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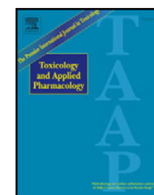

A novel topoisomerase 2a inhibitor, cryptotanshinone, suppresses the growth of PC3 cells without apparent cytotoxicity

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ABSTRACT

DNA topoisomerase 2, which is ubiquitously expressed in eukaryotic cells, is an essential nuclear enzyme that promotes cell survival by regulating DNA topology and chromatid separation. This enzyme has been validated as a target for anticancer agent screening. It can be poisoned by common chemotherapeutics, such as etoposide and doxorubicin, which leads to the accumulation of cytotoxic enzyme-linked DNA double-stranded breaks. However, recent studies have suggested that the topoisomerase 2a isozyme is predominantly responsible for the carcinogenic side effects associated with etoposide and doxorubicin chemotherapy. Thus, we need to find a promising topoisomerase 2-targeting anticancer agent that avoids these carcinogenic side effects. Recent studies have found that cryptotanshinone has obvious anticancer activities against diverse cancer cells. Here, we demonstrate that cryptotanshinone markedly decreases the steady-state mRNA level of topoisomerase 2a, thereby decreasing the protein and activity levels of this enzyme. Moreover, cryptotanshinone exhibited dramatic *in vitro* and *in vivo* antitumor activity with low toxicity to normal tissues. Collectively, our findings support the development of cryptotanshinone as a promising candidate for treating cancer by targeting topoisomerase 2a.

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1. Introduction

DNA topoisomerases (topos) are enzymes that regulate the superhelical density of DNA by transiently breaking and rejoining DNA strands (Wang, 1985; Lee and Hong, 1998). The activities of DNA topoisomerase 1 (topo 1) and topoisomerase 2 (topo 2) are essential for chromosomal segregation and DNA replication, transcription, recombination and repair in mammalian cells (Wang, 1987; Liu, 1989; Leteurtre et al., 1994), and these enzymes have been proposed as intracellular targets for cancer chemotherapy (Liu, 1989; Lee and Hong, 1998). There are two isomers of topo 2: topo 2a (170 kDa) is expressed specifically in proliferating and tumor cells, plays important roles in cell cycle events, and is expressed at low levels in quiescent cells (Heck and Earnshaw, 1986; Hsiang et al., 1988); in contrast, topo 2b (180 kDa) is expressed in all cells, including quiescent cells (Woessner et al., 1990; Lyu et al., 2007; Patra et al., 2011).

Both isoenzymes are targeted by the topo 2-directed anticancer drugs, etoposide (VP-16), doxorubicin (Adriamycin), amsacrine, and mitoxantrone (Cornarotti et al., 1996; Willmore et al., 1998; Lyu et al., 2007). It has been well established that these topo 2-targeting drugs exert their antitumor activities by inhibiting the breakage-reunion reaction of topo 2. They stabilize a DNA-cleavable complex formed between DNA and the enzyme, leading to the accumulation of DNA double-stranded breaks. Such drugs are thus called topo 2 poisons. Other topo 2-targeting drugs act by blocking the overall catalytic activity of the enzyme. For example, novobiocin inhibits the interaction between ATP and the enzyme, and ICRF-193 blocks recycling of the enzyme (Roca et al., 1994; Hashimoto et al., 1997). Of the topo 2-targeting agents, topo 2 poisons act by generating topoisomerase-mediated DNA breaks and are more efficient as chemotherapeutics. However, most topo 2 poisons exhibit severe side effects, including life-threatening cardiotoxicity, multidrug resistance, and the development of secondary malignancies, such as leukemia (Felix, 1998; Azarova et al., 2007). Hence, we urgently need to identify novel small molecules that are capable of targeting topo 2 while showing low toxicity and overcoming these limitations (Shen et al., 2014).

Cryptotanshinone is one of the chief active ingredients isolated from dried roots of Salvia miltiorrhiza Bunge (Danshen), which is commonly used in Oriental medicine for the treatment of circulatory disorders, cardiovascular disease, hematological abnormalities, hepatitis, hyperlipidemia, and chronic renal failure (Kim et al., 2007). Cryptotanshinone

Abbreviations: topo, topoisomerase; VP-16, etoposide; AChE, acetylcholinesterase; STAT 3, signal transducer and activator of transcription 3; AMPK, AMP-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; HRP, horseradish peroxidase; RT-PCR, reverse transcription-polymerase chain reaction; H&E, hematoxylin and eosin; PI, propidium iodide.

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has been shown to possess multiple pharmacological activities, including anticholinesterase, antibacterial, anti-inflammatory, and antioxidative properties (Kang et al., 2000; Hur et al., 2005; Ge et al., 2012). It also improves cognitive impairment in Alzheimer's disease transgenic mice by inhibiting acetylcholinesterase (AChE) and reducing Aβ peptide generation (Zhou et al., 2011), and it exerts antitumor activity by inhibiting signal transducer and activator of transcription 3 (STAT 3) activity (Shin et al., 2009). In a previous study, we showed for the first time that cryptotanshinone exerts anti-diabetic and anti-obesity effects *via* activation of AMPK (AMP-activated protein kinase) (Kim et al., 2007), and further demonstrated the anticancer activity of cryptotanshinone.

Here, we hypothesized that cryptotanshinone may be a novel alternative to the existing topo 2-targeting anticancer drugs. To support this notion, we examined the effects of cryptotanshinone on topo 2 in human prostate cancer PC3 cells and other human cancer cell lines. Cryptotanshinone displayed potent antiproliferative effects against various human cancer cells, and markedly decreased the mRNA, protein, and enzyme activity levels of topo 2a in PC3 cells (IC50 > 40 μM at 24 h and 30 ± 0.18 μM at 48 h). Moreover, we confirmed that cryptotanshinone has *in vivo* antitumor effects with minimal adverse effects (loss of body weight or damage to normal tissues) in a nude mouse xenograft model. In summary, our data suggest that cryptotanshinone deserves further investigation as an anti-cancer agent, especially given that the identification of a novel topo 2a inhibitor may significantly contribute to the treatment of cancer.

2. Materials and methods

2.1. Materials

Cryptotanshinone and the other chemicals used in the buffer solutions were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The reagents utilized for the DNA decatenation assay were purchased from TopoGEN (Port Orange, FL). The antibodies against topo 1, topo 2a, topo 2b, STAT 3, U170S, GAPDH, and α-actinin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary anti-rabbit and anti-mouse antibodies were purchased from Santa Cruz Biotechnology.

2.2. Cell culture

The HeLa (human cervical cancer), HepG2 (human liver cancer), WiDr, HCT116 (both human colorectal cancer), A549 (human lung cancer), PC3, DU145, LNCap (all human prostate cancer), MCF7 (human breast cancer), U2OS (human osteosarcoma), and AGS (human gastric cancer) cell lines were obtained from the American Type Culture Collection (Rockville, MD). HeLa cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. HCT116, A549, PC3, DU145, LNCap, MCF7, and AGS cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HepG2, WiDr, and U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere (95% air, 5% CO_2) at 37 °C.

2.3. Cell proliferation assay

Cell proliferation was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) assay kit (Roche Diagnostics, Mannheim, Germany). Proliferation assays were done based on the protocol manual. Data are expressed as a percentage of the growth of control cells calculated from absorbance, corrected for background absorbance. The IC50 value (the drug concentration that inhibited growth to 50% of the vehicle-treated control) was calculated from sigmoidal analysis of the dose-response curve, using Origin version 6.1 (OriginLab Corp., Northampton, MA).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the QIAzol reagent (Qiagen, Hilden, Germany) and quantified by formaldehyde-agarose gel electrophoresis. Single-strand cDNA was synthesized from RNA (2 μg) using 0.27 μg of oligo dT and the amfiRivert reverse transcriptase (GenDEPOT, Katy, TX). The desired cDNA fragments were PCR amplified using the following specific primer pairs: STAT 3, 5′-TCA AGA GTC AAG GAG ACA TG-3′ (sense) and 5′-AGT CTT TGT CAA TGC ACA CT-3′ (antisense); topo 1, 5′-ACA GAG CCA CGG AGA GCA GCA-3′ (sense) and 5′-TTT GCC CGA GGA GCC ACA GC-3′ (antisense); topo 2a, 5′-ACA GAG CCA CGG AGA GCA GCA-3′ (sense) and 5′-TTT GCC CGA GGA GCC ACA GC-3′ (antisense); topo 2b, 5′-TGC TTA GGA GAG CAG CAG CCG-3′ (sense) and 5′-CCG ACC GGT TCG TGG CAG AG-3′ (antisense); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-TGC TGA GTA TGT CGT GGA GTC TA-3′ (sense) and 5′-AGT GGG AGT TGC TGT TGA AGT CG-3′ (antisense).

2.5. Western blot analysis

Total cell lysates were prepared in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% [v/v] Triton X-100, 1% [w/v] sodium deoxycholic acid, 30 mM Na_2HPO_4, 50 mM NaF, and 1 mM Na_3VO_4) containing a freshly added protease inhibitor cocktail (GenDEPOT). Cytoplasmic and nuclear extracts were prepared using a NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, USA). Extracts (30 μg) were mixed with SDS sample buffer, boiled for 5 min, separated by 8% or 10% (w/v) SDS-PAGE and transferred to nitrocellulose membranes (Millipore, USA). Blots were incubated with primary antibodies, exposed to an appropriate HRP-conjugated secondary antibody, and examined using an ECL chemiluminescence detection system (Amersham Biosciences, NJ).

2.6. DNA decatenation assay

Topoisomerase 2 was extracted from the nuclei of PC3 cells, and its specific decatenation activity was calculated based on the ability of a given nuclear extract (containing the alpha and beta isoforms) or a defined amount of purified enzyme to completely decatenate a given amount of catenated input DNA (100 ng kDNA) in a particular amount of time. Reactions were started by adding ATP (final concentration, 450 μM), the samples were incubated 37 °C for 20 min, and the reactions were terminated on ice with 4 μl stop/gel loading buffer. Samples (20 μl) were resolved by 1% agarose gel electrophoresis, the gels were analyzed under a UV transilluminator, and the decatenated kDNA products were quantified using the ImageJ software.

2.7. Mouse tumor xenografts and treatments

PC3 cells (2×10^6) were suspended in 100 μl PBS and injected subcutaneously into the flanks of 8-week-old 12 male athymic nude mice (Athymic NCr-nu/nu; KOATECH, Gyeonggi-do, Korea). Tumor volumes were calculated using the formula, $V = \text{length} \times (\text{width})^2/2$. When the tumor volume reached approximately 100 mm^3, mice were treated with intratumoral injection of vehicle (3% dimethyl sulfoxide and 30% polyethylene glycol; $n = 3$) or cryptotanshinone (5 mg/kg; $n = 3$) three times weekly for 2 weeks. Body weight and tumor volume were measured twice a week. On day 16, the mice were killed and the tumors were removed, photographed, fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Kidneys and livers were also removed and fixed in 10% buffered formalin, and paraffin sections were prepared for histologic analysis. All animal experiments were conducted

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