Tanshinone IIA Induces Mitochondria Dependent Apoptosis in Prostate Cancer Cells in Association with an Inhibition of Phosphoinositide 3-Kinase/AKT Pathway

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Tanshinone IIA (Tan IIA; 14,16-epoxy-20-nor-5(10),6,8,13,15-abietapentaene-11,12-dione), a phytochemical derived from the roots of Salvia miltiorrhiza Bunge, has been reported to possess anti-angiogenic, anti-oxidant, anti-inflammatory and apoptotic activities. However, the cancer growth inhibitory/cytocidal effects and molecular mechanisms in prostate cancer cells have not been well studied. In the present study, we demonstrate that Tan IIA significantly decreased the viable cell number of LNCaP (phosphate and tensin homolog (PTEN) mutant, high AKT, wild type p53) prostate cancer cells more sensitively than against the PC-3 (PTEN null, high AKT, p53 null) prostate cancer cells. Tan IIA significantly increased TdT-mediated dUTP nick-end labeling (TUNEL) positive index and sub-G1 DNA contents of treated cells, consistent with apoptosis. Tan IIA treatment led to cleavage activation of pro-caspases-9 and 3, but not pro-caspase-8, and cleavage of poly (ADP ribose) polymerase (PARP), a caspase-3 substrate. Additionally, Tan IIA treatment induced cytochrome c release from the mitochondria into the cytosol and reduced mitochondrial membrane potential and suppressed the expression of mitochondria protective Bcl-2 family protein Mcl-1L. Tan IIA reduced the expression of phosphoinositide 3-kinase (PI3K) p85 subunit, and the phosphorylation of AKT and mammalian target of rapamycin (mTOR) in a concentration-dependent manner. Moreover, the combination of Tan IIA and LY294002, a specific PI3K inhibitor, enhanced PARP cleavage of LNCaP and PC-3, but not in MDA-MB-231 breast cancer cells which do not contain detectable active AKT. The findings suggest that Tan IIA-induced apoptosis involves mitochondria intrinsic caspase activation cascade and an inhibition of the PI3K/AKT survival pathway.

Key words Tanshinone IIA; apoptosis; mitochondria; phosphoinositide 3-kinase/AKT; LNCaP

Apoptosis, also called programmed cell death, is morphologically characterized by cell shrinkage, membrane remodeling, cell blebbing, chromatin condensation and DNA fragmentation with apoptotic bodies.1–3) Apoptosis activation has been considered a good target in cancer therapies.4,5) In general, apoptosis is regulated by pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family and executed through caspases (or cysteine-aspartic proteases), chiefly via two major and inter-related pathways, i.e., the mitochondria-dependent “intrinsic” cytochrome c/caspase-9 pathway and the death receptor-mediated “extrinsic” caspase-8 pathway.6–8) Additionally, apoptosis is controlled by various cell signaling pathways such as the phosphoinositide 3-kinase (PI3K)/AKT survival pathway, regulating apoptosis chiefly by blocking caspase-9 and the mitochondria damaging Bcl-2 family protein, Bad, through phosphorylative inactivation.9)

Tanshinone IIA (Tan IIA, 14,16-epoxy-20-nor-5(10),6,8,13,15-abietapentaene-11,12-dione, Fig. 1A), one of the phytochemical compounds isolated from the Chinese medicinal herb Danshen (root of Salvia miltiorrhiza Bunge), has been reported to exert diverse biological properties including anti-oxidative,3,10,11) anti-angiogenic,12) and anti-inflammatory activities.13) More importantly, anti-cancer activities have been reported including human hepatoma,14–17) breast cancer18) and leukemia.19) However, there is no report of anticancer activity of Tan IIA on prostate cancer cells. Thus, in the present study, we investigated the cytocidal/apoptosis effect against prostate cancer cell lines of different pathogenic make-up: LNCaP (p53 null, phosphate and tensin homolog (PTEN) null-high AKT, androgen sensitive) and PC-3 (p53 null, PTEN null-high AKT, androgen receptor null). Our mechanistic investigations suggested an involvement of mitochondria-intrinsic caspase activation cascade and inhibition of PI3K/AKT pathway.

MATERIALS AND METHODS

Tanshinone IIA Isolation The procedure of Tan IIA (Fig. 1A) isolation is as reported by Choi and colleagues.20) Cell Culture Human prostate cancer cells LNCaP (ATCC CRL 1740, p53 wild type, PTEN mutant-high AKT) and PC3 (ATCC CRL 1435, p53 null, PTEN null-high AKT), and breast cancer cells MDA-MB-231 (ATCC HTB 26, p53 mutant, wild type PTEN-low AKT) were obtained from American Type Culture Collection (ATCC) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2 mmol/l l-glutamine, 10 mmol/l N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES), 1 mmol/l sodium pyruvate, and 4.5% d-glucose without antibiotics.

Cytotoxicity Assay The cytotoxicity of Tan IIA was assessed by a tetrazolium salt, 2,3-bis[2-methyloxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay. LNCaP or PC3 cells were seeded onto 96-well microplates at a density of 1×10^4 cells per well in 100 μl of complete medium. After incubation for 24 h, cells were exposed to var-

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ious concentrations of Tan IIA (0, 12.5, 25, 50 or 100 \mu M) for 24 or 48 h and 40 \mu l of XTT (1 mg/ml in phosphate buffered saline (PBS) with 10 \mu l PMS) was then added to each well for 2 h. The optical density was measured using microplate reader (Tecan Group Ltd., Switzerland) at 450 nm (reference wave length 650 nm). Cell viability was calculated as a percentage of viable cells in Tan IIA treated group versus untreated control by following equation. Cell viability (%) = [optical density (OD) (Tan IIA)−OD (blank)/OD (control)−OD (blank)]×100.

Estimation of Sub G1 Fraction by Flow Cytometric Analysis LNCaP or PC3 cells were treated with or without Tan IIA (50 \mu M) for 24 or 48 h. Then, cells were collected, washed with cold PBS and fixed in 75% ethanol at −20 °C. The fixed cells were then stained with propidium iodide (50 \mu g/ml after treatment with RNase (5 \mu g/ml). The stained cells were analyzed for the DNA content using FACSCalibur (Becton Dickinson Bioscience, U.S.A.).

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay Apoptotic cell death was observed by using Dead-End™ fluorometric TUNEL assay kit following manufacturer’s instructions. Briefly, LNCaP cells treated with (or without, vehicle control) Tan IIA (50 \mu M) for 48 h, plated onto the poly-l-lysine-coated slide, fixed with 4% paraformaldehyde for 15 min and incubated in TdT enzyme buffer containing fluorescein-12-dUTP for 1 h at 37 °C. After washing in PBS, cells were stained with propidium iodide (PI) solution for 15 min at room temperature and visualized under an Axio vision 4.0 fluorescence microscope (Carl Zeiss Inc., Germany) using a standard fluorescein filter set to view the red fluorescence of propidium iodide at >620 nm.

Western Blotting Cells (4×10^5 cells/ml) treated with various concentrations were lysed by using lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na3VO4, 1 mM NaF; protease inhibitor cocktail). The extracts were incubated on ice for 20 min, at 14000 ×g for 20 min at 4 °C and supernatants were collected. Protein concentrations were determined by Bradford assay (Bio-Rad), and proteins were separated by electrophoresis on 4—12% NuPAGE Bis-Tris gels (Novex, U.S.A.), transferred to a Hybond ECL transfer membrane and analyzed with the antibodies of anti-PARP, caspase-9, caspase-8, caspase-3, Mcl-1L, Bcl-2, Bax, phospho-AKT, phospho-mTOR (Cell Signaling, U.S.A.), P13K (Upstate Biotechnologies, U.S.A.) and β-actin (Sigma, U.S.A.).

Isolation of Cytosolic Fraction for Cytochrome c Detection LNCaP cells (4×10^5 cells/ml) treated with various concentrations of Tan IIA (0, 10, 25 or 50 \mu M) for 48 h. Cells were lysed in 40 \mu l of lysis buffer (20 mM HEPES/NaOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol (DTT), protease inhibitor cocktail) for 30 min on ice and homogenized by 10 passages through a 22-gauge needle. The homogenates were centrifuged at 25000 ×g for 30 min at 4 °C and the protein contents were measured using a Bio-Rad DC protein assay kit II. The lysate containing 25 \mu g of protein were analyzed for cytochrome c (BD Biosciences) by Western blotting.

Measurement of Mitochondrial Membrane Potential LNCaP cells (4×10^5 cells/ml) were treated with or without of Tan IIA (50 \mu M) for 48 h and stained with tetramethylrhodamine ethyl ester (TMRE) for 30 min at 37 °C. Mitochondrial membrane potential was detected by flow cytometry (FACSCalibur, Becton Dickinson, U.S.A.) at 582 nm.

Statistical Analysis All data were expressed as means± standard deviation (S.D.). The statistically significant differences between control and Tan IIA groups were calculated by the student’s t-test.

RESULTS

Tanshinone IIA Decreased Viable Cell Number of LNCaP and PC-3 Cells To examine whether Tan IIA is cytotoxic against LNCaP or PC3 cells, XTT assay was per-
formed. Cells were treated with various concentrations of Tan IIA (0, 12.5, 25, 50 or 100 μM) for 24 or 48 h. As shown in Fig. 1B, Tan IIA dramatically decreased the viability of LNCaP cells in a concentration-dependent manner. The treatment for 48 h revealed more significant effect on the cytotoxicity than for 24 h (IC50; ca. 90 μM at 24 h and ca. 35 μM at 48 h). In contrast, the cytotoxicity of Tan IIA in androgen-independent PC3 prostate cancer cells was lower than that in LNCaP cells (ca. 100 μM at 48 h) (Fig. 1C).

**Tanshinone IIA Induced Apoptosis in LNCaP Cells**

To determine whether cytotoxicity of Tan IIA involved apoptosis, we evaluated sub-G1 fraction by flow cytometry analysis and TUNEL staining of LNCaP cells treated with or without Tan IIA (50 μM). In flow cytometry analyses, Tan

![Flow cytometry analysis of sub G1 apoptotic DNA fraction of LNCaP cells treated with Tan IIA (50 μM) for 24 or 48 h. After fixation in 75% ethanol, cells were stained with PI and analyzed by flow cytometry. (B) Flow cytometry analysis of sub G1 apoptotic DNA fraction of LNCaP and PC3 cells treated with or without 100 μM Tan IIA for 24 h. (C) TUNEL detection of apoptotic LNCaP cells treated with or without Tan IIA (50 μM) for 48 h. PI was used for nuclear staining. Bar graphs represent % of TUNEL positive cells.

![Fig. 2. Tanshinone IIA Induced Apoptotic Cell Death in LNCaP Cells](image-url)
IIA significantly increased the sub-G1 DNA content from 0.63 to 9.59% at 24 h and to 27.35% at 48 h (Fig. 2A). In comparison, Tan IIA had no significant effect on induction of this apoptosis indicator even at 100 μM treatment for 24 h in PC3 cells (1.44% vs. 17.4% of the sub-G1 contents in PC-3 and LNCaP, respectively) (Fig. 2B). Consistent with the results of flow cytometric analysis in LNCaP cells, Tan IIA significantly increased the number of TUNEL positive apoptotic cells compared with untreated control. These results suggest that Tan IIA induced apoptotic cell death in LNCaP cells (Fig. 2C).

**Tanshinone IIA Induced Mitochondrial Events Associated with Apoptosis in LNCaP Cells**

Bcl-2 family proteins play an important role in regulating apoptosis.21) LNCaP cells were treated with various concentrations of Tan IIA (0, 10, 25 or 50 μM) for 48 h and analyzed for the expression of the Bcl-2 family protein members including Mcl-1L, Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) by Western blotting. As shown in Fig. 3A, Tan IIA reduced the level of Mcl-1L in a concentration-dependent manner. There was no significant change on the expression of Bcl-2 and Bax in LNCaP cells.

Caspase family proteins are critical enzymes to execute apoptosis.22) Of caspase family members, caspase-3 is a key executioner for apoptosis in mammalian cells.23) Caspase-3 can be activated by upstream initiator caspsases such as caspase-8 or -9 through two distinct pathways, i.e., the death receptor-mediated extrinsic caspase-8 pathway or the mitochondria dependent-cytochrome c/caspase-9 intrinsic pathway, respectively.6—8) In this study, Western blot analysis showed that Tan IIA reduced the abundance of pro-caspase-9 and -3, and increased cleaved caspase-3, but did not affect...

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**Fig. 3.** Tanshinone IIA Induced Mitochondrial Events Associated with Apoptosis in LNCaP Cells

Western blotting analyses for (A) abundance of Mcl-1L, Bcl-2 and Bax, and (B) PARP pro-caspase-9, -8, -3, cleaved caspase-3 in whole cell extracts after the LNCaP cells had been treated with Tan IIA for 48 h. (C) Western blotting analyses for cytochrome c in cytosolic extract of Tan IIA-treated cells, 48 h. (D) Detection of mitochondrial membrane potential (MMP) by flow cytometry. LNCaP cells were treated with or without Tan IIA (50 μM) for 48 h and were stained with tetramethylrhodamine ethyl ester (TMRE) for 30 min at 37°C and subjected to flow cytometry. Loss of TMRE dye accumulation indicated dissipation of membrane potential.
that of procaspase-8 (Fig. 3B), suggesting a likely involvement of mitochondria-dependent cascade for caspase activation.

A significant role of mitochondria in the apoptosis pathway is the release of cytochrome c from the mitochondria into the cytosol, which is often the consequence of a decrease of mitochondrial membrane potential. LNCaP cells treated with Tan IIA were found to have increased cytochrome c in the cytosol in a concentration-dependent manner (Fig. 3C). Consistent with mitochondrial leakage, the mitochondrial membrane potential (MMP) was reduced by Tan IIA treatment from 80.13 to 49.58% compared with untreated control (Fig. 3D). Taken together, these results plus the decreased mitochondria protective Mcl-1 suggested that Tan IIA induced mitochondria-dependent apoptosis in LNCaP cells.

**Tanshinone IIA-Induced Apoptosis Was Associated with a Downregulation of PI3K/AKT Signaling**

The PI3K/AKT/mammalian target of rapamycin (mTOR) pathway is involved in various cellular processes including apoptosis, survival, proliferation and differentiation. To evaluate whether Tan IIA-induced apoptosis is related to the PI3K/AKT/mTOR pathway, LNCaP cells were treated with various concentrations of Tan IIA for 48 h and analyzed for PI3K/AKT/mTOR by Western blotting. As shown in Fig. 4A, Tan IIA reduced the expression of PI3K p85 subunit and the phosphorylation of AKT and mTOR compared with untreated cells.

To further test the contribution of the PI3K/AKT pathway to Tan IIA-induced apoptosis, LNCaP cells were treated with LY294002, a specific PI3K inhibitor, in the absence or presence of Tan IIA for 48 h. Consistent with the results of Fig. 4A, Tan IIA decreased the level of phospho-AKT in the cells. LY294002 also completely inhibited the phosphorylation of AKT. Notably, the combined treatment with Tan IIA and LY294002 showed a synergistic effect on PARP cleavage compared with the treatment with Tan IIA or LY294002 alone in LNCaP cells (Fig. 4B). In agreement with the PARP data, flow cytometry analysis displayed also a synergistic effect of Tan IIA and LY294002 on increasing the sub-G1 DNA contents (Fig. 4C).

In addition, we evaluated whether the synergistic effect of Tan IIA on apoptosis was specific for LNCaP cells. PC3 (androgen-independent prostate cancer) and MDA-MB-231 (breast cancer) were treated with Tan IIA and LY294002 and analyzed PARP cleavage. Consistent with the results of previous reports, prostate cancer cells LNCaP and PC3 revealed more activated level of phospho-AKT due to the
mutation or loss of phosphatase and tensin homolog (PTEN),\textsuperscript{26,27} compared with MDA-MB-231 (Fig. 4D). The combination of Tan IIA and LY294002 induced PARP cleavages were induced in both LNCaP and PC3, but not in MDA-MB-231 cells. These results suggest that the synergistic effect of Tan IIA and LY294002 was more prominent in cancer cells with activated AKT due to PTEN functional loss. Rapamycin, a specific mTOR inhibitor, in combination with Tan IIA did not have additive or synergistic increase of PARP cleavage compared with treatment with Tan IIA or rapamycin alone (Fig. 4E). These results suggest that AKT downstream targets other than mTOR likely contributed more to the apoptosis signaling induced by Tan IIA.

**DISCUSSION**

Tanshinone IIA (Tan IIA) is a major phytochemical of a well-known Chinese herbal medicine called ‘Danshen’, the dried root of *Radix salvia miltiorrhiza*,\textsuperscript{28,29} Tan IIA has many reported biological effects, such as anti-cancer, anti-oxidant and anti-inflammatory activities in mammalian cells. Regarding to anti-cancer activity, Tan IIA has been reported to induce apoptosis in A549 lung cancer cells through the induction of reactive oxygen species and decreasing the mitochondrial membrane potential.\textsuperscript{30} Tan IIA also induced apoptosis in Colo-205 colon cancer cells through both mitochondrial-mediated intrinsic and Fas-mediated extrinsic caspase cell-death pathways.\textsuperscript{31} In addition, Tan IIA inhibited invasion and metastasis of HepG2 hepatocellular carcinoma cells *in vitro* and *in vivo*, partly by the inactivation of MMP-2 and -9 and the activation of the nuclear factor-kappa B (NF-κB) pathway.\textsuperscript{12} However, to our knowledge, our study is the first to report the cytocidal effect of Tan IIA on the prostate cancer cells.

The treatment with Tan IIA showed a significant cytotoxic effect against LNCaP (wild type p53) cells in a concentration and a time dependent manner and much more sensitive than against PC-3 (p53 null) cells. Since p53 activation was observed in LNCaP cells,\textsuperscript{33,34} we suspect that this contributed to the greater sensitivity of the LNCaP cells to Tan IIA than the PC3 cells. Our results revealed that Tan IIA increased the sub G1 DNA contents and induced DNA fragmentation and apoptotic bodies in the cells, supporting apoptosis induction. The role of p53 pathway in Tan IIA-induced apoptosis signaling is currently being investigated.

Regarding the caspase-activation pathway(s), our results were collectively consistent with mitochondria intrinsic pathway being primarily involved. First, we observed that Tan IIA activated caspase-3 and -9, but not caspase-8, in a concentration-dependent manner in the cells. Consistent with caspase-3 activation, Tan IIA caused the proteolytic cleavage of PARP with accumulation of the 85 kDa fragments in LNCaP cells. Second, Bcl-2 family proteins are known to be involved in mitochondrial apoptotic pathway. The balance of pro-apoptotic (Bax, Bad and Bid) and anti-apoptotic members (Bcl-2, Bcl-X\textsubscript{L} and Mcl-1) controls the sensitivity of cells to apoptosis activators. The translocalization of apoptotic proteins from the cytosol to the surface of the mitochondria leads to the release of cytochrome c and second mitochondria-derived activator of caspases/direct inhibitor of apoptosis (IAP) binding protein with low PI (SMAC/DIABLO) from the mitochondria into the cytosol.\textsuperscript{35,36} The release of cytochrome c is often the consequence of the decrease of mitochondrial membrane potential (MMP),\textsuperscript{24,25} the integrity of which is necessary for production of energy (ATP) and preservation of cellular homeostasis.\textsuperscript{37} We observed that Tan IIA decreased the expression of anti-apoptotic Mcl-1, and increased the cytosolic level of cytochrome c and caused the loss of MMP in LNCaP cells, consistent with mitochondria dependent apoptosis. Similarly, Tan IIA has been reported to induce apoptosis *via* mitochondria pathway in A549 lung cancer and Colo-205 colon cancer cells.\textsuperscript{30,31} We should note that our results did not conclusively exclude an involvement of the extrinsic death receptor-mediated caspase 8 pathway nor the cross-talk with this extrinsic cascade. The PI3K/AKT signaling pathway plays a crucial role in regulation of proliferation and survival in cancer cells\textsuperscript{38,39} and activation of the pathway is associated with resistance to apoptosis induction and cell growth.\textsuperscript{40} In prostate cancer, PTEN loss is a frequent and early event during carcinogenesis, leading to activation of AKT.\textsuperscript{41} Interestingly, Kaarbo and colleagues recently reported that the PI3K/AKT/mTOR pathway is dominant over androgen receptor related signaling in prostate cancer cells, the latter being a favorite target for developing anti-cancer agent for prostate cancer.\textsuperscript{42} Morgan and colleagues also reviewed the importance of the PI3K/AKT/mTOR pathway in prostate cancer.\textsuperscript{43} In the present study, our results showed that Tan IIA significantly reduced the expression of PI3K p85 subunit and the phosphorylation of AKT and mTOR in a concentration dependent manner in LNCaP cells. Additionally, Tan IIA showed synergistic apoptosis effect in combination with PI3K inhibitor LY294002 not only in LNCaP cells, but also in PC3 cells, both with activated AKT due to the functional loss of PTEN.

The lack of high AKT in MDA-MB231 breast cancer cells was associated with a lack of synergistic apoptosis action in this cell line to Tan IIA/LY294002 combination. Since the combined treatment with Tan IIA and rapamycin, a specific mTOR inhibitor, had no synergistic apoptotic effect, our results suggest downstream targets of AKT other than mTOR contributed more to Tan IIA-induced apoptosis.

Taken together, this study suggests that Tan IIA induces p53 activation and mitochondrial dysfunction, leading to caspase-9/caspase-3 mediated apoptosis. Tan IIA seems to inhibit PI3K/AKT survival pathway to contribute to apoptosis induction signaling. Further mechanistic investigations and *in vivo* anti-cancer efficacy studies using prostate xenograft models are in progress.

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