Inhibition of Proliferation and Induction of Apoptosis by Trimethoxyl Stilbene (TMS) in a Lung Cancer Cell Line

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Abstract

Trimethoxyl stilbene (TMS) is a derivative of resveratrol, a compound shown to inhibit development of a variety of tumor types. We aimed to evaluate the effect of TMS on cell proliferation and apoptosis in the A549 non-small cell lung cancer cell line. Growth inhibition rate and colony formation was measured and apoptosis was determined with Hoechst 33258 staining. Protein expression levels of caspase-3, STAT3, STAT5b, JAK2, NF-κB, and IκB were examined by Western blotting. Furthermore, localization of NF-κB protein was also explored. TMS inhibited proliferation (IC50 8.6 μmol/L) and induced apoptosis of the cells in a concentration-dependent manner, also inducing apoptosis accompanied by up-regulated expression and cleavage activation of caspase-3, up-regulation of IκB and down-regulation of NFκB, STAT3, STAT5b, and JAK2 signal transduction. TMS has potential as a new drug for treatment of non-small cell lung cancer patients with anti-proliferation and apoptosis inducing effect of TMS to A549 cells apparently related to its inhibitory effect on STATs and NF-κB signal transduction. Up-regulation of caspase-3 further supports the potential clinical use of TMS for the treatment of non-small cell lung adenocarcinoma.

Key words: Trimethoxyl stilbene - resveratrol - lung cancer cell line - adenocarcinoma - anti-cancer

Introduction

Lung cancer is a common malignancy that causes substantial mortality and morbidity (Wang et al., 2008). It can be divided into two general types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The majority of lung cancer patients (80%-85%) have NSCLC (Herbst et al., 2008). Because after resection, treatment may still fail in some patients due to recrudescence and metastasis, post-operative auxiliary chemotherapy to eliminate the residual micrometastases in the body can reduce the risk of tumor recurrence and metastasis and improve the prognosis.

Resveratrol (RES, chemical name 3,5,4′-trihydroxyl stilbene) is a type of active non-flavone phenol that was first isolated from the root of veratrum grandiflorum in 1940. It has been shown to inhibit significantly the occurrence and progression of many tumor types, including breast cancer, colon cancer, prostate cancer, endometrial cancer, and ovarian cancer (Subbaramaiah et al., 1998). The anti-cancer activity and mechanism of action of RES vary according to the cell type treated (Windmill et al., 1997; Chang et al., 2001; Ciolino and Yeh, 2001; Dorrie et al., 2001; Potter et al., 2002; Liu et al., 2004; Schlegel and Williamson, 2005; Song et al., 2005). Because RES contains 3 phenolic hydroxyls, it is easily oxidized and is therefore unstable. To improve stability, researchers have tried to protect these phenolic hydroxyls with chemical modifications, methylation being the modification most frequently reported. The trimethylated derivative, cis-3,5,4′-trimethoxyl stilbene (TMS), has been shown to produce a significant anti-mitotic effect by inhibiting polymerization of tubulin (IC50 4 μmol/L) in a dose-dependent manner, and to have a strong inhibitory effect on human colon cancer Caco-2 cells, with an inhibition of 80% at a dosage of 0.3 μmol/L, and 100% at a dosage of 0.4 μmol/L. This inhibitory activity is 100 times stronger than that of the trans-isomer (Schneider et al., 2003). However, the trans-isomer has a strong anti-angiogenesis effect, and is 30-100 times stronger than RES in terms of inhibiting endothelial cell proliferation, growth, and collagen invasion, and has a strong vascular targeting effect (Belleri et al., 2005). Cardile et al (2005) studied the inhibition of 18 types of RES derivatives on human prostate cancer cells, and found that these derivatives had stronger or similar effects compared to RES. Of these compounds, the trimethyl derivative had the strongest activity, with a GI50 of 2192 μmol/L compared to a GI50 of 24109 μmol/L for RES.

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It is thought that in addition to increasing its stability, methylation of RES increases its lipophilicity, thus allowing easier entry into the cell, and that the specific position of the substituent groups is very important for activity. However, there are also some contradictory reports. When the apoptosis-inducing activity of RES and its trimethyl derivative were compared in two prostate cancer cell lines, Morris et al. (2002) found that RES showed higher activity than the derivative, and thought therefore that its hydroxyl groups were important for activity. And in another study, Stivala et al. (2001) suggested that the 4'-hydroxyl in RES was necessary for its anti-oxidant and anti-proliferative effects.

However, it should be noticed that in other anti-cancer experiments, RES had no effect on some tumor types. For instance, Gao et al. (2002) found that RES could not produce an inhibitory effect in the transplantable mouse leukemia model, and Sato et al. (2003) found that RES had no inhibitory effect on breast cancer induced by N-methyl-N-nitrosourea, and suggested that the anti-cancer effect of RES was selective for individual tumor types. The exact reasons for these differences need further investigation.

NF-κB protein was first discovered in a study of the regulation of immunoglobulin gene expression in B cells carried out by Sen and Baltimore (1996). This protein, a member of the NF-κB/Rel transcription factor family (Chen et al., 1998), has since been found in many types of cells and plays an important role in the regulation of many physiological and pathological processes, such as inflammation, immune response, cell proliferation and differentiation. NF-κB also plays an important role in regulating apoptosis, cell cycle progression, inflammation and tumor formation (Chen et al., 2001), and is reported to produce an activating effect in various malignant metastases (Bargou et al., 1997; Wang et al., 1999). Although in previous studies, it has been found that the activity of NF-κB is mainly anti-apoptotic, it can also promote apoptosis (Keles et al., 1995; Uberti et al., 2004). In the present study we tried to discover whether TMS could cause decrease A549 cell proliferation and increase apoptosis through an action involving the NF-κB signal transduction pathway.

Materials and Methods

Materials

2.1.1 The human non-small cell lung cancer A549 cell line was obtained from American Type Culture Collection (Manassas, VA, USA)

2.1.2 Major reagents: TMS powder (provided by Prof. Jiang Xinyu from School of Chemical Engineering, Central South University) was prepared as a 20 mmol/L mother liquid with DMSO (Sigma, St. Louis, MO, USA) and stored at -20˚ until use. RPMI-1640 powder (Gibco, Grand Island, NY, USA) was prepared with ultrapure water followed by filtration to remove any bacterial contamination. L-glutamine, penicillin, and streptomycin was then added into it at final concentrations, respectively, of 200μg/ml, 100U/ml, and100μg/ml. Newborn calf serum was purchased from Siva. Lymphocyte separation medium was purchased from Shanghai Hengxin Chemical Co., Ltd, and preserved in the dark until use. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma and prepared as a 5mg/ml solution for experiments. Agarose (Shanghai Bioengineer Company) was prepared as 5% solution with normal saline, followed by high pressure sterilization. 2.1.3 The major instruments used were the following: HW0301 carbon dioxide incubator (Philip Harris, UK); CK2 inverted microscope and BX-50 photomicroscope (Olympus); SW-CJ-1FD clean bench (Suzhou Antai Airtech Co., Ltd.); high speed centrifuge (Beckman, USA); Multiskan Ascent microplate reader (Thermo Electron Corporation, Waltham, MA, USA).

Cell Culture

A549 cell culture was performed as previously described (Situ and Wu, 2004). In brief, cells were grown in RPMI-1640 culture medium supplemented with 10% newborn calf serum, and kept at 37˚ in a humidified atmosphere containing 5% CO2. Cells in the exponential phase were used for all experiments.

Determination of Growth Inhibition Rate for A549 Cells

A549 cells in the exponential growth phase were digested with trypsin, and seeded into sterile 96-well plates at 5x103 cells/well in 200 μl of medium (six wells for each experiment group) and incubated for 24 h. At this time, the supernatant was removed, the cells washed twice with PBS, and complete medium 1640 containing 0μM, 1.25μM, 2.5μM, 5μM, 7.5μM, or 10μM TMS (concentrations that were selected on the basis of preliminary experiments) was added to the cells, followed by 48h culture. During the 48 h culture period, the medium was replaced at 24 h, but the TMS concentration was not changed. At 44 h, 20 μl 5g/L MTT solution was added to each well, mixed gently, and the culture continued for another 4 h. The plates were then removed from the incubator and the supernatant removed carefully from each well. 150 μl DMSO was added to each well, and the plate placed on a microplate shaker for approximately 10 minutes of low speed vibration in the dark to dissolve the blue-violet formazan crystals. The plate was then placed onto a microplate reader to measure the absorbance at 490 nm (A490) of each well. The blank control well was used to zero the reader. The cell growth inhibition rate was calculated according to the following formula:

\[
\text{Cell growth inhibition rate} (\%) = 1- \frac{A_{490\text{ mean of treatment group}}}{A_{490\text{ mean of control group}}} \times 100%. 
\]

The mean of 3 wells was calculated, the measurement repeated three times, and the results averaged. After
Smears were then observed and photographed with a room temperature, the smears were rinsed and air-dried. Solution was added, and after 10 min incubation at with PBS. One hundred µl Hoechst 33258 working 1 ml 4% formaldehyde for 10 min, and washed twice with cold PBS. Smears were prepared, fixed with the remaining supernatant. The cells were then washed eight hours later, part of the supernatant was carefully removed, followed by centrifugation and removal of the supernatant. The cells were then washed 1 ml 0.3% agarose medium in each well. Following solidification under room temperature, the plate was placed into an incubator (5% CO2, saturated humidity, 37˚) for 14 days. At day 14, colony number of experiment group/colony number of control group)

Colony inhibition rate (%) = 1-(colony number of experiment group/colony number of control group) x100.

The measurements were repeated 3 times and the results averaged.

**Apoptosis Determination with Hoechst 33258 Staining**

A549 cells in the exponential growth phase were added into RPMI-1640 medium supplemented with 10% sterile newborn calf serum to prepare a 3.0x105/ml single cell suspension, which was then transferred into a 6-well plate, and the plate placed under room temperature until solidification occurred. 10% sterile newborn calf serum medium RPMI-1640 was added to A549 cells of different groups to prepare single cell suspensions (940µl containing 1000 cells), and 60µl of the remaining supernatant was added. The cells were then placed into a 1.5 ml sterile microtubes, and the proteins isolated with Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology , Jiangsu,China) according to manufacturer’s instructions.

**Extraction of Cytoplasmic and Nuclear Proteins**

A549 cells were prepared in a 3.0 x105 single cell suspension, and inoculated into sterile culture flasks. TMS of different concentrations but the same volume was added and mixed well, to make final concentrations of 0 µmol/L, 4 µmol/L, 8 µmol/L, 16 µmol/L, 32 µmol/L, 64 µmol/L. For the control group, DMSO of the same volume was added. At the end of a 48 h incubation period, cells were collected, washed once with PBS, centrifuged, and the supernatant removed. The cells were then placed into a 1.5 ml sterile microtubes, and the proteins isolated with Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology , Jiangsu,China) according to manufacturer’s instructions.

**Immunofluorescence Localization of NF-κB Protein**

To study changes in expression and localization of NF-κB during TMS-induced apoptosis, we treated A549cells with the NF-κB activator TPA (100nmol/L) for 2 h, followed by addition of 32 µmol/L TMS, and a further 2 h incubation. The fluorescence intensity and distribution of NF-κB protein were then examined.

The protocol for this experiment was as follows: A549 cells in the exponential phase were added into 10% sterile newborn calf serum medium RPMI-1640 to prepare a 3.0x105/ml single cell suspension. Ten ml were inoculated into each sterile culture flask. The following groups were used: control group, TMS group, TPA group, and TPA+TMS group. Five µl DMSO were added to the control and TMS groups for 2h. A further 5 µl DMSO was then added to the control group, and 5µl TMS (final concentration 32
µmol/L) to the TMS group, followed by cell collection 2h later. For the TPA and TPA+TMS groups, 5µl TPA (final concentration 100 nmol/L) was added to each group, and after treatment for 2h, 5µl DMSO was added to the TPA group, and 5µl TMS (final concentration was 32 µl/L) to the TPA+TMS group, followed by cell collection 2h later. The collected cells from each group were then washed with PBS, centrifuged and the supernatant removed. They were then collected into a 1.5 ml sterile polyethylene tube, followed by indirect immunofluorescence staining analysis of the cell smears.

The A549 cell smears were soaked in 2% paraformaldehyde for 15 min, and then soaked with 0.2% Triton-X100 for 5 min in order to permeabilize the cell membranes. For the experimental group, rabbit anti-human NF-κB polyclonal antibody (diluted in 1:50) was added and the preparation incubated in a 37˚ wet box for 60 min. For the primary antibody control group: rabbit IgG (diluted in 1:50) was added, and the cells also incubated in a 37˚ wet box for 60 min. Goat anti-rabbit IgG (diluted in 1:500) was then added, labeled with FITC, and the cells incubated in a 37˚ wet box for 30 min, after which time the smears were air-dried and mounted. The samples were then observed and photographed under fluorescent microscopy.

Statistical Processing
The concentration effects of colony count, colony inhibition rate, cell growth inhibitory ratio, apoptosis and protein expression were determined using a linear mixed model with contrast. Pair-wise multiple comparisons between different concentration were tested using Bonferroni procedure with type-I error adjustment. To determine the time effect of cell growth inhibition a linear mixed model was used.

Data are presented as mean ± standard deviation. All statistical assessments were two-sided and evaluated at the 0.05 level of significance. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS Inc, Chicago, IL).

Results

TMS inhibits A549 cell proliferation
Figure 1-1 shows that as TMS concentration increased (from 0 µmol/L to 64 µmol/L), the cell growth inhibitory ratio increased in a concentration-dependent manner (P<0.05), with an IC50 of 8.6 µM/L. In addition, the longer the TMS intervention time, the higher the cell growth inhibition rate, that is, time dependence was also seen (P<0.05). Table 1 shows that as TMS concentration increased, colony number decreased and colony inhibition rate increased compared to control (P<0.01). And Figure 1-2 shows that as TMS concentrations increased, the diameter of the TMS-treated cell colonies also decreased.

TMS induces A549 Cell Apoptosis
Histological staining showed TMS treatment to induce features characteristic of apoptosis. In cells in the control group, the nuclear boundary can be seen clearly and blue-green fluorescence is distributed evenly. Chromatin is also distributed evenly in round or oval shaped nuclei (Figure 2A). In some cells treated with TMS, chromatin is aggregated in clusters of grains and shrunken nuclei are seen, and in other cells the nucleus has split into multiple spherical fragments, as seen in apoptosis (Figure 2B).

The apoptosis rate of A549 cells (Figure 3) increased dramatically in a concentration dependent manner as TMS concentrations increased (P<0.05). No significant difference from control was seen after treatment with

Table 1. Inhibition by TMS of Colony Formation of A549 Cells

<table>
<thead>
<tr>
<th>Drug concentration</th>
<th>Control group</th>
<th>4µmol/L</th>
<th>8µmol/L</th>
<th>16µmol/L</th>
<th>32µmol/L</th>
<th>64µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony count (colonies/well)</td>
<td>63.3±4.8</td>
<td>34.0±4.5*</td>
<td>18.4±2.8*</td>
<td>11.3±2.3*</td>
<td>7.5±2.1*</td>
<td>0*</td>
</tr>
<tr>
<td>Colony inhibition rate(%)</td>
<td>0</td>
<td>45.5±5.8*</td>
<td>62.4±3.5*</td>
<td>78.6±4.5*</td>
<td>81.7±3.5*</td>
<td>100*</td>
</tr>
</tbody>
</table>

*P < 0.05 compared to the control group.

Figure 1. a) Relationship between A549 cell growth inhibition rate, TMS concentration and treatment time; b) Inhibition by TMS of Colony Formation. Panels A, B, C, D, E and F represent, respectively, the 2µmol/L, 4µmol/L, 8µmol/L, 16 µmol/L, 32 µmol/L, 64µmol/L TMS concentrations (×200)
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Figure 2. Control (left) and TMS Group (right) Fluorescence

Figure 3. A549 Cell Apoptosis (%) after Treatment with Different Concentrations of TMS with the Hoechst 33258 Staining method (n=3)

Figure 4. Western Blotting Results. Lanes 1, 2, 3, 4, 5 and 6 represent, respectively, 0 µmol/L, 4 µmol/L, 8 µmol/L, 1.6 µmol/L, 32 µmol/L and 64 µmol/L TMS

Figure 5. Immunofluorescence Localization for NF-κB protein in A549 cells. Treatment with no drugs (Control Group), 100 µM TPA (TPA Group), 32 µM TMS (TMS Group) and 100µM TMS followed by 32 µM TPA (TPA+TMS Group) (x 400)

Inhibition of proliferation and induction of apoptosis by Trimethoxyl Stilbene in a lung cancer cell line was observed. 2 µmol/L TMS. However, A549 cell apoptosis rates at 32µmol/L TMS and 64µmol/L TMS were 6.75 and 8.34 times that of the control group, respectively (P<0.001).

Caspase-3 expression and cleavage activation increased as TMS concentrations rose from 8 µmol/L to 64 µmol/L (Figure 4). This result indicates that TMS up-regulates caspase-3 expression and cleavage activation in a concentration dependent manner, and that the apoptosis of A549 cells induced by TMS is associated with a caspase-mediated signal transduction pathway.

Figure 6. A549 Cell Apoptosis (%) after Treatment with Different Concentrations of TMS with the Hoechst 33258 Staining method (n=3)

Impact of TMS on Expression of Proteins in A549 Cells

In addition to up-regulation of caspase-3, protein expression of IκB was up-regulated and that of NF-κB, STAT3, STAT5b and JAK2 were down-regulated compared to the internal standard as TMS concentrations increased (Figure 4). The down-regulation of JAK2, however, was less than that of STAT3 and STAT5b.

Localization of NF-κB Protein in A549 Cells

The fluorescence signal for NF-κB in A549 cells in the control group was distributed mainly in the cytoplasm, with a small amount in the nucleus. In group treated with the NF-κB activator, TPA, the fluorescent signal was significantly increased compared to that of the untreated control group, and NF-κB distribution in the nucleus was increased. In the TMS-treated A549 cells (no activator used), 32µmol/L TMS made both the cell nucleus and cell body shrink, and only weak fluorescent intensity of NF-κB to be seen. In the cells treated with 100 nmol/L of the NF-κB activator for 2h followed by 32µmol/L TMS for 2h, NF-κB fluorescent signal intensity was reduced compared to cells treated with the activator alone, and almost no fluorescence was seen in the nucleus. These results show TMS could inhibit not only the activation of NF-κB in A549 cells, but also the NF-κB activating effect of TPA in these cells (Figure 5).

Discussion

RES has anti-proliferative activity in many types of tumor cells. In the current study, in a non-small cell lung cancer cell line treated with a methylated RES analog, MTT, we observed a decrease in cell proliferation, an increase in apoptosis, activation of caspase 3, an increase in protein expression of IκB and a decrease in protein expression and activation of NF-κB, a decrease in protein expression of STAT3 and STAT5b, and a smaller decrease in protein expression of JAK2.

TMS inhibited A549 cell proliferation with an IC50 of 8.6 µmol/L. It also decreased both the number and size of the A549 cell colonies formed in a concentration-dependent manner. In another study, TMS was more potent than the parent compound, RES, in inhibiting two human breast adenocarcinoma and one hepatoma cell lines, but the IC50 required was >150 µM (Alex et
al., 2010). However it was also more potent than RES in inhibiting bFGF-stimulated proliferation of human umbilical vein endothelial cells, with an IC50 of 3.75 μM (Alex et al., 2010) and more potent than RES in inhibiting colony formation of HT29 colon cancer cells, causing complete inhibition at a concentration of 25 μM (Saiko et al., 2008).

In the current experiment, TMS treatment induced the morphological changes characteristic of apoptosis in A549 cells. Most anti-tumor drugs can induce apoptosis in sensitive cells, and their anti-cancer activity is related to their ability to induce apoptosis in tumor cells (Steller, 1995; Hannun, 1997). Treatment for tumors should not be limited to killing tumor cells and inhibiting cell division and proliferation, but should also focus on initiating and enhancing apoptosis mechanisms in order to speed up apoptotic tumor cell death (Johnson et al., 2001). Therefore, the strategy of inducing and regulating the tumor apoptosis has become a current focus of tumor treatment (Jiang et al., 2002). The observation that TMS induces apoptosis makes it an attractive potential addition to cancer treatment.

In our experiment, as little as 4 μmol/L TMS was able to up-regulate caspase-3 protein expression and activate its cleavage in A549. Apoptosis signal transduction pathways play an important role in the process of apoptosis, and can be divided into endogenous and exogenous pathways (Kiechle and Zhang, 1998; Reed, 2000; Müllauer et al., 2001; Zimmerman et al., 2002). Because caspase-3 is involved in both endogenous and exogenous pathways, it plays a critical role in apoptosis (Ueda et al., 2002) and because TMS increases apoptosis, its up-regulation of caspase-3 is therefore not unexpected. Pan et al (2008) found TMS to up-regulate caspase-3 in COLO 205 colon cancer cells, and linked this event to an upstream TMS-induced decrease in expression of the anti-apoptotic protein, Bcl-2, and an increase in expression of the pro-apoptotic protein, BAX. We did not investigate these two proteins in the current study.

The binding of tumor necrosis factor (TNF) to its membrane receptor can activate nuclear transcription factor NF-κB, a major control for prevention of apoptosis. Manna et al (2000) found that RES could inhibit TNF-induced NF-κB activation, NF-κB p65 subunit phosphorylation, nuclear translocation and NF-κB dependent reporter gene transcription. It also inhibited NF-κB activation induced by phorbol esters, lipopolysaccharide, H2O2, and ceramide. Further study showed that RES inhibited IκB kinase, an enzyme that activates NF-κB by releasing it from inhibition by IκB. Therefore, at least part of the anti-tumor effect of RES is achieved by inhibiting IκB kinase activity and thus preventing NF-κB activation (Holmes-McNary and Baldwin, 2000). In our study, the RES derivative TMS down-regulated NF-κB and up-regulated and IκB protein expression was up-regulated. These results taken together suggest that the anti-proliferative and apoptosis-inducing effects of TMS in A549 cells might also be related to inhibited degradation of IκB and activation of NF-κB.

The JAK/STATs signaling pathway plays an important role in the growth and development, and regulation of hemopoiesis and the immune response, and is closely related to cell proliferation, differentiation and apoptosis. Abnormal activation of this pathway can cause abnormal proliferation and malignant transformation of cells. Continuous expression of STATs-specific signaling, especially STAT3 and STAT5, is directly related to tumor formation through stimulating the proliferation and inhibiting the apoptosis of cells. In our study, treatment with TMS caused reduced expression of STAT3 and STAT5b proteins. Although A549 cell proliferation was inhibited by TMS, only a minor decrease in JAK2 was seen, a result that suggested that the anti-proliferative effect of TMS in A549 cells was not necessarily through the “JAK/STATs” signal pathway, but might perhaps be through an inhibition of STAT that did not involve the JAK2 pathway. A question that needs further investigation is whether the anti-proliferative and apoptosis inducing effect of TMS is caused, at least partially, by blocking the “Bcr/abl-STAT” signaling pathway.

In conclusion, in summary, this study has shown that the trimethylated derivative of resveratrol, TMS, produces a significant apoptosis-inducing effect in A549 cells, and suggests that TMS can produce its anti-tumor effect by inducing apoptosis. Through our research, it can be seen that TMS is able to inhibit proliferation and promote apoptosis of A549 cell significantly, and this effect parallels its inhibition of signal protein STATs and NF-κB in cell, which suggests that the anti-proliferation and apoptosis inducing effect of TMS to A549 cell is related to its inhibitory effect on STATs and NF-κB signal transduction. Up-regulation and cleavage activation of caspase-3 could also be seen during the process of TMS-induced A549 cell apoptosis, providing the experimental evidence to support the potential clinical use of TMS for the treatment of non-small cell lung adenocarcinoma.

Acknowledgments

The authors declare no conflict of interest.

References


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