Original Research Article

Inhibitory effect of tetramethylpyrazine combined with propranolol on murine hemangioma endothelial cells

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Abstract

Purpose: *To study the inhibitory effect of different doses of tetramethylpyrazine (TMP) combined with the beta-blocker, propranolol (Pro) on hemangioma endothelial (EOMA) cells.*
Methods: *EOMA cells were cultured in vitro with varying doses of TMP and Pro (5, 10, 20 and 40 uM). The effect of treatments on cell proliferation was assessed by MTT assay, while cell apoptosis was assayed by flow cytometry. The expressions of Bcl-2, Bax, p-mTOR), total-mammalian target of rapamycin (t-mTOR, p-p70S6) and total-p70 ribosomal protein S6 (t-p70S6) proteins were determined using Western blot.*
Results: *MTT data showed that when used alone, TMP had no significant inhibitory effect on EOMA cells (p > 0.05). However, when TMP was combined with propranolol, there was significant inhibition of EOMA cells, and that the inhibition is dependent on TMP dose. Flow cytometry results showed that the combination of TMP and Pro induced EOMA cell apoptosis dose-dependently (p < 0.05). Moreover, TMP dose-dependently inhibited the phosphorylation of mTOR and p70S6 in EOMA cells, and enhanced Bax expression, but downregulated Bcl-2 (p < 0.05).*
Conclusion: *These results suggest that TMP enhances the inhibitory influence of Pro p-mTOR and p-p-70S6 in EOMA cells in a dose-dependent manner. Thus, TMP may enhance Pro-induced inhibition of the growth of endothelial cells, and promote apoptosis through suppression of activation of PI3K/AKT signal route. These findings provide a theoretical basis for the clinical application of TMP/Pro combination for the treatment of hemangioma.*

Keywords: *Hemangioma, Propranolol, Tetramethylpyrazine, Mouse hemangioma endothelial cells, Mammalian target of rapamycin, p70 ribosomal protein S6*

INTRODUCTION

Hemangioma is a congenital benign tumor or vascular malformation that often occurs in the skin and soft tissues of infants and young children. In general, it occurs in the mouth or maxillofacial region, especially in oral mucosa, facial skin and facial subcutaneous tissues [1]. Most cases of hemangioma can resolve on their own, but may give rise to such problems as scars and pigmentation after self-resolving [2]. A small number of hemangioma may affect tissue or organ function due to their specific location or excessive volumes [3]. Thus, the disease may be

life-threatening, which makes active treatments necessary. Apart from surgery, medication is one of the main means of treating hemangioma. Currently, drugs including β-blockers, hormones, interferons and cytotoxic agents are used for treating hemangioma. However, these drugs have certain adverse effects which limit their application in clinical practice [4,5]. Consequently, research has focused on the development of safer and more effective drugs for treating hemangioma.

Chuangxiong (*Ligusticum chuanxiong* Hort.) refers to the dried radix of the umbelliferous plant, with the original name of *XiongQiong*. It is native to Sichuan and is currently grown in several provinces in China. Modern pharmacological studies have shown that *chuanxiong* has antioxidant, neuroprotective, anti-inflammatory and antibacterial properties [6,7]. There are more than 100 pharmacologically active compounds in *chuanxiong*. These biologically active components are classified into four categories i.e. phenols and organic acids (e.g. ferulic acid), benzoquinones (e.g. ligustilide), alkaloids (e.g. tetramethylpyrazine), and polysaccharides [8,9].

Tetramethylpyrazine (TMP, Figure 1) is an alkaloid derived from plant and microbial fermented products. The chemical name of TMP is 2, 3, 5, 6-tetramethylpyrazine, and it can be synthesized in the laboratory. Pharmacological studies have shown that TMP exerts anti-thrombotic effects, and is involved in preventing and treating cerebral ischemia, improving microcirculation, protecting coronary arteries, protecting cardiomyocytes and preventing myocardial ischemia and reperfusion injury [10 – 12]. As a new angiogenesis inhibitor, it controls the formation of tumor blood vessels [13]. There are limited reports on the effect of TMP on hemangioma endothelial cells in China and elsewhere. Therefore, the effect of combination of TMP and Pro on EOMA cells, and the possible mechanism involved were investigated in this study.
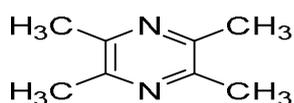

Figure 1: Structure of tetramethylpyrazine

EXPERIMENTAL

Cell lines, drugs and reagents

Mouse hemangioma EOMA cells were supplied by American Type Culture Collection (ATCC);

tetramethylpyrazine was from China Institute for the Control of Pharmaceutical and Biological Products, while propranolol was product of Sigma. Fetal bovine serum (FBS), penicillin, streptomycin, trypsin and high-dose DMEM medium were purchased from GIBCO BRL, while D-Hanks was purchased from Hylcone. Penicillin-streptomycin and trypsin cell digestive fluid were obtained from Biyuntian Biotechnology Research Institute; Bcl-2, Bax, p-mTOR, T-mTOR, P-p70S6, T-p70S6 and β-actin antibodies were products of Abcam; goat anti-IgG (horseradish peroxidase label) was purchased from Proteinech Group, while PVDF membrane and ECL luminescence kits were obtained from Abcam. Other reagents were analytically pure.

This research received approval from the Animal Ethical Committee of Department of Dermatology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (approval no. 201713409). It was conducted according to the guidelines of "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [14].

Cell culture

The EOMA cells were seeded in DMEM/F-12 high glucose medium containing 10 % FBS, and maintained in a 5 % CO_2 atmosphere at 37 °C and saturated humidity. The cells were then conventionally passaged. Cells in logarithmic growth phase were selected for use in the experiments.

MTT assay

The cells in logarithmic growth phase were collected and made into a single cell suspension of density 2×10^7 cells/L, and were then inoculated into a 96-well cell culture plate at 200 μL per well. A blank control group was set up with culture medium but without cells. The Pro group contained EOMA cells with Pro alone, while the treatment groups comprised TMP culture media (200 μl/well) containing different TMP concentrations (5, 10, 20 and 40 μM), with or without Pro (5 μM). Four replicate wells were set up for each group. After incubating for 24 h, the supernatant was aspirated; D-Hanks was added to each well, and thereafter was aspirated.

Low-sugar DMEM was added (200 μL per well), and was aspirated after culturing for 24 h, followed by drug addition. Each group was cultured for 48 h. Subsequently, MTT (20 μL, 0.5 g %) was put into every well, followed by culturing for 4 h, after which the medium was

removed and replaced with DMSO (150 µL), with shaking, to stop the reaction. The 96-well plate was moved into a plate shaker and shaken horizontally for 10 min to fully dissolve the formazan crystals formed. The absorbance (A) of each well was measured against blank at a wavelength of 490 nm (4 parallel wells per group, repeated 3 times independently) [15]. Inhibition (H) of tumor cell proliferation was calculated using Eq 1.

$$H\ (\%) = (1 - A_{expt})/A_{control}\ x\ 100 \ldots\ldots (1)$$

where A_{expt} and $A_{control}$ are the absorbance of the experimental and control groups, respectively.

Determination of apoptosis

The EOMA cells were seeded at a density of 1×10^9 cells/L in a 6-well culture plate with medium (1.2 mL per well). After the cells were attached, the culture medium was discarded and TMP was added. As before, the concentrations of TMP were set at 5, 10, 20 and 40 µM, and were combined with 5 µM Pro. Three duplicate wells were set up in each group. After culturing for 48 h, cells in each well were collected, rinsed thrice in ice-cold PBS, and re-suspended in binding buffer (BF, 200 µL), followed by adding 10 µL of membrane-protein V-FITC and 5 µL of PI. After gentle mixing, the reaction was allowed to proceed for 0.25 h, after which BF (300 µL) was introduced. The degree of apoptosis (%) was measured using flow cytometry.

Western blot

Cells at the logarithmic growth phase were cultured with TMP for 24 h, washed 3 times with pre-cooled PBS, and treated with 100 µL of cell lysate solution (PBS containing 1 % Nonidet P-40, 5 g/L Na deoxycholate, 0.1 g/100 mL sodium dodecyl sulphate, 0.1 g/L PMSF and 10 mg/L aprotinin) at 4 ° C for 60 min. Following lysis, the mixture was subjected to centrifugation at 11 000 × g at the same temperature for 600 sec, followed by protein assay on the clear supernatant using BCA protein assay kit in line with the kit protocol.

After routine SDS-PAGE electrophoresis and transfer to PVDF membrane, the membrane was incubated with antibodies for Bcl-2, Bax, P-mTOR, T-mTOR, P-p70S6, T-p70S6 and β-actin. After washing with blocking solution, horseradish peroxidase-labeled secondary antibody was added and allowed to react at 37 °C for 1.5 h. Thereafter, the membrane was washed, ECL

was added, and the resultant images were captured with gel imaging system. β-Actin was used as an internal reference to correct the relative expression of the target protein [16].

Statistical analysis

The statistical software, Graphpad Prism 7.0 was used for data analysis. Numeric data are expressed as mean ± SD, and comparison between groups was performed using one-way analysis of variance. Comparison between multiple means was performed with Tukey's procedure. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of combination of TMP and propranolol on proliferation of EOMA cells

Results from MTT assay showed that the use of TMP alone at various concentrations had no appreciable suppressive influence on the proliferation of EOMA cells, while propranolol alone had a certain inhibitory effect on the proliferation of EOMA cells, relative to the blank group. When TMP was combined with Pro, the inhibition of Pro on EOMA cells increased significantly with increase in TMP dose (Figure 2, $p < 0.05$).
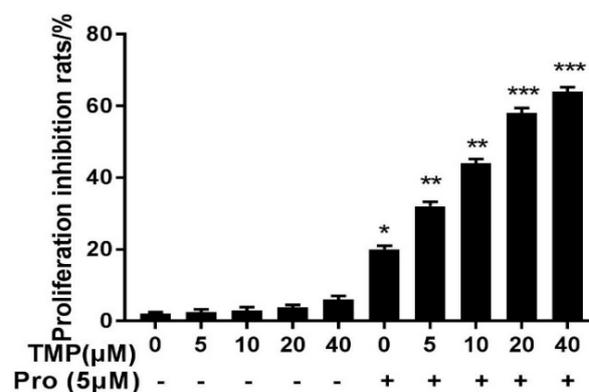

Figure 2: Effect of TMP-Pro combination on EOMA cell proliferation. Data are shown as mean ± SD from three independent experiments

Effect of combination of TMP and Pro on apoptosis of EOMA cells

As shown in Figure 3, results from flow cytometry revealed that the combination TMP with Pro significantly and dose-dependently induced EOMA cell apoptosis ($p < 0.05$, Figure 3).

Influence of combination of TMP and Pro on the expressions of Bcl-2, Bax, P-mTOR, T-mTOR, P-p70S6 and T-p70S6 proteins in EOMA cells

Western blot assay showed that treatment of hemangioma endothelial cells with combination of TMP and Pro, decreased P-Mtor/T-mTOR and P-p70S6/T-p70S6 ratios. In addition, TMP dose-dependently reduced mTOR and p70S6 phosphorylation in EOMA cells (Figures 4 A and 4B) ($p < 0.05$). At the same time, the expression of apoptosis-related Bax-2 related was upregulated, while Bcl-2 was significantly downregulated (Figure 4C).
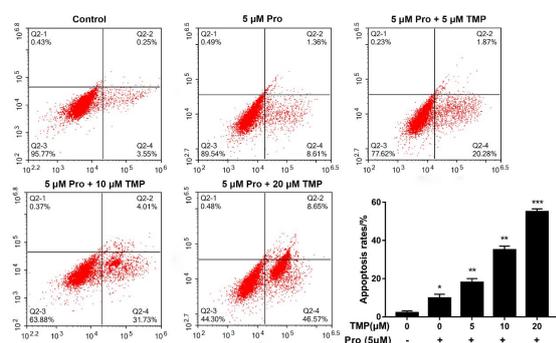

Figure 3: Effect of TMP-Pro combination on apoptosis of EOMA cells. Data represent mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, relative to control
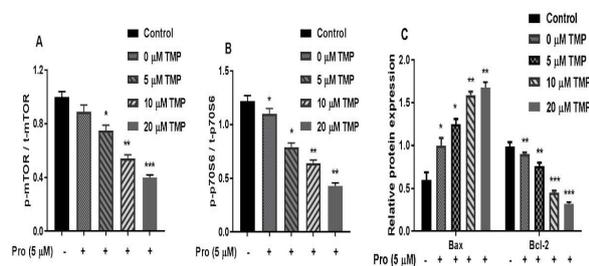

Figure 4: A: Effect of TMP/Pro combination on the expression of p-mTOR; B: Effect of TMP/Pro combination on the expression of p-p70S6; C: Effect of TMP/Pro combination on the expressions of BAX and Bcl-2 (*$p < 0.01$; **$p < 0.05$, compared with control group)

DISCUSSION

Hemangioma is the most common tumor in infants and young children, with incidence up to 3 – 8 %. Some hemangioma can cause serious complications, resulting in body dysfunction, severe damage to physical appearance, or even threat to life of the patient. The cause of hemangioma and the factors affecting its growth are still unclear. Thus, the treatment of hemangioma presents a difficult problem in clinical practice. Although surgical resection and medication offer good therapeutic outcomes, these therapies have some side effects which limit their applications [4, 5]. Therefore, research efforts on hemangioma have focused on the development of safer and more effective drugs for treating the disease.

Chuanxiong, the dried radix of the umbelliferous plant *Ligusticum chuanxiong* Hort, is warm in nature and pungent in taste, and *enters* liver, gallbladder and pericardium *meridians*, with good and thick smell. It can unblock blood vessels, promote blood circulation and remove blood stasis. Tetramethylpyrazine (TMP) is the main active component of *Chuanxiong.* It is popularly used in the treatment of kidney, liver, cardio-cerebrovascular and respiratory diseases [17]. It is a new angiogenesis inhibitor, and studies have shown that it inhibits the formation of tumor blood vessels [13]. However, there are few reports about the effect of TMP on hemangioma endothelial cells. In this study, the *in vitro* inhibitory effect of different doses of TMP combined with propranolol on mouse hemangioma endothelial cells (EOMA) was investigated. The findings of this study suggest that TMP enhanced the inhibitory effect of Pro on the growth and apoptosis of mouse hemangioma endothelial cells in a dose-dependent fashion.

Tumor cell growth and metastasis depend on formation of tumor blood vessels, and the process of tumor angiogenesis is complex, involving regulation by multiple genes and signaling pathways. The PI3K/AKT pathway is an important signaling pathway in this process, with AKT as the core protein of the pathway. Activated AKT is involved in the biological processes of tumor cells through direct or indirect mediation in downstream-related proteins and regulation of tumor angiogenesis [18,19]. The serine/threonine acid protein kinase, mTOR, is widely involved in several cellular events including autophagy and transcription [20,21]. The p70S6K protein belongs to the family of phosphatidylinositol 3-kinases [22].

It is known that Bax and Bcl-2 belong to the same family; Bax exerts a strong antagonistic effect against Bcl-2, and it can directly promote apoptosis. Studies have shown that Bax and Bcl-2 co-regulate cell proliferation and apoptosis [23]. Moreover, mTOR, p70S6, Bcl-2 and Bax are downstream genes related to AKT, and they play important roles in the development of hemangioma [24,25]. The PI3K/AKT signaling pathway is abnormally activated in infantile hemangiomas, and its inhibition can significantly promote apoptosis of vascular endothelial cells.

The results of this study revealed that treating hemangioma endothelial cells with combination of TMP and propranolol upregulated the apoptosis-related Bax, and decreased Bcl-2 expression. The combination treatment also decreased P-Mtor/T-mTOR and P-p70S6/T-p70S6 ratios. Moreover, TMP dose-dependently inhibited mTOR and p70S6 phosphorylation in EOMA cells. These findings suggest that TMP may suppress the growth of hemangioma endothelial cells and promote their apoptosis through suppression of activation of PI3K/AKT signal route.

CONCLUSION

The findings of this study indicate that the use of varying doses of TMP in combination with the β-blocker propranolol, significantly inhibits proliferation of mouse EOMA cells. These findings, for the first time, demonstrate that the mechanism involved in the inhibition of the growth of hemangioma cells by TMP/Pro combination may be related to inhibition of the activation of PI3K/AKT signal route. The results of this investigation provide a theoretical background for the treatment of hemangioma using TMP and propranolol.

DECLARATIONS

Acknowledgement

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Wan-Wan Jin and Jian-Ming WU conceived and designed the study. Wan-Wan Jin, Jian-Ming WU, Yi Tong, Ji-Cong Jiang, Hehe Quan and Yu Gao collected and analyzed the data, while Yu Gao wrote the manuscript. Wan-Wan Jin and Jian-Ming WU contributed equally to this work and should be considered as co-first authors.

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