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RESEARCH ARTICLE

A benzoxazine derivative specifically inhibits cell cycle progression in p53-wild type pulmonary adenocarcinoma cells

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Abstract A fundamental aspect of cancer development is cancer cell proliferation. Seeking for chemical agents that can interfere with cancer cell growth has been of great interest over the years. In our study, we found that a benzoxazine derivative, (6-tert-butyl-3,4-dihydro-2H-benzo[b][1,4]oxazin-3-yl) methanol (TBM), could inhibit cell growth and caused significant cell cycle arrest in pulmonary adenocarcinoma A549 and H460 cells with wild-type p53, while not affecting the cell cycle distribution in *p53*-deleted H1299 lung adenocarcinoma cells. Since P53 plays an important role in regulating cell cycle progression, we analyzed the protein level of p53 by Western blot, and detected a significant elevation of p53 level after TBM treatment in A549 and H460 cells. The data suggested that TBM might specifically inhibit the proliferation of p53 wild-type lung adenocarcinoma cells through a p53-dependent cell cycle control pathway. More interestingly, results indicated that TBM might serve as a useful tool for studying the molecular mechanisms of lung cancer cell growth and cell cycle control, especially for the biologic process regulated by P53.

Keywords (6-tert-butyl-3,4-dihydro-2H-benzo[b][1,4] oxazin-3-yl) methanol, lung adenocarcinoma cells, cell cycle arrest, p53

1 Introduction

Lung cancer is the leading cause of cancer death worldwide, causing more than one million fatalities each year (Berns, 2005; Hung et al., 2008). Fighting for a cure of this disease relies on discovery of promising new agents that can be integrated into current methods of treatment and on the elucidation of the mechanisms by which lung cancer cells undergo growth, proliferation, differentiation, apoptosis and metastasis (Thatcher, 2008).

In pursuing these goals, chemical genetics, in which small molecules are utilized to alter cell phenotypes, has been making significant impact (Diamandis et al., 2007; Kolev et al., 2008; Merrick et al., 2008; Thatcher, 2008). Moreover, identification of novel small molecules with specific biologic properties, but unknown intracellular targets, can also provide powerful tools for elucidating the molecular mechanisms underlying the biologic processes (Burkard et al., 2007; Diamandis et al., 2007; Fraser and Hupp, 2007; Larochelle et al., 2007). The 2,3-dihydro-1,4-benzodioxine system has been widely used as a substructure of several biologically interesting agents (Capilla et al., 2001). Furthermore, the 2,3-dihydro-1,4-benzoxazine derivatives with the oxygen atom replaced by nitrogen have been synthesized, and various pharmacological activities have been reported with this class of molecules (Kajino et al., 1991; Bourlot et al., 1998). However, it remains unknown if these agents can interfere with tumor cell growth or survival. Therefore, we have synthesized a series of benzoxazine derivatives and investigated their effects on cancer cell growth or cell death, along with the molecular mechanisms underlying the effects of the agents on cellular proliferation, apoptosis or autophagy. In previous work, we found that some of these benzoxazine derivatives could effectively inhibit the growth of A549 lung cancer cells (Jiao et al., 2006). By investigating the phenotypic alterations in combination with the molecular pathways that are targeted by these agents, we have gained valuable

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insights into the molecular mechanisms of lung cancer cell growth and survival.

In the present work, we studied one of the benzoxazine derivatives, (6-tert-butyl-3,4-dihydro-2H-benzo[b][1,4] oxazin-3-yl) methanol (TBM), in pulmonary adenocarcinoma cell lines A549, H460 and H1299 to investigate the mechanism of TBM's effects on cell growth and survival. Among them, A549 and H460 cells carry wild-type *p53* gene, but in H1299 cells *p53* is deleted (Kawabe et al., 2000).

It is well known that P53 plays a pivotal role in controlling multiple aspects of cellular processes, including cell growth, survival, senescence and stress response (Vogelstein et al., 2000; Pietsch et al., 2008; Vazquez et al., 2008). Defects of p53 have been found in at least 50% of human cancers (Shangary and Wang, 2008). In one word, the purpose of our study is to explore the effects of TBM on cell growth and survival and to clarify if TBM posed its effects via p53-dependent pathway.

2 Materials and methods

2.1 Cell culture

A549, H460 and H1299 lung adenocarcinoma cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) bovine calf serum at 37°C with 5% CO_2.

2.2 Preparation of TBM

The structure of TBM is shown in Fig. 1. TBM was synthesized as described previously, and it was shown to effectively inhibit A549 cell growth at 200 μmol/L (Jiao et al., 2006). Therefore, in this study we treated the three cell lines with 200 μmol/L TBM to investigate its action mechanism. TBM stock solution (200 mmol/L) was prepared with DMSO as solvent. Not more than 0.1% (v/v) TBM stock solution or DMSO (as control) was applied in medium in all the experiments.

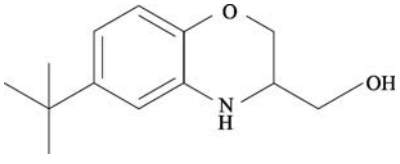

Fig. 1 Chemical structure of TBM

2.3 Reagents

RPMI 1640 was obtained from Gibco BRL Co. (Grand Island, USA). Bovine calf serum was provided by DingGuo Biotechnology (Beijing, China). Hoechst 33258 was obtained from Sigma (St. Louis, MO, USA).

2.4 Antibodies

Anti-p53 mouse monoclonal antibody (sc-126) and anti-β-actin mouse monoclonal antibody (sc-47778) were obtained from Santa Cruz Biotechnology (USA). Polyclonal rabbit anti-mouse immunoglobulins/HRP (code No. P 0161) were provided by DakoCytomation (USA).

2.5 SRB viability assay

Cell viability was determined by SRB assay as described previously (Lian et al., 2009).

2.6 Flow cytometry analysis of cell cycle distribution

A549, H460 and H1299 cells were incubated in media with the presence or absence of 200 μmol/L TBM for 48 h. Cells were harvested and then fixed with 70% ethanol, stained with 50 μg/mL propidium iodide (PI) containing 10 μg/mL Rnase A at 4°C for 1 h. The stained cells were analyzed using FACSCalibur flow cytometer (BD Bioscience, USA). Cell cycle distribution was analyzed by ModiFit software (BD Bioscience, USA) .

2.7 Hoechst 33258 staining to detect apoptosis

Living cells were stained with 10 μg/mL Hoechst 33258 in the medium for 10 min at 37°C. Subsequently, the cells were gently washed once with PBS, and were then observed under an inverted fluorescence microscope (Nikon, Japan). Cells with condensed DNA identified by intense local staining in the nucleus should be regarded as apoptotic ones, while those with diffused staining of DNA should be identified as normal cells. Each experiment was performed in triplicate.

2.8 Cell nuclear staining with acridine orange

Cell culture medium was removed and then cells were stained with 5 μg/mL acridine orange (AO) in PBS for 1 min at room temperature, washed twice with PBS, and observed and photographed under an inverted fluorescence microscope (Nikon, Japan).

2.9 Western blot analysis

Western blot analysis was performed as described previously (Lv et al., 2008). The relative quantity of proteins was analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA) and normalized to that of β-actin.

2.10 Data analysis

Data from three independent experiments were analyzed with SPSS software and were expressed as the mean±SE,

and analyzed by *t*-test. Differences at $P < 0.05$ were considered statistically significant.

3 Results

3.1 TBM caused cell cycle arrest in A549 and H460, but not in H1299 cells

To screen for novel chemical agents with the potential of inhibiting cancer cell growth or inducing cancer cell death, we have designed and synthesized a series of benzoxazine derivatives. In the study, we tested the effects of TBM, one of the benzoxazine derivatives, on pulmonary adenocarcinoma cell lines A549, H460 and H1299. In A549 and H460, *p53* is wild type, whereas in H1299, *p53* gene is deleted. A549, H460 and H1299 cells were incubated in media containing 200 μmol/L TBM or without TBM (as control) for 24 or 48 h. An obvious growth inhibition was observed in A549 and H460 cells ($P < 0.05$) treated with TBM, while no effects were observed in H1299 cells (Fig. 2).

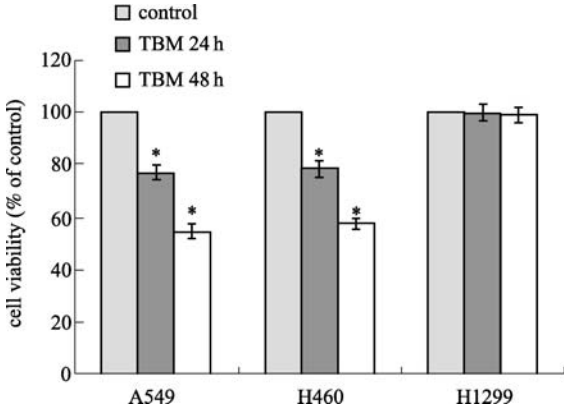

Fig. 2 TBM inhibited cell growth in A549 and H460 cells but not in H1299 cells. A549, H460 and H1299 cells were incubated in the presence or absence of 200 μmol/L TBM for 24 or 48 h. Cell viability was determined by SRB assay. Results are represented as mean±SE; $n = 3$. *: $P < 0.05$ *vs*. control.

Cell cycle progression is the key to cell proliferation; therefore we analyzed the cell cycle distribution of the above cell lines in the presence or absence of 200 μmol/L TBM after 48 h by flow cytometry. A significant increase of the proportion of cells in G1 phase and decreases in both S and G2/M phase cells were observed in A549 and H460 cells treated with 200 μmol/L of TBM. For A549 cells, G1 cells increased by 36% ($P < 0.01$) while S phase and G2/M phase cells decreased by 58% ($P < 0.01$) and 27% ($P < 0.05$), respectively. For H460 cells, a 27% increase ($P < 0.01$) of G1 cells, a 28% decrease ($P < 0.01$) of S phase cells and a 22% decrease ($P < 0.01$) of G2/M phase cells were observed. In contrast, the cell cycle distribution of H1299 cells was hardly affected by TBM (Fig. 3A, B and C).

Sometimes, an agent could pose inhibitory effects on cellular proliferation through interfering with multiple aspects of cell life. To rule out the possibility that TBM inhibits the proliferation of A549 or H460 cells also by inducing apoptosis or autophagic cell death, Hoechst 33258 and acridine orange staining was carried out to detect the DNA condensation profile and acidic vesicles, respectively. The results showed that 200 μmol/L TBM treatment for 48 h did not induce DNA condensation or increased volumes of acidic vesicles typical of apoptosis and autophagic cell death, respectively, in A549, H460 or H1299 cells (Figs. 4 and 5). These implicate that TBM inhibits the proliferation of A549 and H460 cells through arresting cell cycle progression, without simultaneously inducing apoptosis or autophagic cell death.

3.2 TBM elevated the protein level of p53 in A549 and H460 cells

Since p53 has been found to be involved in regulating G1/S transition (Kawabe et al., 2000; Pietsch et al., 2008), we suspect that TBM might induce G1/S cell cycle arrest in A549 and H460 cells through affecting the level of p53. Therefore we performed Western blot analysis to detect the level of p53. The result showed that in A549 ($P < 0.01$) and H460 ($P < 0.05$) cells a significant elevation of p53 protein level was induced by TBM treatment; and not surprisingly, p53 was not detected in H1299 cells (Fig. 6). These results implicate that p53 might be involved in the cell cycle arrest induced by TBM in A549 and H460 cells.

4 Discussion

P53 is a key player in cell cycle surveillance, especially in regulating G1/S progression (Kawabe et al., 2000; Pietsch et al., 2008). In response to stress signals including hypoxia, nutrient depletion, heat shock, and DNA damage (Pietsch et al., 2008; Shangary and Wang, 2008), it is activated and the protein level is elevated or stabilized, and subsequently it activates the transcription of p21, an inhibitor of cyclin-dependent kinases (CDKs) responsible for the progression of cell cycle from G1 to S phase (Kawabe et al., 2000; Pietsch et al., 2008). In our experiments, it was found that the benzoxazine derivative TBM could specifically stimulate an increase of p53 protein level while inducing significant cell cycle arrest in lung adenocarcinoma cell lines A549 and H460 with normal *p53*, but not affecting the cell cycle progression of the *p53*-deleted H1299 lung adenocarcinoma cells. In other words, TBM seems to be a specific growth inhibition agent for p53 wild-type lung carcinoma cells, and it may exert this effect through upregulating p53 protein level and subsequently activate a p53-mediated cell cycle control

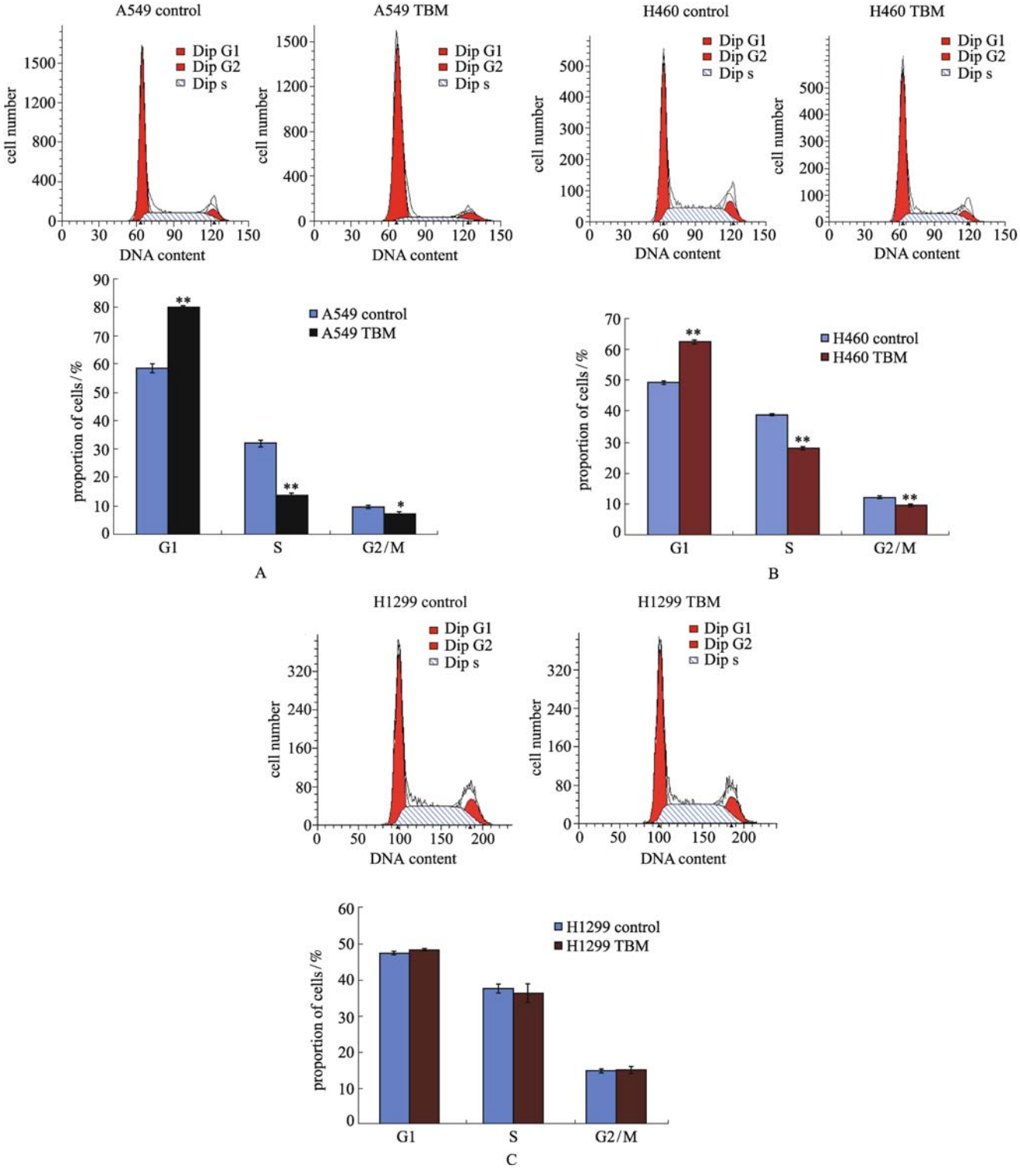

Fig. 3 TBM caused cell cycle arrest in A549 and H460 while not affecting H1299 cells. A549, H460 and H1299 cells were incubated in the presence or absence of 200 μmol/L TBM for 48 h, and were then harvested, fixed, stained with PI, and analyzed for DNA content by flow cytometry. Cell cycle distribution was illustrated in a column figure. Results are represented as mean±SE; $n = 3$. *: $P < 0.05$ *vs.* control; **: $P < 0.01$ *vs.*. control. A: The effects of TBM on the cell cycle progression in A549 cells; B: The effects of TBM on the cell cycle progression in H460 cells; C: The effects of TBM on the cell cycle progression in H1299 cells.

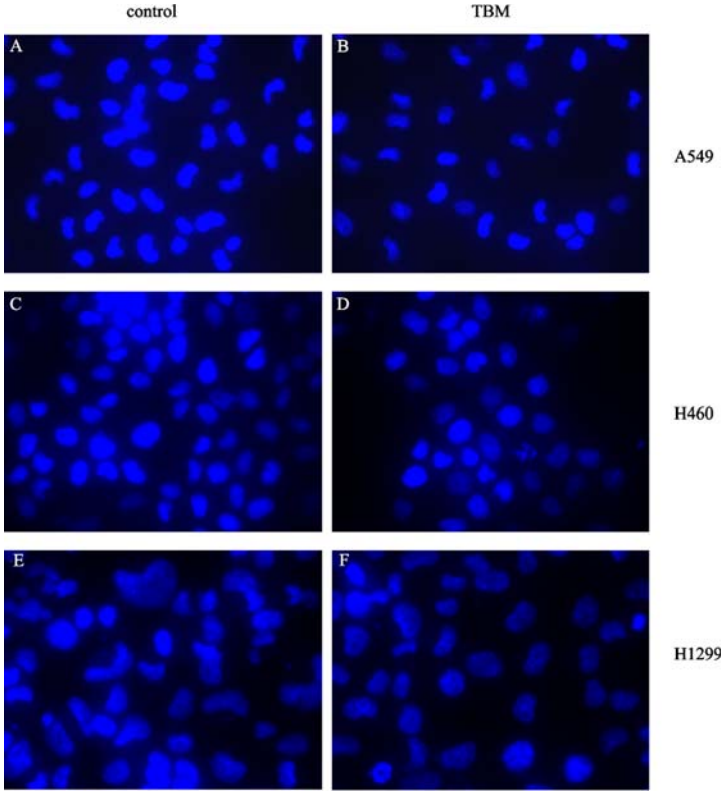

Fig. 4 TBM did not cause apoptosis in A549, H460 or H1299 cells. A549 (A: control; B: TBM), H460 (C: control; D: TBM) and H1299 (E: control; F: TBM) cells were incubated in media containing 200 μmol/L of TBM or without TBM for 48 h, and were then stained with Hoechst 33258 and observed under an inverted fluorescence microscope (400 ×) .

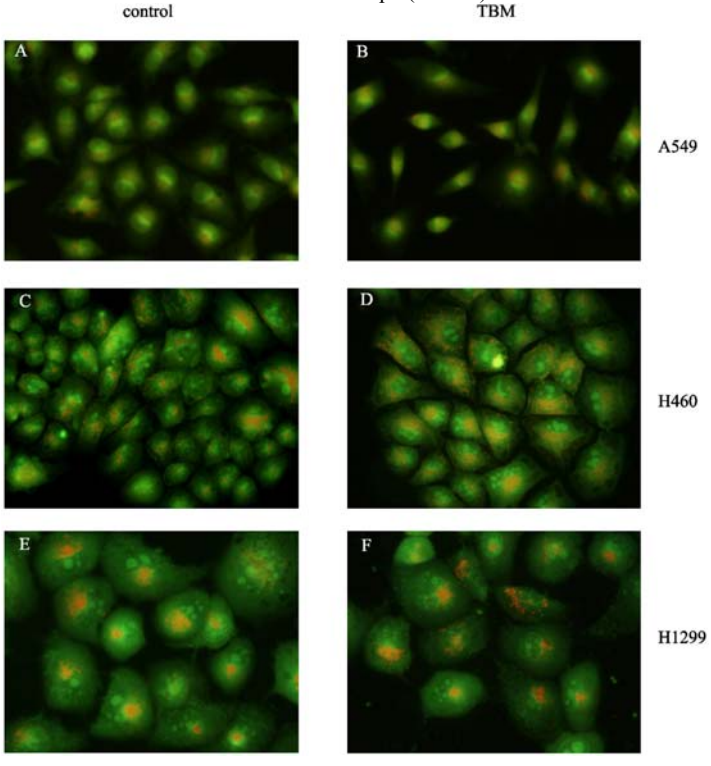

Fig. 5 TBM did not induce autophagic cell death in A549, H460 or H1299 cells. A549 (A: control; B: TBM), H460 (C: control; D: TBM) and H1299 (E: control; F: TBM) cells were incubated in the presence or absence of 200 μmol/L TBM for 48 h, and were then stained with acridine orange and observed under an inverted fluorescence microscope (400 ×).

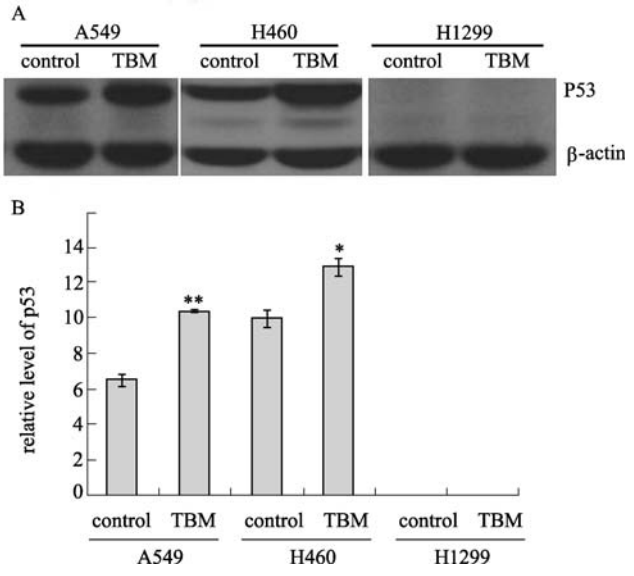

Fig. 6 TBM elevated the protein level of P53 in A549 and H460 cells. A549, H460 and H1299 cells were incubated in media containing 200 μmol/L TBM or no TBM for 48 h. Western blot experiments were carried out to detect the protein level of P53 (A). Relative protein levels of P53 versus β-actin were quantified and illustrated in a column figure (B). Results are represented as mean±SE; $n = 3$. *: $P < 0.05$ *vs*. control; **: $P < 0.01$ *vs*. control.

pathway. TBM might have caused certain cell stress which subsequently activated a p53-dependent cell cycle checkpoint, resulting in a blockage of cell cycle progression. Therefore, TBM might be a useful tool for studying cell cycle control and cancer cell proliferation, especially those cellular processes regulated by p53. Also, it might be a potential anticancer agent for tumor suppression through manipulating p53 protein level specifically in cancer cells with wild-type *p53* gene.

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