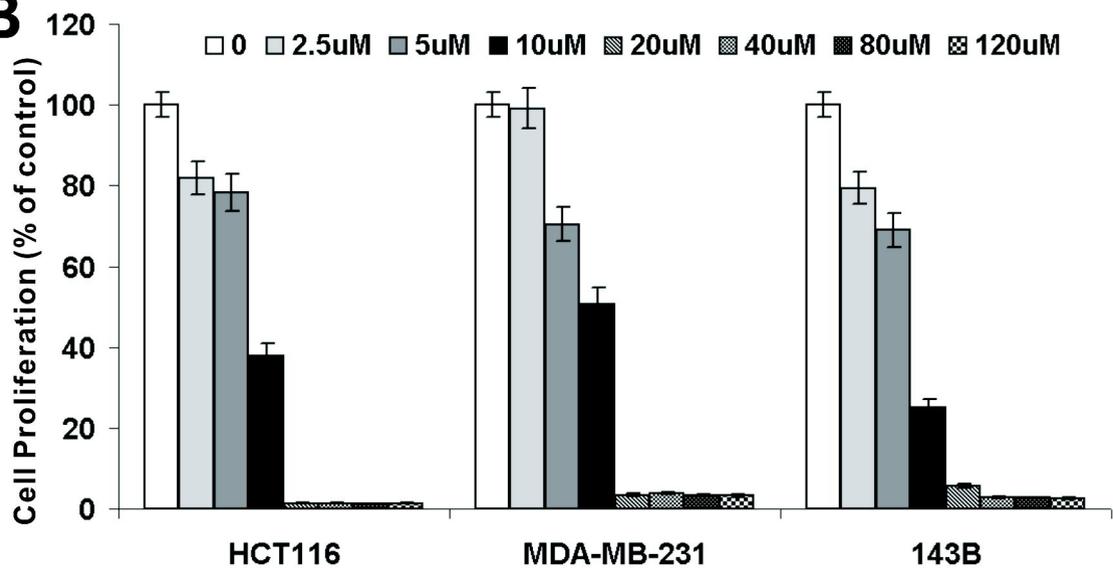
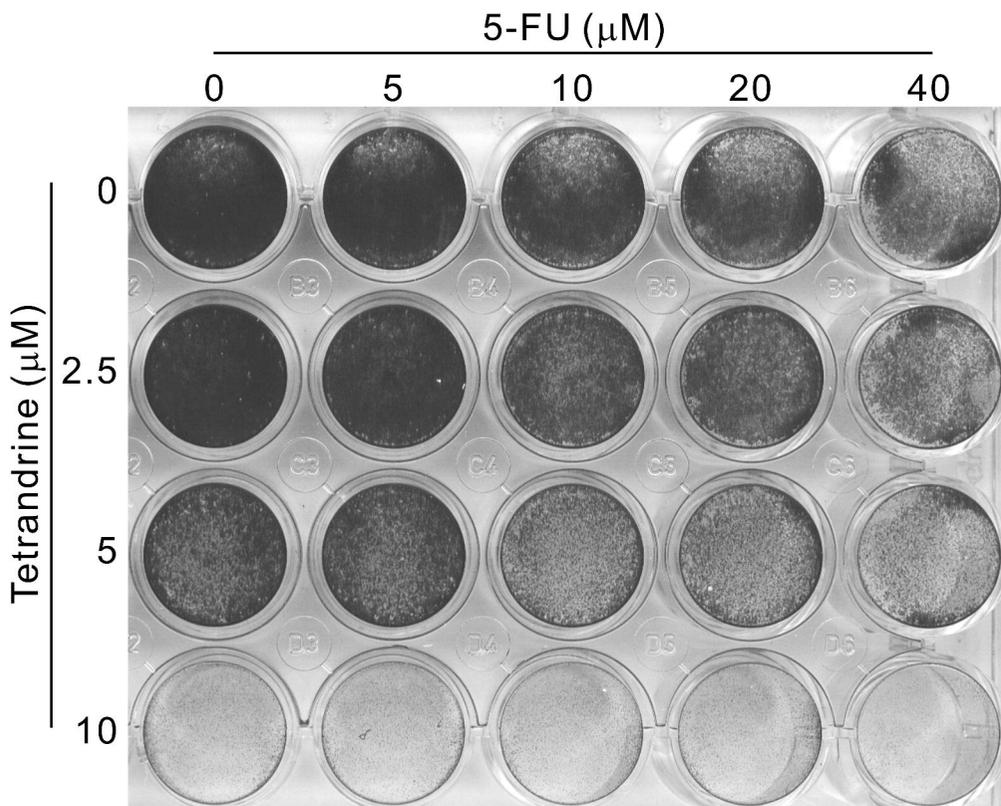
A**B**

Figure 2

A



B

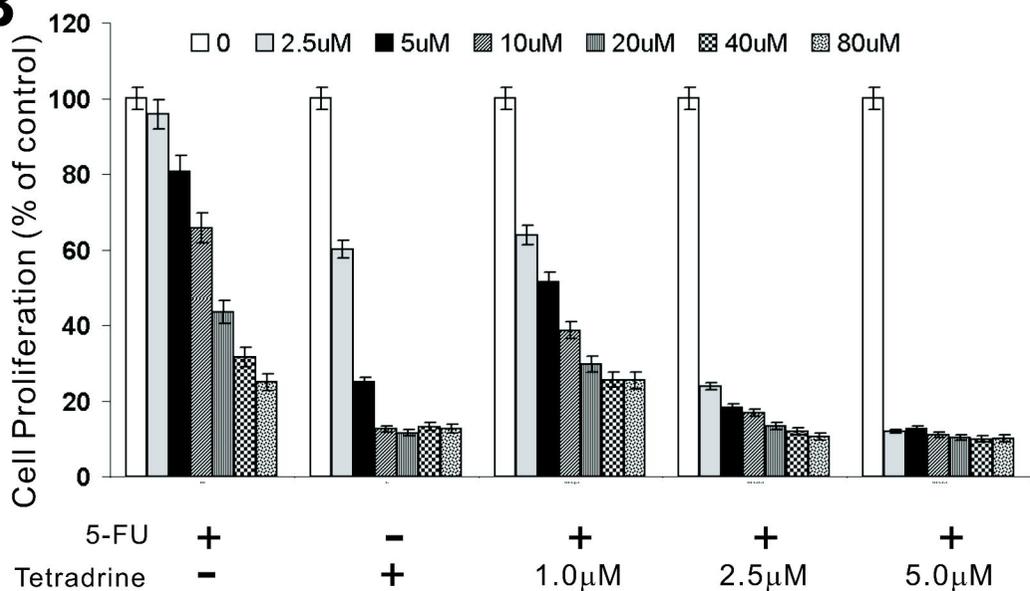


Figure 3

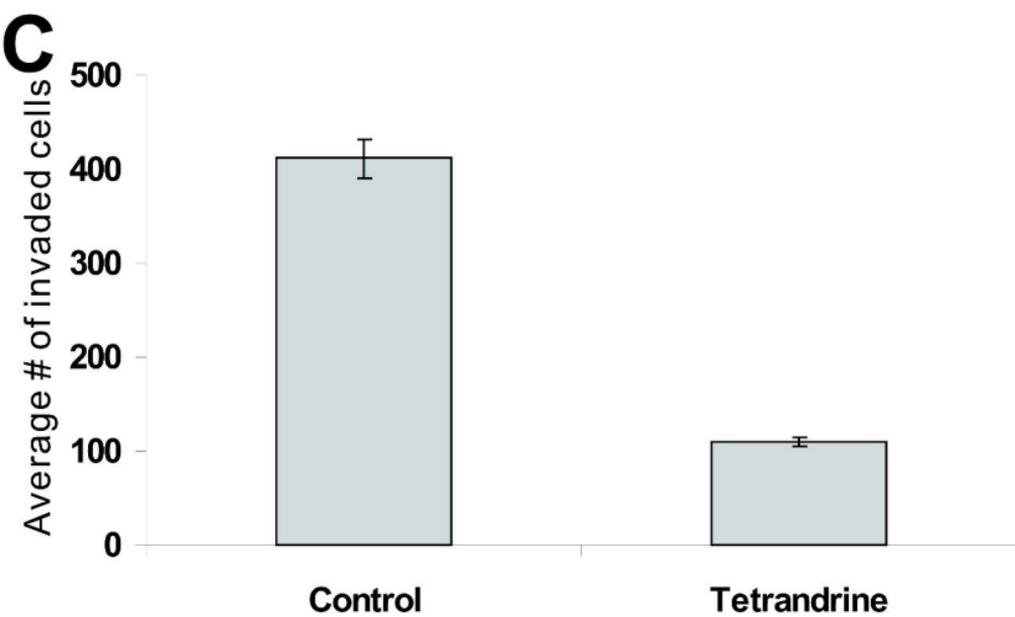
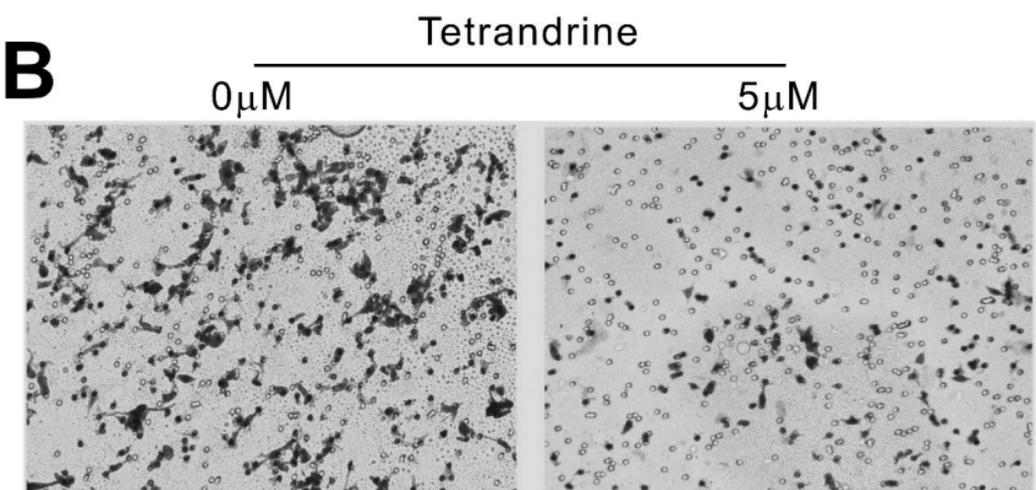
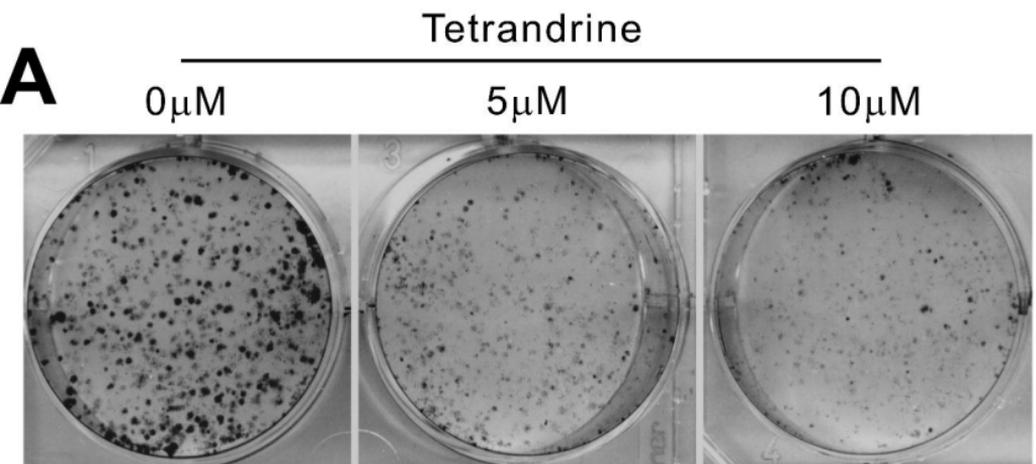


Figure 4

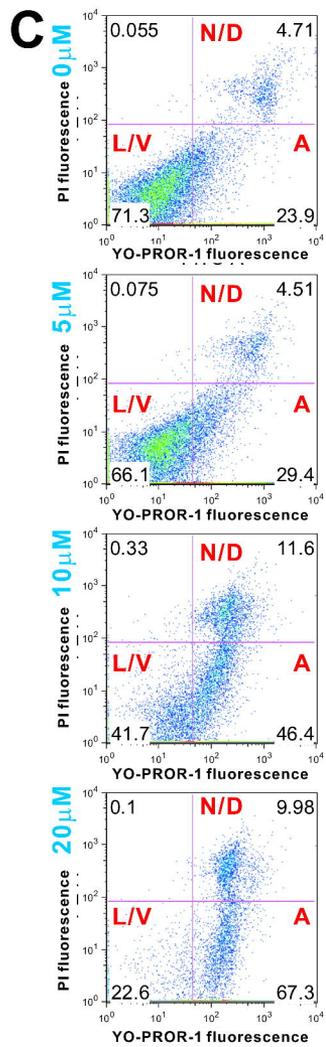
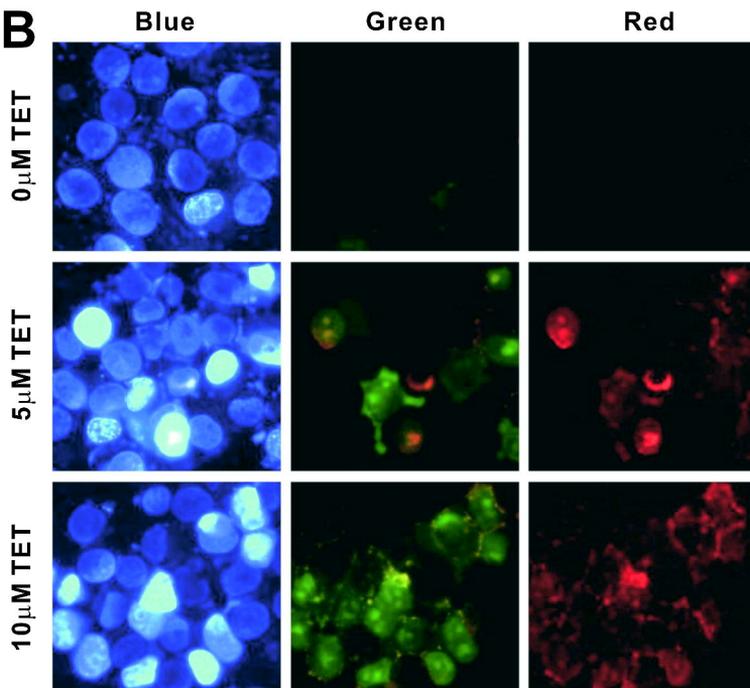
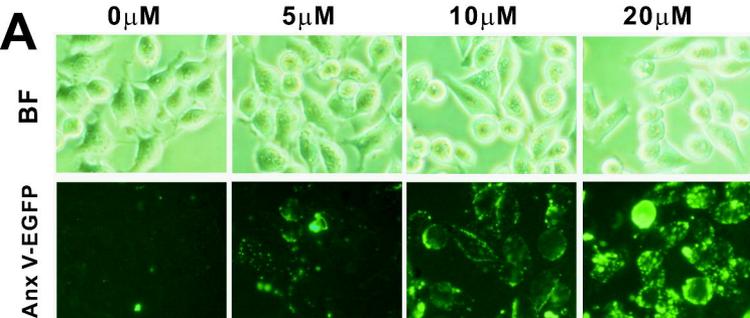


Figure 5

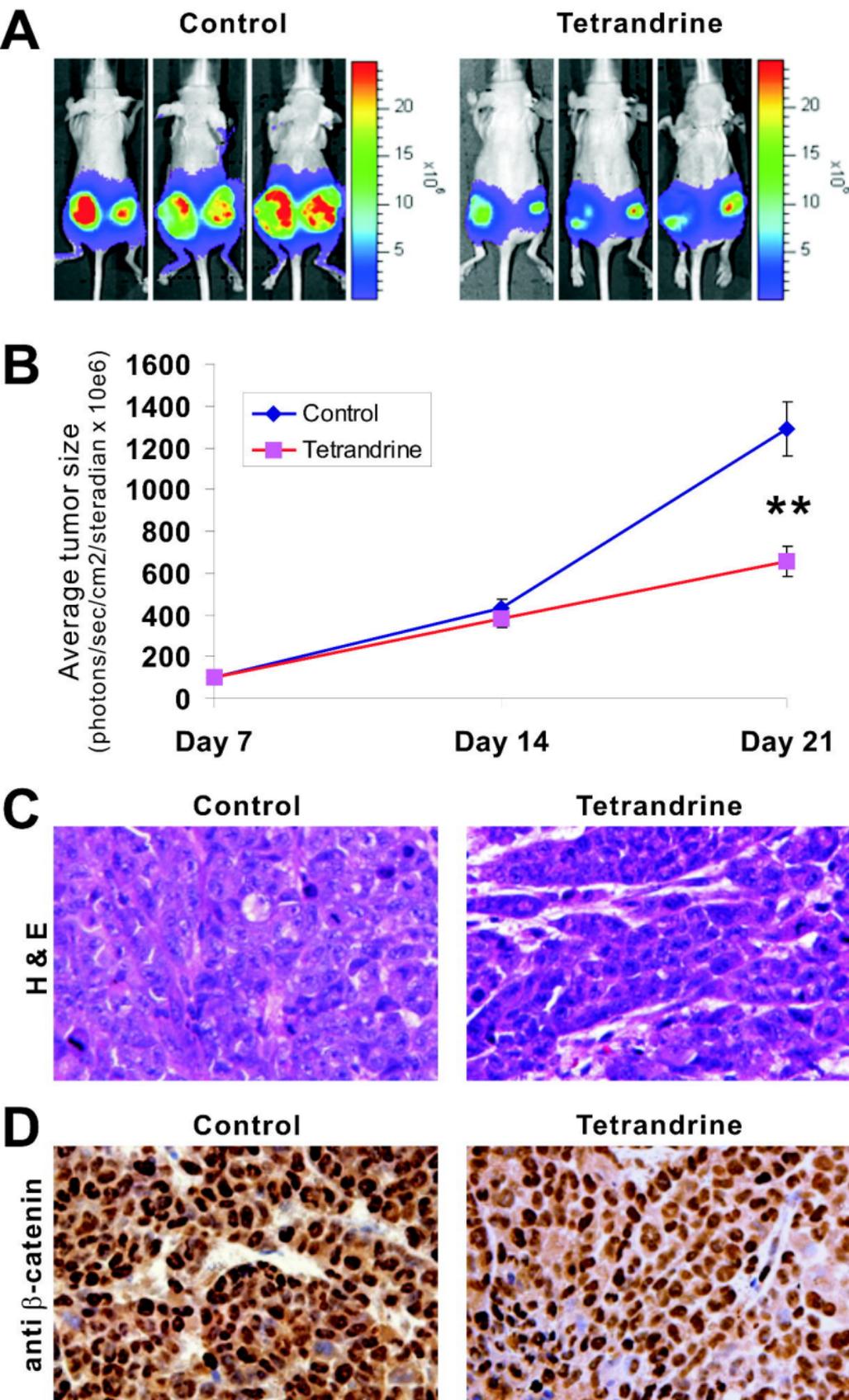


Figure 6

