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Anticancer effects of tanshinone IIA in bladder urothelial carcinoma by down-regulating aurora A, HIF-1α and Bcl-2 both *in vitro* and *in vivo*

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Abstract: The mechanisms of the anticancer effect of Tanshinone IIA (Tan IIA) on Bladder urothelial carcinoma (BUC) remain mostly unknown. In this study, BUC T24 cells were treated with Tan IIA at different concentrations and durations. The apoptosis, proliferation and invasion of T24 cells were evaluated using MTT assays, Annexin V-FITC Staining, Hoechst staining and Trans well assay. One group of T-24 cell xenograft mice was treated with Tan IIA, while the other group received normal saline for 25 days. Subsequently, the size of tumors as well as mRNA and protein expression of Aurora A, HIF-1α and Bcl-2 were measured both *in vitro* and *in vivo*. Tan IIA induced apoptosis, inhibited proliferation, suppressed invasion of T24 cells in a time- and dose-dependent manner *in vitro* and attenuated growth in vivo. The decreasing of mRNA and protein expression of Aurora A, HIF-1α and Bcl-2 in T-24 cells treated with Tan IIA were detected in a time- and dose-dependent manner both *in vitro* and *in vivo*. The pro-apoptotic, anti-proliferative and anti-invasive effects of Tan IIA on T-24 cells may be derived from inhibition of mRNA and protein expression of Aurora A, HIF-1α, and Bcl-2. Tan IIA could potentially serve as a novel potential anti-cancer agent for BUC.

Keywords: TanshinoneII A, bladder urothelial carcinoma, proliferation, apoptosis, invasion.

INTRODUCTION

According to GLOBOCAN estimates, approximately 573,000 new bladder urothelial carcinoma (BUC) cases and 213,000 BUC deaths occurred worldwide in 2020 (Jubber *et al*., 2023). In China, BUC is the 13th most common cancer in terms of incidence, with an estimated 85,694 new cases in 2020 (He *et al*., 2023). BUC is the 10th most common cancer and the 5-year relative survival rate ranges from 97% (stage I) to 22% (stage IV). BUC is the most expensive tumor on a per-patient basis from diagnosis to death, and the economic burden on BUC patients with progression is higher than on patients without progression (Grabe-Heyne *et al*., 2023). BUC mostly consists of urothelial histology, including non-muscle invasive BUC (NMIBC) and muscle-invasive disease (MIBC), and roughly 75% of bladder tumors initially present as NMIBC (Witjes *et al*., 2021). However, MIBC is histologically identified in about 30% of all newly diagnosed patients and distant metastasis is ultimately present in approximately 50% of patients with MIBC (Milowsky *et al*., 2016). Moreover, over 50% of patients with metastatic BUC are ineligible for first-line cisplatin-containing chemotherapy, but small single-arm phase II trials and a single randomized phase III trial do not provide sufficient data to validate second-line chemotherapy (Cathomas *et al*., 2022). Therefore, the investigation of novel anti-cancer agents with fewer side effects and comparable efficacious has always been a focus in the treatment of advanced UBC.

The aberrant expression of Aurora A, HIF1-α (hypoxia-inducible factor 1) and Bcl-2 (B Cell Lymphoma/ Leukemia 2) have been reported to be associated with both unfavorable clinicopathology and poor prognosis in cancer patients (Mou *et al*., 2021, Lee, 2023, Strasser and Vaux, 2020). As a serine/threonine kinase, Aurora A is involved within metaphase and anaphase events of the cell cycle by modulating mitotic entry, which is essential for G2/M phase transition and the loss or over-expression of Aurora A is related to the occurrence of various cancers (Mou *et al*., 2021, Ali and Stukenberg, 2023). HIF1-α, an O_2-labile subunit, is commonly induced by hypoxia, through which many crucial hallmarks of carcinogenesis, including tumor initiation, promotion, malignant conversion and progression, are activated (Rashid *et al*., 2021). Bcl-2 is an anti-apoptotic protein, that participates in an oncogenic function via survival signaling pathways, and its elevated expression of it is associated with advanced-stage tumors and poor differentiation (Suvarna *et al*., 2019).

Tanshinone IIA (Tan IIA; C19H18O3), a phenanthrenequinone derivative, is isolated from Danshen, *Salviae miltiorrhizae* Radix (Zhou *et al*., 2005, Che *et al*., 2004). The anti-inflammatory and

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immunomodulatory effects of Tan IIA have been utilized in cardio- and cerebrovascular therapeutic remedies for many years (Zhou *et al*., 2019, Xie *et al*., 2023). As a multi-targets agent, Tan IIA acts on transcription factors, kinases, apoptosis-related proteins and growth factors, and its anticancer effects with fewer side effects have been reportedly observed in a variety of human cancer cells, such as cholangiocarcinoma, breast cancer, neck squamous cell carcinoma, colorectal cancer, and lung adenocarcinoma (Liu *et al.*, 2021, Liu *et al.*, 2023, Mao *et al*., 2022, Qian *et al*., 2023, Wang *et al*., 2023). Although Tan IIA has been regarded as a potential therapeutic agent for BUC due to inhibition of the invasion and migration of BUC T-24 cells via modulation of STAT3-CCL2 signaling and mitochondria-dependent pathway, its molecular mechanism of anticancer effect on human BUC T-24 cells remains largely unknown (Chiu *et al*., 2014, Huang *et al*., 2017).

The present study investigated the effects of Tan IIA on apoptosis, proliferation, and invasion of T24 cells and measured the messenger RNA (mRNA) and protein expression of Aurora A, HIF-1α and Bcl-2 of ofT24 cells treated with or without Tan II A both *in vitro* and *in vivo*, by which provided a basis for evaluating the value in the clinical application of Tan IIA in treatment for BUC and elucidating additional mechanisms involved within the anticancer effect of Tan IIA.

MATERIALS AND METHODS

Reagents and antibodies

Tan IIA, Dimethyl sulfoxide (DMSO) and [3-(4,5-dimethylthizol-2-yl)-2,5-diphenyl tetrazolium bromide] MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies used in this study were: mouse anti-Aurora Kinase A (Abcam, Cambridge, MA), rabbit anti-HIF1-α (Bioss, Beijing, China), rabbit anti-Bcl-2 (CST, Danvers, MA, USA), and mouse anti-GAPDH (Santa Cruz, CA, USA). polyvinylidene fluoride (PVDF) membranes, BSA protein assay kits and western blot chemiluminescence agent were purchased from Amersham Biosciences (Arlington Heights, IL, USA)

Cell cultures and animals

The human BUCT24cell line was obtained from the Cell Resource Center (Xiangya School of Medicine of Central South University, Changsha, China) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100U/ml penicillin and streptomycin in a 37˚C humidified atmosphere containing 5% CO_2. BALB/c-nu mice purchased from the Experimental Animal Center (Central South University) were used for *in vivo* tumor induction and maintained in cages at room temperature (20-25°C) with stable humidity (55±5%) and provided water and food ad libitum. All animal assays were undergone following the Institutional Animal Ethics Committee and Animal Care Guidelines for Care and Use of Laboratory Animals of Guilin Medical University (GLMC 202003242).

In vivo T24 cell tumor xenograft model

A total of 40 4-week-old male BALB/c-nu mouse hypodermically injected in the back with 0.2ml BUC T24 cells (1×10^6) and sustained in a pathogen-free environment to generate solid tumor *in vivo*.

Those mice were divided randomly into two groups (20 mice/group). 20 mice in one group were injected intraperitoneally with Tan IIA (15mg/kg of body weight, every 3 days), as described previously (Yen et al., 2018). As the control group, 20 mice were treated intraperitoneally with normal saline (0.1ml/10g body weight, every 3 days). Those mice were sacrificed by CO_2 inhalation on day 25. Following xenograft transplantation, mice exhibiting tumors were monitored and tumor size was measured every 3 days according to the formula: Tumor volume (mm^3)=$L\times W^2/2$, where L is the tumor length and W is the width, with the final volume of xenograft tumors were measured 25 days after T24 cells inoculation. The mRNA and protein expression of Aurora kinase A, B-cell lymphoma-2 (Bcl-2), and Hypoxia-inducible factor 1-alpha (HIF1-α) were assessed by PCR and western blot analysis.

MTT assay

BUC T-24 cells ($1x10^4$/well) were plated onto 96-well microtiter plates and incubated for 24h in 100µl medium. After incubation overnight, cells were treated with different concentrations of Tan IIA (0.5mg/L, 2mg/L, 5mg/L, and 10mg/L) for 24, 48 and 72 hours. MTT [50 µl (0.5mg/L)] was added to T-24 cells, which were then cultivated for a further 4h at 37°C. Following the removal of the supernatant fluid, 150µl of DMSO was added to each well. The optical density (OD) was measured at 570 nm and was detected using an ELISA reader (Mindray, Shenzhen, China) and the percentage of cell viability was calculated as OD drug/OD control x 100%. The untreated T-24 cells served as controls. MTT assay was repeated three times according to the manufacturer's instructions. The absorbance for DMSO-treated cells was considered 100%.

Annexin V-FITC staining

BUC T-24 cells were cultured and were treated with Tan IIA (0.5mg/L, 2mg/L, 5mg/L and 10mg/L) for 24, 48 and 72 hours. The vehicle control groups were treated with 0.2% DMSO. Apoptotic cell death was examined using annexin V-FITC detection kits according to the manufacturer's instructions (Mbchem, Shanghai, China). The analysis was carried out using CXP software.

Cell invasion assay

The trans well assay was performed using Hanging inserts (Corning Life Sciences, Tewksbury, MA, USA) with an

8µm polycarbonate membrane in a 6-well plate. Cells were seeded in 6 well plates and treated without or with Tan IIA (0.5mg/L, 2mg/L, 5mg/L and 10mg/L) for 24, 48 and 72 hours. Cells were then detached and seeded (5×10^4) in the Hanging inserts filled with culture medium. The upper side of the hanging inserts was coated with Matrigel basement membrane matrix (BD Biosciences, San Jose, CA, USA) at a concentration of 3.9µg/µl. Culture medium supplemented with 10% FBS was added in the lower chamber. Incubation was carried out at 37°C for 24 hours. The hanging inserts were washed with PBS, and cells on the upper filter surface were gently removed with a cotton swab. The inserts were subsequently fixed with 10% formalin for 10 min at room temperature, stained with 0.2% w/vcrystal violet, washed with PBS, and the remaining cells counted under a light microscope (Novel, Nanjing, China) operating at 200×magnification. The migration cell numbers of the control group were considered 100%.

Hoechst 33258 staining

The condensation of chromatin of cells is a mark of cytotoxicity. BUC T-24 cells were stained with Hoechst-33258 after being incubated with Tan IIA (0.5mg/L, 2mg/L, 5mg/L and 10mg/L) for 24, 48 and 72 hours and rinsed with PBS. Cells were then stained with Hoechst-33258 for 15 min at room temperature. The morphology of cells was observed under a fluorescence microscope operating at 200×magnification (Leica, Wetzlar, Germany).

Reverse transcription-polymerase chain reaction

Total RNA of T24-cells, which were treated with Tan IIA (0.5mg/L, 2mg/L, 5mg/L, and 10mg/L) for 24, 48 and 72 hours and samples of xenograft tumors were extracted using the Trizol reagent according to the manufacturer's instructions (Invitrogen Co., Carlsbad, CA, USA). Total RNA isolated from cells and tumor samples was reverse transcribed to cDNA using oligo-dT and random primers. Reverse transcription was done and the cDNA was amplified by PCR using the following specific primers. Primers were designed by the primer analysis software Primer Express v3.0 (Applied Biosystems). The cycling conditions were one initial cycle of 95°C for 5 min followed by 40 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30s.

Western Blot Analysis

Total protein product in solid tumor samples and T-24 cells, which were treated with Tan IIA (0.5mg/L, 2mg/L, 5mg/L, and 10mg/L) for 24, 48 and 72 hours, and total protein lysate containing protease and phosphatase inhibitors was extracted following the manufacturer's instructions (Pierce, Thermo Scientific, Rockford, IL, USA). The protein concentration of the supernatant was measured by a bicinchoninic acid protein concentration assay kit. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by loading 20µg protein per lane. Resolved proteins were then transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% nonfat milk for 2 hours at room temperature. The proteins were incubated with anti-Aurora A, HIF-1α, Bcl-2 and GAPDH-antibodies at 4°C for 2 hours followed by anti-rabbit or anti-mouse immunoglobulin G-horseradish peroxidase conjugated secondary antibodies overnight. After the PVDF membrane was washed three times for 10 min with washing solution at room temperature, the resolved protein bands were detected using Western Lightning ™Chemiluminescence Reagent Plus (Amersham Biosciences, Arlington Heights, IL, USA).

STATISTICAL ANALYSES

All data were expressed as mean ± standard deviation (SD) of at least three independent experiments. Differences between the two groups were analyzed using the student's t-test or with ANOVA if more than two groups were assessed. Differences were considered statistically significant at $P<0.05$. SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for all calculations.

RESULTS

Anti-proliferation effect of Tan IIA on T-24 cells

To determine whether Tan IIA affected cell viability, T-24 cells were treated with different concentrations (0, 2, 5, and 10mg/l/L) of Tan IIA for various times (24, 48 and 72 hours). As present in fig. 1, Tan IIA significantly attenuated cell viability in a concentration- and time-dependent manner ($P<0.01$). The half maximal inhibitory concentration (IC_{50}) was 10.40, 4.47 and 2.64 mg/L at 24, 48 and 72h, respectively (fig. 1)

Pro-apoptosis effect of Tan IIA onT-24 cells

The effect of Tan IIA on apoptosis was determined through Annexin V-FITC Staining. Results obtained by flow cytometry showed that the apoptosis rate (annexin-V+/PI-) was increasing with treated times and concentrations (fig. 2) and the apoptosis rate was significantly increased in a time- and dose-dependent manner (fig. 3).

Anti-invasion effect of Tan IIA onT-24 cells

The Tran swell test conducted on T-24 cells treated with different concentrations and durations of Tan IIA demonstrated a significant decrease in the number of invasive cells in a time- and dose-dependent manner ($P<0.01$). However, there was no significant decrease in T-24 cells observed between 48h and 72h, and between 2mg/L and 10mg/L at 48h and 72h respectively ($P>0.05$) (fig. 4 and fig. 5).

Morphological observation of cell apoptosis

The morphological changes of apoptotic cells can be observed after treatment with Tan IIA (fig. 6 A-D). In the

control group, untreated T-24 cells were dyed green and had smooth and intact cell membranes (fig. 6A). After exposure to Tan IIA (2mg/L, 24h), the nuclei of T-24 cells became mildly hyperchromatic and shrank (fig. 6B). In T-24 cells treated with 5mg/L Tan IIA for 48h, denser hyperchromatism, more obvious shrinking and characteristic membrane blebbing were observed (fig. 6C). Particularly at 10mg/L Tan IIA for 72h, the most extensive cellular apoptosis and dying were indicated (fig. 6D).

Effects of Tan IIA on the mRNA and protein expression of Aurora A, HIF-1αand Bcl-2 in T-24 cells

The T-24 cells were treated with different concentrations (0.5, 2 and 10mg/L) and time (24, 48 and 72 hours) and the mRNA and protein expression of Aurora A, HIF-1α and Bcl-2 in T-24 cells were evaluated by PCR and western blot analysis respectively to explore modulation effect of Tan IIA on these carcinogenesis markers of T-24 cells. As the concentration and time of Tan IIA increased, the protein and mRNA expression of Aurora A, HIF-1α and Bcl-2gradually and significantly increased in a time- and dose-dependent manner, except for the protein expression of Aurora A and HIF-1α in T-24 cells treated with 0.5mg/ml Tan IIA ($P>0.05$) (fig. 7 A-D and 8 A-D).

Effects of Tan IIA on the growth of T-24 cell tumor xenograft in BALB/c-nu mice.

According to tumor volume curves (fig. 9), on the 13th day after inoculation, the size of tumors in xenograft BALB/c-nu mice treated with Tan IIA was significantly smaller compared with the saline group. On the last day (25 days), the tumor volume in the Tan IIA treatment group was 223.2±19.7mm^3, while that of the saline group increased to 450.0±48.64 mm3. Therefore, there was a 50.0% inhibition of tumor growth ($P<0.01$) (fig. 10).

Effect of Tan IIA on the mRNA and protein expression of Aurora A, HIF-1αand Bcl-2 in T-24 cells tumor xenograft in BALB/c-nu mice

As shown in fig. 11A-C, Tan IIA significantly inhibited the mRNA and protein levels of Aurora A, HIF-1α and Bcl-2 in xenograft tumor samples after 25 days of inoculation compared to the saline group, except for the protein expression of Bcl-2 ($P= 0.547$). It was suggested that Aurora A, HIF-1α and Bcl-2 were down-regulated at the transcription and translation levels after treatment with Tan IIA in vivo.

DISCUSSION

The discovery of the ability of Tan IIA to effectively inhibit carcinogenesis with low side effects and multi-targets has raised attention on evaluating the potential capacity in the treatment of BUC and exploring the underlying molecular mechanisms. In the present study, we found that Tan IIA effectively inhibited proliferation, induced apoptosis and impaired invasive ability in BUC T-24 cells. Additionally, a decrease in both mRNA and protein expression levels of Aurora A, HIF-1α, and Bcl-2 was observed to be associated with the inhibition of growth in BUC T-24 cells both in *vitro* and in *vivo.*

Deregulation of checkpoints responsible for guaranteeing the accurate coordination of cell cycle events has been regarded as an important contributor to Chromosomal instability (CIN) and aneuploidy leading to genetic heterogeneity and cancer progression, in which Aurora Kinase family, including Aurora A, B and C, has been deemed as a novel oncogenic family of mitotic serine/threonine kinases and a key checkpoints essential for chromosomal stability in mammals (Ali and Stukenberg, 2023, Kim et al., 2022). The aberrant Aurora A has been implicated in carcinogenesis, inhibition of apoptosis, and promotion of proliferation and invasion in cancer cells (Willems et al., 2018). Up-regulation of Aurora A is reportedly associated with high aggressiveness and poor outcomes in various carcinomas, including leukemia, colorectal and ovarian cancer (Naso et al., 2021). In the present study, down-regulation of Aurora A mRNA and protein expression in T-24 cells treated with Tan IIA suggested that the anticancer effects of Tan IIA might be attributed to targeting checkpoints of the cell cycle. However, more research is needed to investigate the mechanisms underlying these effects.

However, over the past decade, no less than 13 inhibitors of Aurora kinases have been developed by pharmaceutical companies and research institutions, in which the only drugs that progressed to phase III clinical trials are MLN8237 (Aurora A selective) and AZD1152 (Aurora B selective) (Jing and Chen, 2021). Therefore, there has been an increasing recognition that the underlying mechanisms need to be explored to clarify the causes of trial failures, as Aurora A is not limited to centro some maturation and bipolar spindle assembly during different stages of interphase (Pradhan et al., 2021). Given the pleiotropy of Aurora A, the present study indicated that Tan IIA might not only directly inhibit the kinase activity but also regulate other pathways, which could contribute to anticancer effects with fewer side effects compared to conventional inhibitors of Aurora kinases mentioned above.

In the last two decades, the investigation of the hypoxia-inducible transcription factors (HIFs) family, which has been proven to play an essential role in ensuring mitochondria function, has promoted extensively the elucidation of mechanisms involved within hypoxia-induced carcinogenesis (Xin, 2023). As a transcription factor, Hypoxia-inducible factor-1 (HIF-1) modulates the expression of erythropoietin (EPO) in response to hypoxia in the blood and HIF-1α, as one of HIF-1 subunits (α and β), is expressed due to imbalance between O_2 supply and usage in tissue (Taylor and Scholz, 2022).

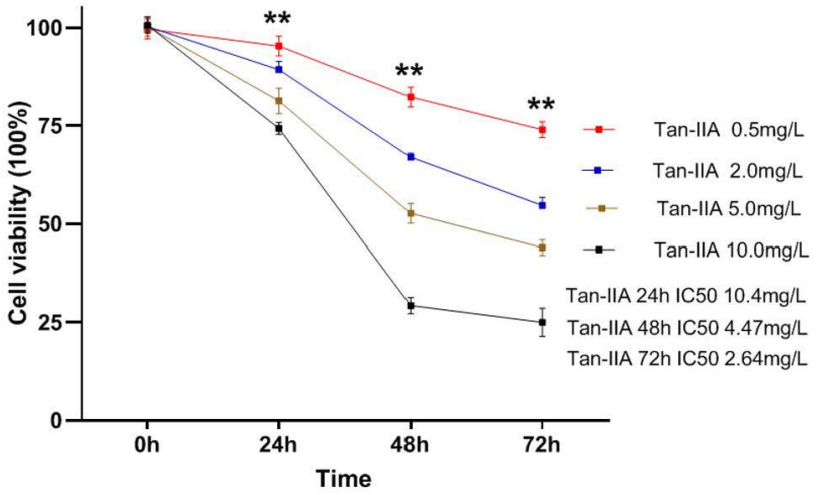

Fig. 1: Cytotoxic effects of Tanshinone IIA (Tan IIA) on T-24 cells. The cytotoxic effects of Tan IIA on T-24 cells were determined by the MTT assay. Each point is the mean ± standard deviation of three independent experiments. Differences between groups were statistically significant (P<0.01) (n=3). (**p<0.01)

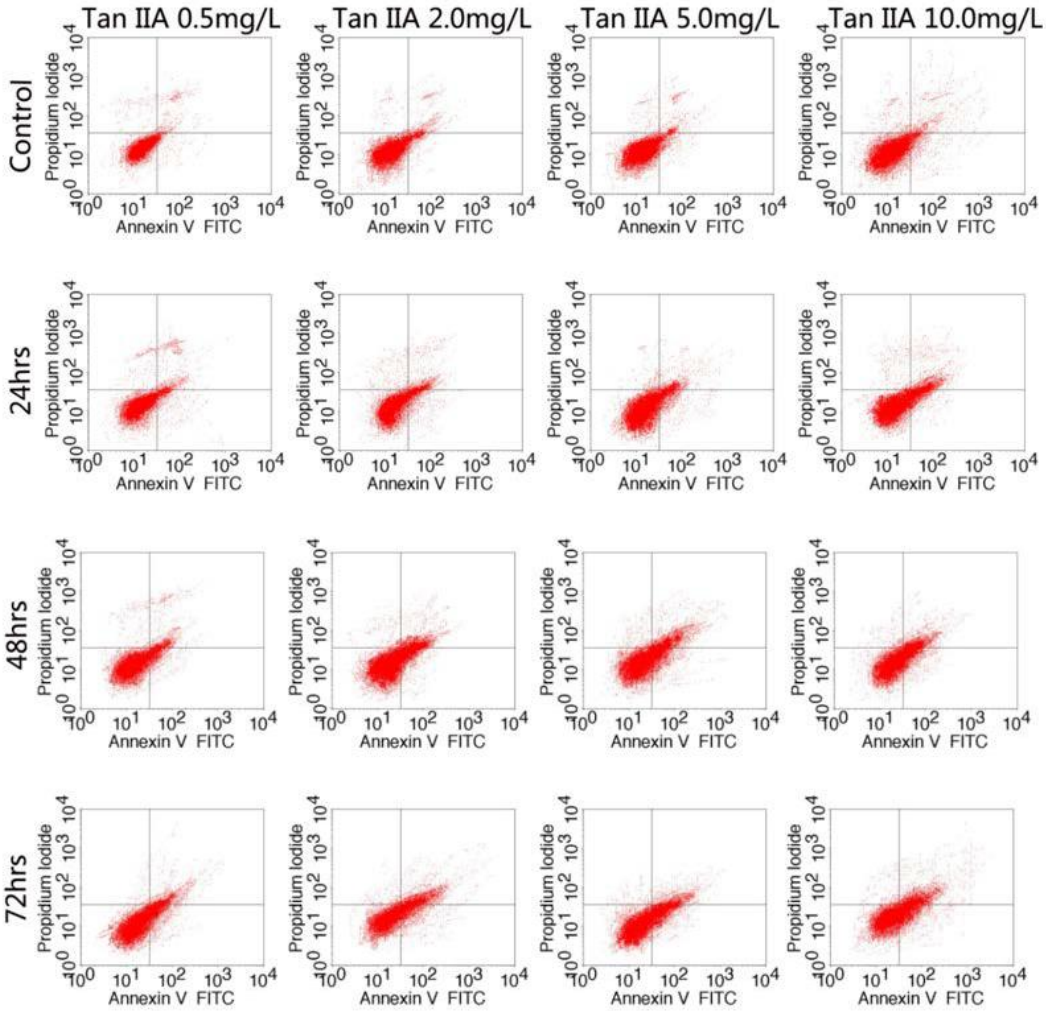

Fig. 2: Annexin V-FITC assay was performed to examine the apoptosis of T-24 cells treated with Tan IIA at various durations and concentrations, and the results obtained by flow cytometry show that the apoptosis cells (annexin-V+/PI−) were increasing with durations and concentrations (n=3). The upper left quadrants of each panel show the dead cells, the lower left quadrants of each panel show the viable cells, the upper right quadrants contain the late apoptotic cells, and the lower right quadrants represent the early apoptotic cells.

Table 1: Primers used in the present study

Symbol	Forward primer	Reverse primer	Product	TA°C
Aurora A	ACCGCAATCCTACCAGTGTC	CGTCTTCTTCACCAGCTTCC	160bp	55
HIF1-α	TCTCGGCGAAGCAAAGAGTCTGAA	TCTCGGCGAAGCAAAGAGTCTGAA	159bp	55
Bcl-2	ATTGTGGCCTTCTTTGAGTT	CAAACTGAGCAGAGTCTTCA	231bp	56
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	450bp	56

TA: annealing temperature.

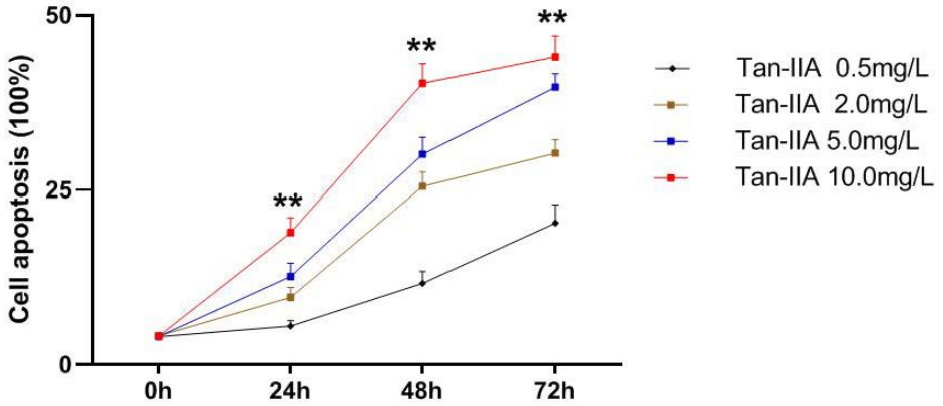

Fig. 3: The apoptosis rate of T-24 cells treated with Tan IIAelevated significantly, in a dose- and time-dependent manner, which was detected by Annexin V-FITC Staining($p<0.01$) (n=3). (****** $p< 0.01$)

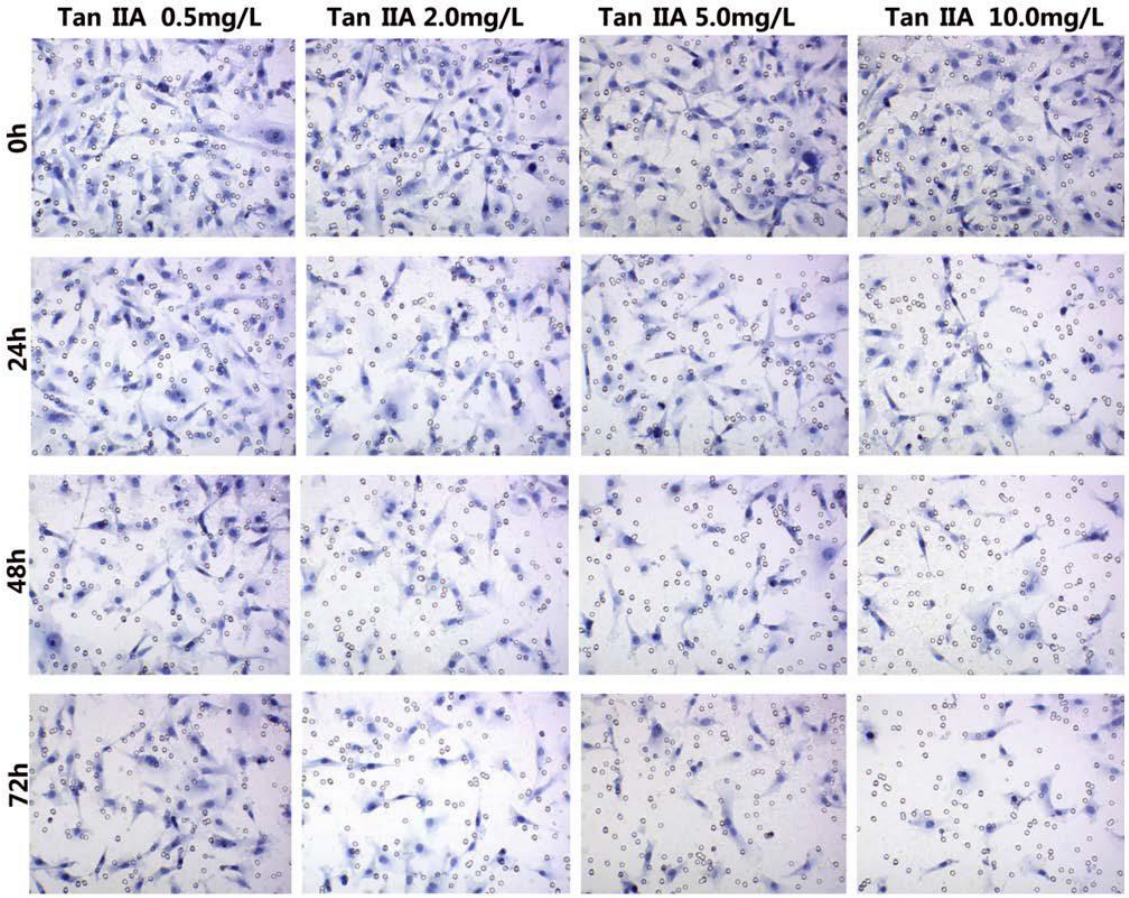

Fig. 4: Human bladder cancer T-24 cells were treated with Tan IIA at various concentrations and durations. The Tran swell test showed that the number of invasive cells was decreased gradually in a dose- and time-dependent manner (×200) (n=3).

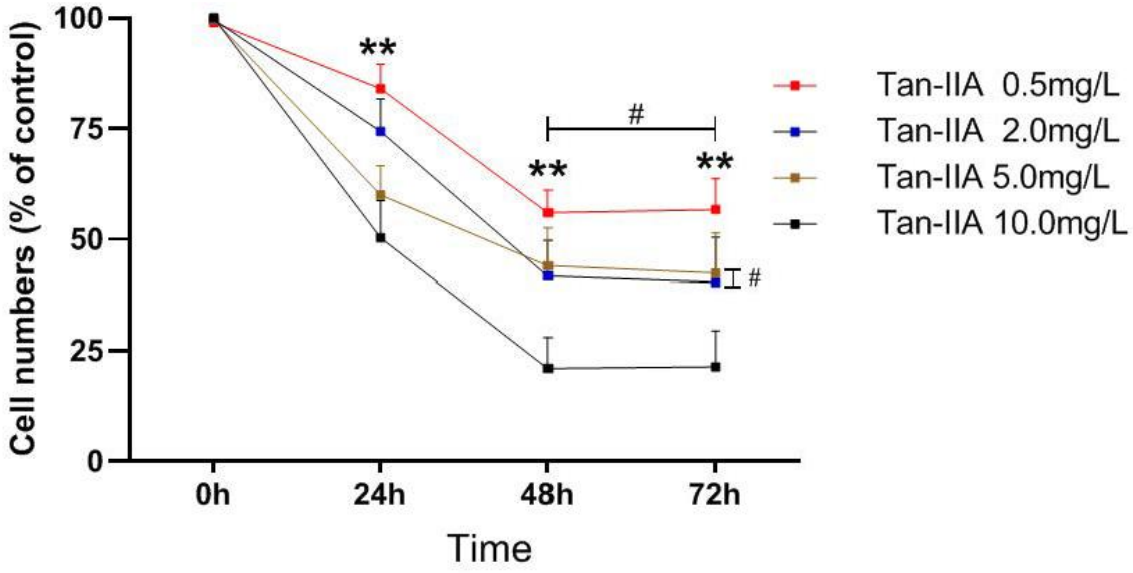

Fig. 5: The significant decrease of invasive T-24 cells number was found by the Tran swell test in a dose- and time-dependent manner($p<0.01$) but was not seen between 48h and 72h and between 2mg/L and 10mg/L at 48h and 72h respectively ($P>0.05$). (n=3) (*******p*< 0.01; #*p*>0.05)

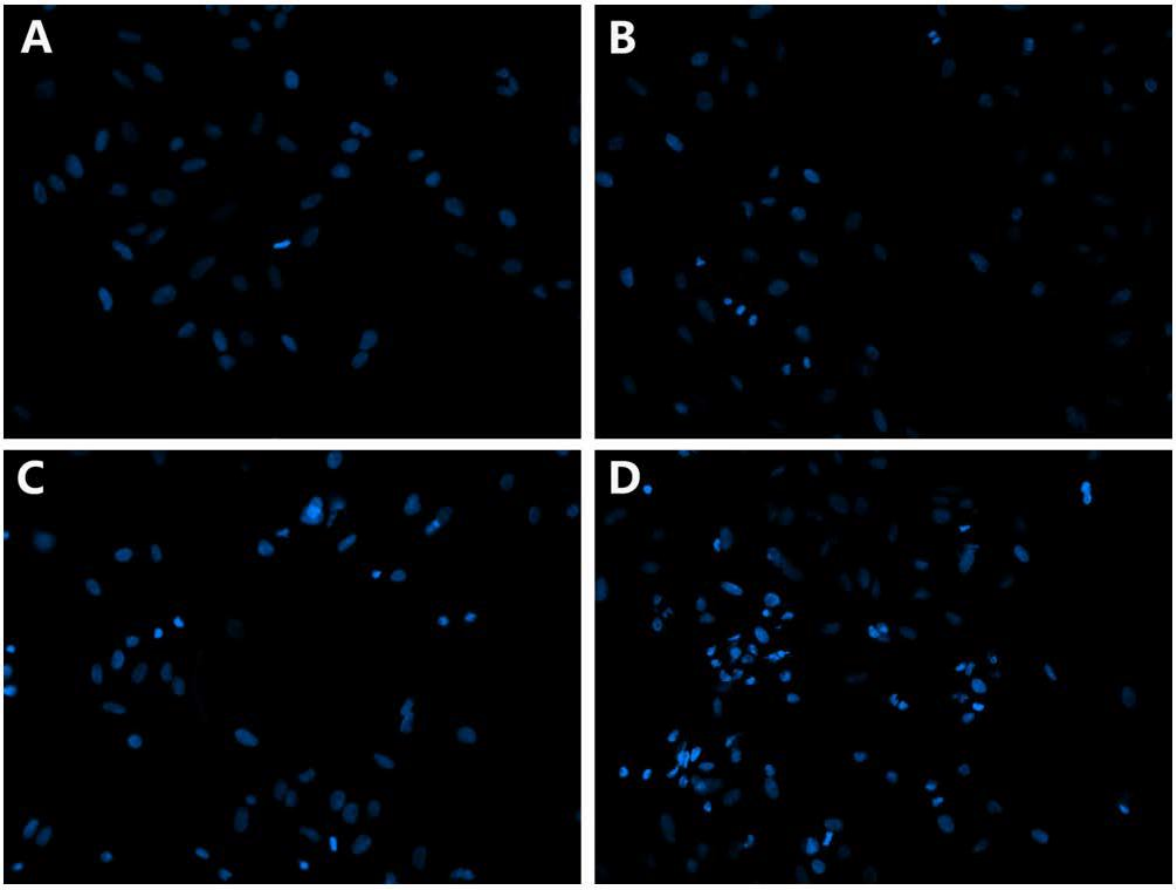

Fig. 6: The morphological changes (×200) of apoptotic cells could be observed after being treated with Tan IIA. (A) Untreated T-24 cells were dyed green and had a smooth and intact membrane; (B) Nuclei of T-24 cells became mild hyperchromatic and shrunk after exposure to Tan IIA (2mg/L, 24h); (C) More dense hyperchromatic, more obvious shrunk and characteristic membrane blebbing was detected in T-24 cells treated with 5mg/L Tan IIA for 48h; (D) Most extensive cellular apoptosis and dying at 10mg/L Tan IIA for 72h (fig. 6D).

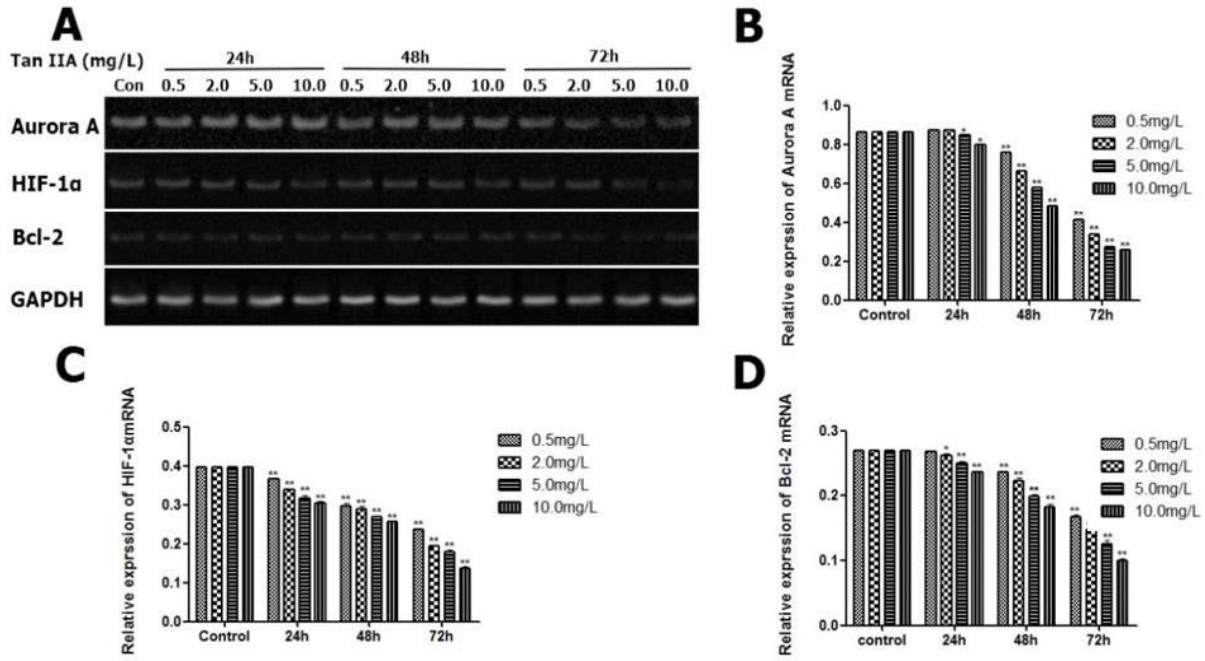

Fig. 7: The T-24 cells were treated with different concentrations (0.5mg/L, 2mg/L, 5mg/L, 10mg/l/L) and durations (24, 48 and 72 hours) of Tan IIA and the mRNA expression of Aurora A, HIF-1α and Bcl-2in T-24 cells was evaluated by PCR (A). With an increase in the concentration and duration of Tan IIA, the mRNA expression levels of Aurora A (B), HIF-1α(C) and Bcl-2 (D) gradually and significantly increased in a time- and dose-dependent manner($p<0.01$) (n=3) (* $p<0.05$: ** $p<0.01$).

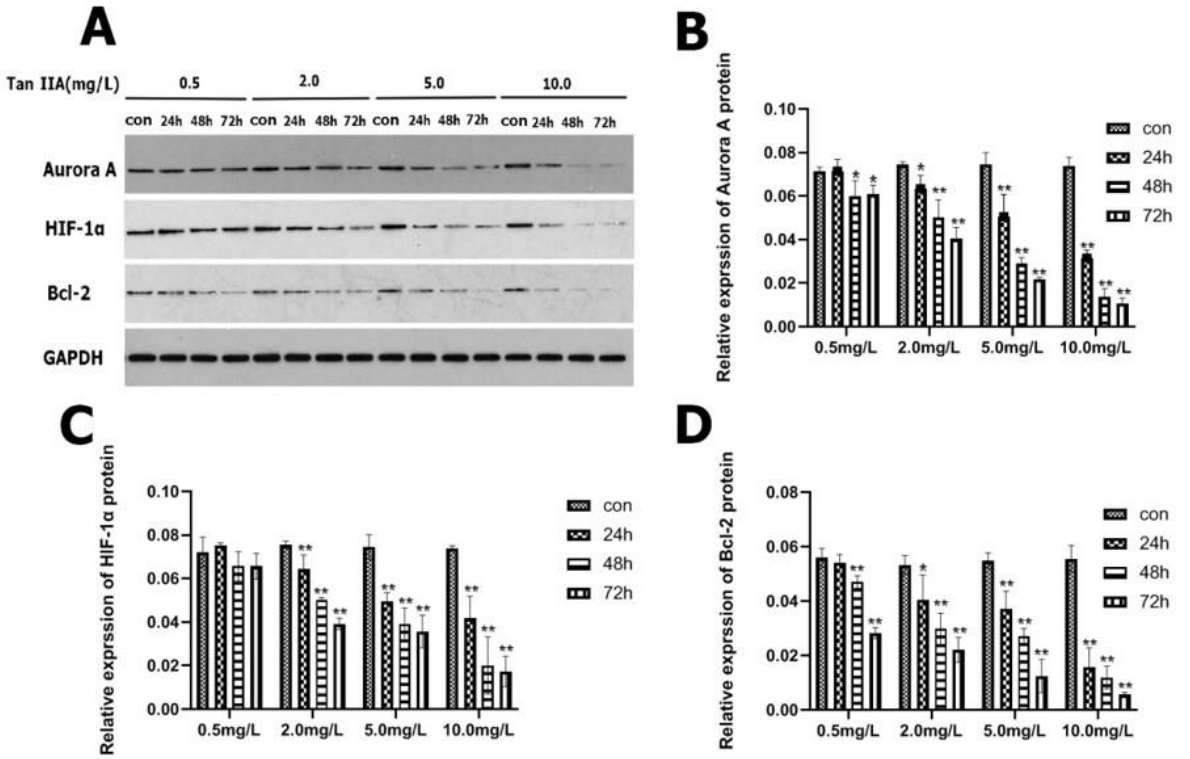

Fig. 8: The T-24 cells were treated with different concentrations (0.5mg/L, 2mg/L, 5mg/L, 10mg/l/L) and durations (24, 48 and 72 hours) of Tan IIA, and the protein expression of Aurora A, HIF-1α and Bcl-2 in T-24 cells were measured by western blot. The protein expression levels of Aurora A (A), HIF-1α(B) and Bcl-2 (C) gradually and significantly decreased in a time- and dose-dependent manner($p<0.01$), except for the protein expression of HIF-1α in T-24 cell treated with 0.5 mg/ml Tan IIA ($P>0.05$) (n=3) (* $p<0.05$: ** $p<0.01$).

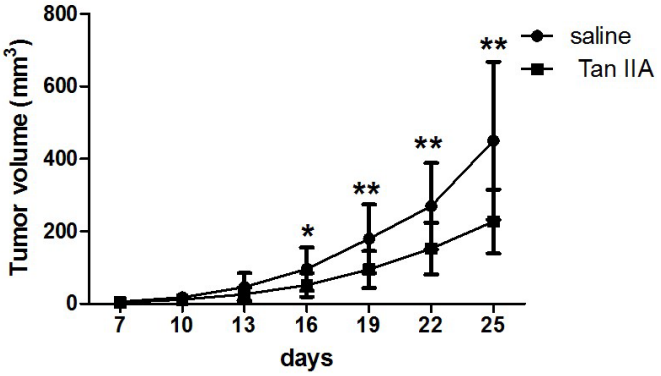

Fig. 9: Treatment with Tan IIA led to significant growth inhibition of xenograft tumors of T24 cells compared to the saline group (*p<0.05： **p<0.01).

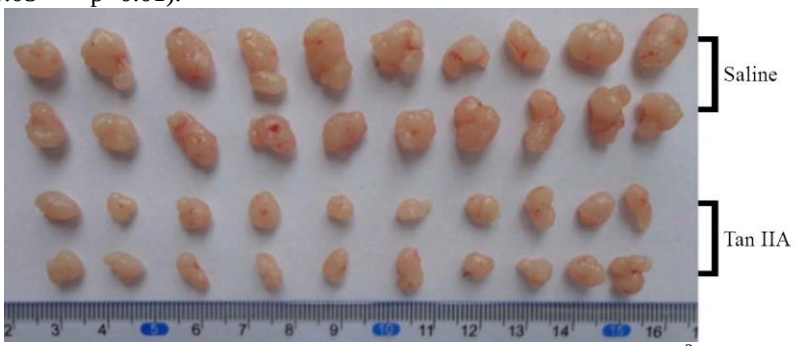

Fig. 10: The final tumor volume in the Tan IIA treated group was 223.2±19.7mm^3, whereas the saline group increased to 450.0±48.64 mm^3 and the tumor growth was attenuated by 50.0% (P<0.01).

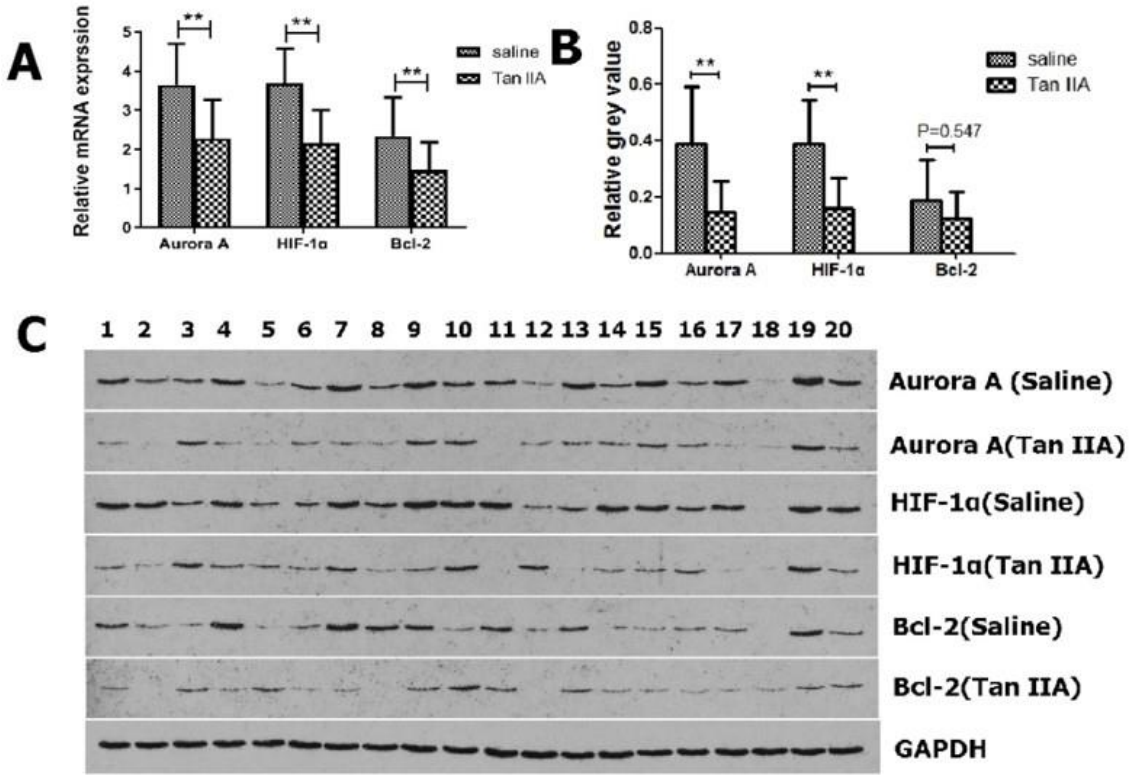

Fig. 11: The mRNA (A)and Protein expression (B, C) of Aurora A, HIF-1αand Bcl-2 were significantly inhibited, except for the protein expression of Bcl-2 (P=0.547), by Tan IIA in xenograft tumor samples after inoculation of 25 day detected respectively by Western Blot Analysis and RT-PCR, compared to that of saline group (The 20 lanes in fig. C denote 20 samples treated with Tan IIA or saline as fig.10) (*p<0.05: **p<0.01).

Abnormal expression of HIF-1α has been reportedly associated with many properties of carcinogenesis, including cell proliferation, angiogenesis, glucose metabolism, invasion and metastasis of tumors, which has been correlated with poor prognosis of patients (Luo, 2022). In a meta-analysis, Soni *et al* found that HIF-1α has been regarded as a potential anticancer target because it can, as a networking hub, regulate multiple signaling molecules influencing carcinogenesis (Soni and Padwad, 2017). In the present study, pro-apoptosis, anti-proliferation and anti-invasion were possibly associated with suppression of mRNA and protein levels of HIF-1αin T-24 cells treated with Tan IIA both *in vitro* and *in vivo*.

HIF-1α subunit has emerged as a potential target of cancer therapeutics. During a phase III trial, PT2399 (belzutifan) demonstrated efficacy against renal cell cancer (RCC) and other tumors in patients with von Hippel-Lindau syndrome, leading to its recent approval by the Food and Drug Administration (FDA) (Wicks and Semenza, 2022). Although down-regulation of mRNA and protein level of HIF-1α in T-24 cells was detected in the present study, a low dose of Tan IIA (0.5mg/L) did not affect protein expression of HIF-1α, suggesting that different concentrations of Tan IIA might trigger varying post-transcriptional modulation.

As a family of protease enzymes, Caspases play essential roles in apoptosis and can be activated by both the 'extrinsic' pathways triggered by various cellular stresses and impairment of the integrity of the mitochondrial outer membrane (MOM) (Adams and Cory, 2018). The over-expression of pro-survival Bcl-2 protein in most cancer tissues and cell lines rendered Bcl-2 a novel potential drug target for cancer treatment (Kaloni et al., 2023). Since the 1980s, more than 16 members of the Bcl-2 protein family have been identified and its capacities of inhibiting cell programmed death without promoting proliferation has been reportedly proved by previous studies, through which some kinds of small-molecule compounds, termed "BH3-mimetics", with the abilities of pro-apoptosis by binding to Bcl-2 have been discovered (Hafezi and Rahmani, 2021). Although significant cytotoxicity against a variety of malignancies has been demonstrated in clinical trials, the progression in clinical application of Bcl-2 inhibitors is hindered by unrelated toxicity and side effects, such as thrombocytopenia and neutropenia (Suvarna et al., 2019). In the present study, although Tan IIA suppressed the mRNA and protein expression of Bcl-2 *in vitro*, no significant change was observed in the protein expression of Bcl-2 *in vivo*. Nevertheless, Tan IIA may still be a novel agent of inhibiting Bcl-2 with significant anti-cancer activity and fewer side effects.

CONCLUSION

The present results *in vitro* and *in vivo* demonstrated that the pro-apoptosis, anti-proliferation, and anti-invasion effects of Tan IIA on BUC T-24 cells may arise from inhibiting the mRNA and protein expression of Aurora A, HIF-1α and Bcl-2 in a time- and dose-dependent manner. The results also confirmed that Tan IIA could be a new anti-cancer drug with the ability of 'multi-targets' and provided a basis for future research to elucidate the underlying mechanisms of anti-cancer involved in checkpoints of the cell cycle, hypoxia induction and the balance between anti- and pro-apoptosis.

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