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Bioactive tanshinone I inhibits the growth of lung cancer in part via downregulation of Aurora A function

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Abstract

Lung cancer is the leading cause of cancer death in the world, and the searching for novel efficacious and safe agents for lung cancer prevention remains the top priority of lung cancer research. In the present study, we evaluated the effect of bioactive tanshinones from a Chinese herb *Salvia Miltiorrhiza*, Cryptotanshinone (CT), Tanshinone I (T1) and Tanshinone IIA (T2A), on the proliferation inhibition of lung cancer cell lines. Tanshinones inhibited the lung cancer cell proliferation in vitro, with T1 the most potent, via cell cycle arrest and apoptosis induction. Gene function assay showed that Aurora A knockdown by siRNA dramatically eliminated the T1 activity in vitro, suggesting that Aurora A is an important functional target for T1. We further evaluated the effectiveness of T1 on the growth of H1299 non-small lung cancer cell in a mouse model. Tanshinone I inhibited the growth of H1299 lung tumor in a dose-dependent manner. Tanshinone I at 200mg/kg body weight significantly reduced final tumor weight by 34% ($P<0.05$) associated with inhibiting proliferation and inducing apoptosis of lung cancer cells by 54% ($P<0.001$) and 193% ($P<0.001$) respectively, inhibiting lung tumor angiogenesis by 72% ($P<0.001$), and reducing Aurora A expression by 67% ($P<0.001$). On the other hand, T1 did not significantly alter food intake or body weight. Our results provided experimental evidence to suggest that T1 may be an efficacious and safe agent for the prevention of lung cancer progression and Aurora A may be an important molecular target for T1 action against lung cancer.

Keywords

lung cancer; tanshinones; apoptosis; angiogenesis; proliferation; Aurora A

INTRODUCTION

Lung cancer is the leading cause of cancer deaths for both men and women in the United States and throughout the world [1]. This cancer has proven difficult to control with conventional therapeutic and surgical approaches, and the mortality rate within 5 years is 80–85%. Although significant progress has been made in our understanding of the molecular mechanisms of lung carcinogenesis, the therapeutic interventions for lung cancer have achieved only modest benefits [2]. Traditional chemotherapy also holds the drawback of

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cytotoxicity to normal tissues. Therefore the searching for efficacious and safe agents to prevent, inhibit, or reverse lung carcinogenesis remains the priority of lung cancer research.

Herbal medicines usually contain multiple bioactive components with specific biological activities and are also used as alternative therapeutic or preventive regimens for individuals with cancer [3,4]. Some of those herbal medicines have been used for centuries without demonstrating significant adverse effects on humans, thus their preparations and/or active ingredients could serve as efficacious and safe candidates for the prevention and/or therapy of cancer.

Danshen (*Salvia miltiorrhiza*) is a Chinese herb that has been widely adopted in the traditional Chinese medicinal preparations. Danshen products have been used for treating coronary heart diseases, such as angina pectoris and myocardial infarction [5]. Along with 20 phenolic acids, 30 diterpene compounds, including the relatively abundant cryptotanshinone (CT), tanshinone I (T1), and tanshinone IIA (T2A), have been isolated from Danshen [5]. In addition to their functions in cardiovascular systems, these abundant tanshinones have been recently shown to possess some activities against human cancer cells. Cryptotanshinone inhibited the growth of hepatocarcinoma cells [6] and breast carcinoma cells [7] in vitro via cell cycle arrest at S or G₁-G₀ phase. Tanshinone I inhibited the growth of leukemia [8–11], lung [12] and breast cancer [13] in vitro in part via induction of apoptosis. Tanshinone IIA inhibited the growth of breast cancer [14,15], nasopharyngeal carcinoma [16], glioma [17], leukemia [8–11,18] and hepatocellular carcinoma [19–21] cells in vitro by induction of apoptosis [16,19], inhibited invasion of lung cancer cells in vitro [22], and inhibited the growth of hepatic carcinoma [23] and breast tumor [14] in vivo. We recently demonstrated that these tanshinones inhibited the proliferation of prostate cancer cells in vitro, with T1 being the most potent agent [24]. We further showed that T1 had the most potent anti-angiogenesis activity in vitro and in vivo, inhibited the growth of prostate tumor in mice, and had minimal adverse effect in vivo [24]. These studies supported tanshinones, especially T1, as candidate preventive and/or therapeutic agents against cancer progression. On the other hand, the effects of tanshinones on lung cancer cells have not been adequately studied, and no in vivo studies have been conducted.

In this study, we determined the effects of tanshinones on the proliferation of lung cancer cell lines in vitro and the effect of T1 on the growth of human non-small cell lung cancer (NSCLC) H1299 tumors in mice. We also determined cellular and molecular biomarkers that were associated with the pharmacologic functions of T1, and identified Aurora A as an important molecular target for T1 function. Our results provided experimental evidence to suggest T1 as an efficacious and safe candidate agent for the prevention and/or therapy of lung cancer.

MATERIALS AND METHODS

Materials

Tanshinones CT, T2A and T1 were purchased from LKT Laboratories (St. Paul, MN), and the purities were verified by high performance liquid chromatography. Propidium iodide (PI) was from Sigma (St. Louis, MO); RNase A and 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were from Promega (Madison, WI). Antibodies used in Western blot against human antigens were cyclin B1, cdc2, p-cdc2 (Thr 161) and Bax (Oncogene Research Products, Boston, MA), Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), Aurora A and survivin (Cell Signaling, Beverly, CA), CDK2 (Selleck Chemicals, TX, USA), and β -actin (Merck Co., Darmstadt, Germany). Antibodies used for immunohistochemistry against human antigens were Ki-67 and Factor VIII (Dako North America, Inc., Carpinteria, CA). Biotinylated anti-mouse/anti-rabbit IgG,

Vectastain ABC kit and DAB substrate kit were from Vector Laboratories Inc. (Burlingame, CA).

Cell Culture and Cell Proliferation Assay

Human NSCLC (H1299, H23, A549) cell lines and mouse Lewis lung carcinoma (LLC) cell line were purchased from the American Type Culture Collection (ATCC, Bethesda, MD), and were maintained in DMEM (Life Technologies, Grand Island, NY), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Grand Island, NY) and antibiotics at 37°C in 5% CO₂. The effect of tanshinones on cell proliferation was determined by using Cell Titer 96 Aqueous One Solution Reagent, MTS, as we previously used [25]. Briefly, cells were cultured in 96-well plates and allowed to attach overnight. Cells were then treated with tanshinones at desired concentrations or dimethyl sulfoxide (DMSO) as the vehicle and incubated for 72 hours. MTS was added and incubated for 2 to 4 hours at 37°C in 5% CO₂ and light absorbance of formazan was measured at 490 nm in a microplate reader (VersaMax, Molecular Device, Sunnyvale, CA). The experiments were independently performed at least thrice, each in triplicate.

Clonogenic Survival Assay

The effects of tanshinones on clonogenic survival of cancer cells were determined by a colony-forming assay following the method we described before [25]. In brief, cells (200 cells) were plated in 35-mm tissue culture disc and allowed to attach overnight, treated with desired concentration of tanshinones or DMSO, and incubated for 10 days. The colonies composed of >50 cells were counted. The experiments were independently performed at least twice, each in duplicate.

Cell Cycle Analysis

Cells treated with different concentration of tanshinones were harvested, stained with propidium iodide (PI) and then analyzed by flow cytometry (Becton Dickinson, Immunocytometry Systems, Mountview, CA) for cell cycle distribution according to the protocol we used before [26]. Stained cells were analyzed by using FACScans (Becton Dickinson, Mountview, CA) for fragmented DNA and cell cycle using programs provided by Becton Dickinson. The experiments were independently performed at least twice, each in duplicate.

Cell Apoptosis Detection

Cell apoptosis after tanshinones treatment was determined by Annexin V-PI apoptosis detection kit (Chemicon International Inc, Billerica, MA) following the protocol we described before [24]. In brief, treated cells were resuspended in Annexin V solution and incubated at room temperature for 15 min, then PI was added for another 5-min incubation in the dark. Apoptotic cells were analyzed by flow cytometry (Becton Dickinson, Immunocytometry Systems, Mountview, CA). The experiments were independently performed at least twice, each in duplicate.

Aurora A Silencing by siRNA

The Aurora A silencing by siRNA followed the method described by Lentini and coworkers [27] with appropriate modifications. Briefly, 8×10^4 H1299 cells were seeded in a six-well plate and incubated for 24h. The silencer negative control and siRNA for Aurora A (Ambion, Austin, TX) were diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA) and transfected with Lipofectamine 2000 according to the manufacturer's instructions. The final concentration of siRNA added to the cells was 33nM. The duplex siRNA sequence for Aurora A was as follows: 5'-AUGCCUGUCUUACUGUCATT-3'.

Animal Study

Female severe combined immune-deficient (SCID) mice (six-week-old) were purchased from Taconic (Germantown, NY), and fed the AIN-93M diet for one week of adaptation. All animals were maintained under specific-pathogen-free conditions. To establish the prevention model, mice were randomly assigned into 3 experimental groups and pretreated with the vehicle (100 μ l corn oil), T1 at 80mg/kg body weight (BW) or 200 mg/kg BW with 100 μ l corn oil by oral gavage daily for 10 days. Each mouse was then injected subcutaneously with 2×10^6 of H1299 cells and continued the treatment throughout the entire experiment for 32 days. Food intake and body weight were measured weekly. The tumor diameter was measured by a caliper weekly. At the end of experiment, the mice were sacrificed, and primary tumors were excised and weighed. A tumor slice from each primary tumor tissue was carefully dissected and fixed in 10% buffer-neutralized formalin, paraffin-embedded, and sectioned at 4 μ m thickness for immunohistochemistry. Other tumor tissues were snapped frozen in liquid nitrogen and further saved at -80°C . The animal experiment was performed in accordance with NIH guidelines [28] and approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Western Blot Analysis

Cells treated with different concentrations of tanshinones or tumor tissues in different experimental groups were prepared for cell lysates, and protein levels were determined following the procedures as we previously described [25,29]. Equal amounts of protein samples were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane. The membrane was soaked in PBS-Tween buffer containing 5% low-fat milk for 60 min with gentle shaking and then incubated with specific antibody overnight followed by washing and incubation with a second antibody and the final chemiluminescence ECL (Thermo Scientific, IL, USA) detection of band. Protein bands were quantitated by densitometric analysis using the NIH image analysis software and expressed as percentages of the control after being normalized with β -actin. The primary antibodies used were Aurora A (1:1000), cyclin B1 (1:2000), Cdc-2 (1:500), p-cdc2 (1:1000), CDK2 (1:1000), Bax (1:500), Bcl-2 (1:300), survivin (1:1000) and β -actin (1:10,000).

In Situ Detection of Apoptotic Index

Apoptotic cells were determined by a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay (Chemicon International Inc, Billerica, MA) following our described protocols [25,29]. In brief, sections were treated with proteinase K and 3% H_2O_2 -methanol. Sections were then incubated with dUTP labeled by biotin and with antidigoxigenin peroxidase for 30 min at room temperature, washed, stained with 3,3'-deaminobenzidine substrate, counterstained with methyl green, and mounted. Six representative areas of each section without necrosis were selected, and apoptotic cells were counted. Apoptotic index was expressed as a percentage of control.

Immunohistochemical Determination of Tumor Growth and Tumor Angiogenesis

The tumor growth was determined by immunohistochemical staining of Ki-67 to quantitate growth index, and tumor angiogenesis was determined by Factor VIII staining to quantitate microvessel density (MVD), by following our previous protocols [25,26]. Briefly, sections were incubated with trypsin (for Factor VIII) or boiling citrate buffer (for Ki-67) for antigen retrieval and quenched with 3% H_2O_2 -methanol, then blocked with 10% normal goat serum. The sections were treated with human Ki-67 or Factor VIII antibody for 1h, followed by the secondary antibody incubation and staining procedures. Six representative areas of each section without necrosis were selected, and Ki-67 or Factor VIII positive cells were counted.

Ki-67 expression level and MVD were expressed as a percentage of control. Representative images were captured and the data was analyzed by the Ivision imaging software (Biovision, Exton, PA).

Statistical Analysis

Results were expressed as group means $\pm$ SEM and analyzed by analysis of variance followed by Fisher's protected least-significant difference based on two-side comparisons among experimental groups by using Statview 5.0 program (SAS Institute, Inc., Cary, NC). A $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Tanshinones on the Proliferation and Clonogenic Survival of Lung Cancer Cells in Vitro

Tanshinones CT (Fig. 1A), T1 (Fig. 1B) and T2A (Fig. 1C) inhibited lung cancer cell proliferation in a dose dependent manner. The IC_{50} 's of CT, T1 and T2A are around 8–12 μ M, 3–8 μ M and 3.5–10 μ M, respectively, for different lung cancer cell lines (except for T2A treatment of LLC cell line). Interestingly, LLC cell line was not at all sensitive to T2A treatment (Fig. 1C), but it was the most sensitive line to T1 treatment (Fig. 1B).

We further examined the effects of CT, T1 and T2A on clonogenic survival of H1299. Tanshinones significantly inhibited the colony formation (Fig. 1D–E). When compared to the proliferation inhibition assay, the colony formation assay was more sensitive (approximately 10 folds) to the treatment.

Effects of Tanshinones on Cell Cycle Progression in Vitro

Cryptotanshinone and T1 inhibited cell proliferation by arresting cell cycle at S phase (Fig. 2A and 2B), but T2A arrested cell cycle at G₂-M phase (Fig. 2C). The measurement of cell cycle-related protein markers showed that CT and T1 treatment dramatically decreased the protein levels of Aurora A and cyclin B, and to the less extent, cdc2 in a dose dependent manner (Fig. 2E, 2F), whereas T2A treatment significantly decreased the protein levels of Aurora A and cyclin B, but did not alter that of cdc2 (Fig. 2F). On the other hand, the functional form of cdc2 (p-cdc2 (Thr 161)) was significantly downregulated by all the tanshinone treatment (Fig. 2H). CDK2 expression was also downregulated by tanshinones with CT the most potent one (Fig. 2E, 2H).

Effects of Tanshinones on H1299 Cell Apoptosis in Vitro

We used Annexin V-PI apoptosis detection kit to determine the effects of tanshinones on apoptosis induction of H1299 cells. As shown in Fig. 2D, CT, T1 and T2A treatments induced apoptosis dose-dependently. Among three tanshinones, T2A was the most potent one in apoptosis induction and increased apoptosis by 5 folds (from 2% to 10%) at the concentration of 2 μ M.

To elucidate the molecular mechanisms of tanshinones actions in apoptosis induction, we measured the expression of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2 and survivin) factors. All tanshinones significantly down-regulated the protein levels of anti-apoptotic factors Bcl-2 and survivin (P at least < 0.05) in H1299 cells, but did not significantly alter the protein level of pro-apoptotic factor Bax (Fig. 2G). All tanshinones also significantly increased the bax/bcl-2 ratio, a more reliable indicator of apoptosis. Cryptotanshinone (5 μ M and 7.5 μ M), T1 (1 μ M and 2 μ M), and T2A (2 μ M and 4 μ M) treatments increased Bax/Bcl2 ratios by 11.6 $\pm$ 3.9, 56.2 $\pm$ 48.0, 11.6 $\pm$ 2.7, 17.7 $\pm$ 11.3, 20.2 $\pm$ 4.4, and 6.8 $\pm$ 2.3 folds, respectively, compared with the control.

Effects of Aurora A Silencing on the Activity of T1

To determine if Aurora A is the functional target of T1, we used Aurora A specific siRNA to inhibit the expression of Aurora A in H1299 cells. Aurora A silencing significantly reduced cell proliferation by 45% (Fig. 3). Moreover, Aurora A silencing significantly decreased the anti-proliferation activity of T1. When Aurora A was silenced, the effect of T1 on cell proliferation was reduced dramatically (Fig. 3). The results support that Aurora A is an important functional target for T1.

Effects of T1 Treatment on H1299 Tumor Growth in Vivo

We further evaluated the effect of T1 on H1299 human lung tumor growth in mice. Tanshinone I reduced tumor volume in a dose- and time-dependent manners (Fig. 4A). Tanshinone I at 80 and 200mg/kg BW reduced the final tumor weight by 27.3% and 34.0% ($P=0.012$) (Fig. 4B), respectively. On the other hand, T1 did not significantly alter either food intake or body weight. In fact, mice treated with T1 (80 or 200mg/kg BW) consumed slightly more total foods (5–10%) than the control mice (Fig. 4C), whereas they had almost the same body weight (Fig. 4D). These results suggest that T1 treatment inhibits the lung tumor growth with limited adverse effect on food intake and/or body weight.

Modulation of H1299 Tumor Proliferation, Apoptosis and Tumor Angiogenesis by T1 Treatment in Vivo

Analyses of cellular markers by immunohistochemical staining showed that the T1 treatment at 200mg/kg BW significantly induced lung tumor cell apoptosis by 193% (Fig. 5A–C, $P<0.001$) and reduced lung tumor proliferation by 54 % (Fig. 5D–F, $P<0.001$). Microvessel density measurement showed that the T1 treatment at 200mg/Kg BW significantly reduced lung tumor angiogenesis by 72% (Fig. 5G–I, $P<0.001$), confirming the anti-angiogenesis activity of T1 in vivo.

We also measured the protein level of Aurora A, Bcl-2 and Bax by Western blot. Results showed that the T1 treatment significantly reduced Aurora A and Bcl-2 protein levels by 67% and 73% (Fig. 6A and 6B, $P<0.001$) respectively, and significantly increased Bax protein level by 15% (Fig. 6A and 6B, $P<0.05$), and the Bax/Bcl-2 ratio by 5 folds (Fig. 6A and 6B, $P<0.01$).

DISCUSSION

In the present study, we found that three major tanshinones from Chinese herb Danshen, CT, T1 and T2A significantly inhibited the proliferation of lung cancer cell lines in vitro via induction of apoptosis and cell cycle arrest. Gene function assay demonstrated that Aurora A was an important molecular target for tanshinone actions. The animal study further confirmed that T1 significantly reduced the final tumor weight associated with inducing lung cancer cell apoptosis and inhibiting lung cancer cell proliferation, and reducing lung tumor angiogenesis, without significant adverse effect on food intake or body weight. This is the first study, to the best of our knowledge, that demonstrated the inhibitory effect of T1 on the growth of lung tumors in vivo and identified Aurora A as an important molecular target for T1 actions.

Cellular mechanism studies showed that tanshinones inhibited the proliferation of lung cancer cells via cell cycle arrest and apoptosis induction (Fig. 2A–2D). Interestingly, CT and T1 arrested the cell cycle progression at S phase in part via down regulation of CDK2, cdc2, cyclin B and Aurora A expression levels (Fig. 2E, 2F, 2H), whereas T2A arrested the cell cycle progression at G₂-M phases with down regulation of cyclin B and Aurora A expression (Fig. 2E, 2F). Cdc2, also known as CDK1 (cyclin dependent kinase), plays an

important role during the cell cycle progress. Cdc2 usually combines with cyclin B and regulates the S and especially G₂-M phase progression. Cdc2 has been considered as an essential molecular target for design of therapeutic anti-cancer drugs [30]. The complex of CDK2-cyclinA holds a key role in the progress of S phase. In this study, we found that the expression of CDK2 was inhibited especially by CT or T1 treatment, the findings that may explain, at least in part, the effects of tanshinones (CT or T1) on arresting cell cycle at S phase. Down-regulation of both cdc2 and cyclin B may also provide an important molecular mechanism that CT or T1 arrests cell cycle progression of lung cancer cells at S phase, whereas down-regulation of cyclin B may play an important role in G₂-M arrest of lung cancer cells by T2A. More investigation is needed to further elucidate the molecular mechanisms by which tanshinones regulate cell cycle progression.

Aurora A was a member of a novel oncogenic family of mitotic serine/threonine kinases. Abundant evidence suggested roles for Aurora A in centrosome maturation [31], spindle formation [32], and G₂-M transition [33]. Aurora A was frequently over expressed in different types of cancers [27,34–37]. Suppression of Aurora A expression and function reduced tumor growth [38–41]. Thus Aurora A has been recognized as an important molecular target for cancer therapy [42–44]. Our studies showed that tanshinones significantly down-regulated Aurora A expression level in vitro (Fig. 2E and 2F) and T1 significantly downregulated Aurora A protein levels in vivo (Fig. 6). Our in vitro function assay also demonstrated that the anti-proliferating activity of T1 was dramatically reduced when Aurora A gene was silenced by siRNA (Fig. 3). These experimental data suggests that Aurora A may be a novel molecular target for tanshinone actions.

Tanshinones also significantly induced apoptosis of lung cancer cells in vitro (Fig. 2D) associated with down regulation of Bcl-2 and survivin gene expression and protein levels (Fig. 2E, 2G) and increased Bax/Bcl-2 ratio, a more reliable indicator for apoptosis [45]. Our results were consistent with that of previous in vitro studies showing that apoptosis induction was an important cellular mechanism of tanshinones actions in inhibiting the cell proliferation of different cancer types [8,13,14,18]. We further provided the in vivo evidence to support that T1 induced apoptosis of lung cancer cells (Fig. 5A–C) associated with down-regulation of anti-apoptotic factor Bcl2 and upregulation of pro-apoptotic factors Bax and Bax/Bcl2 ratio (Fig. 6).

Angiogenesis is a critical step in tumor growth [46]. The growth of all solid tumors depends on angiogenesis and suppression of tumor blood vessel offers a new option for the prevention and treatment of cancer [47]. Our previous study showed that among three tanshinones, T1 had the most potent anti-angiogenesis activity in both the in vitro and in vivo models and inhibited angiogenesis in prostate tumor in mice [24]. In this study, we further demonstrated that T1 inhibited the growth of H1299 tumor associated with inhibition of tumor angiogenesis (Fig. 5G–I). These results provided convincing experimental evidence to support that one of the mechanisms by which T1 inhibits tumor growth is via inhibiting angiogenesis. Further investigation is required to determine the underlying molecular mechanisms that T1 inhibits tumor angiogenesis.

In conclusion, our results provided experimental evidence to suggest that T1 may be efficacious and safe agent for the prevention of lung cancer progression and Aurora A may be an important molecular target for T1 action against lung cancer.

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Abbreviations

BW	body weight
cdc2	cell division cycle-2
CT	cryptotanshinone
CDK	cyclin dependent kinase
DMSO	dimethyl sulfoxide
MVD	microvessel density
NSCLC	non-small cell lung cancer
PBS	phosphate-buffered saline
PI	propidium iodide
SCID	severe combined immune-deficient
T1	tanshinone I
T2A	tanshinone IIA
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling

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Figure 1. Effects of tanshinones on the growth and colony formation of lung cancer cells in vitro. A–C, the dose-dependent effects of CT (A), T1 (B) and T2A (C) on the growth of three human lung cancer cell lines (H1299, H23 and A549) and one mouse lung cancer cell line (LLC). D–F, effects of CT (D), T1 (E) and T2A (F) on colony formation of H1299 cells. Values are mean $\pm$ SEM of three independent experiments in triplicate.

Figure 2.

Effects of tanshinones on cell cycle progression and apoptosis of H1299 cell line. A–C, effects of CT (A), T1 (B) and T2A (C) on cell cycle distribution measured by flow cytometry. D, effects of tanshinones on apoptosis measured by Annexin V-PI staining. E, the representative images of Western blot analyses for protein levels of cell cycle related biomarkers Aurora A, cdc2, p-cdc2, CDK2 and cyclin B, and apoptosis related biomarkers Bcl-2, Bax and survivin. F–H, protein levels of Aurora A, cdc2 and cyclin B (F), Bcl2, Bax and survivin (G), and p-cdc2 and CDK2 (H) as quantified by densitometry after normalization to β-actin. Values are mean ± SEM. Within the panel, the value with a letter is significantly different from that of the corresponding control, a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

Figure 3. Effect of Aurora A silencing by siRNA on the anti-growth activity of T1. Values are mean $\pm$ SEM of three independent experiments in triplicate.

Figure 4.

Effects of T1 treatment at 80mg/kg BW and 200mg/kg BW on the volume of H1299 tumors in mice (A), the final tumor weight (B), cumulative food intake (C) and body weight (D). Values are group mean $\pm$ SEM (n=12/group). Within the panel B, the value with a letter is significantly different from that of the control, a, $p < 0.05$.

Figure 5.

Effects of T1 treatment at 200mg/kg BW on H1299 tumor cell apoptosis index measured by TUNEL assay (A–C), tumor cell proliferation measured by immunohistochemical staining of ki-67 (D–F), and tumor microvessel density measured by Factor VIII staining (G–I). For the analysis of immuno-staining, six representative areas of each section were selected, and the results were analyzed by using the Ivision-Mac imaging software. The data are expressed as the mean percentage of the control $\pm$ SEM. The value with a letter was significantly different from that of the control, c, $p < 0.001$.

Figure 6.

Effects of T1 treatment at 200mg/kg BW on the protein levels of Aurora A, bcl2 and bax in H1299 tumors measured by Western blot (A) and quantified by densitometry after normalization to β -actin (B). The data are expressed as the mean percentage of the control $\pm$ SEM. Within the panel B, the value with a letter is significantly different from that of the control, a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.