# World Journal of *Clinical Cases*

World J Clin Cases 2021 June 26; 9(18): 4460-4880





Published by Baishideng Publishing Group Inc

W J C C World Journal of Clinical Cases

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# **RESPONSIBLE EDITORS FOR THIS ISSUE**

Production Editor: Ji-Hong Lin; Production Department Director: Xiang Li; Editorial Office Director: Jin-Lei Wang.

NAME OF JOURNAL	INSTRUCTIONS TO AUTHORS
World Journal of Clinical Cases	https://www.wignet.com/bpg/gerinfo/204
ISSN	GUIDELINES FOR ETHICS DOCUMENTS
ISSN 2307-8960 (online)	https://www.wjgnet.com/bpg/GerInfo/287
LAUNCH DATE	GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
April 16, 2013	https://www.wjgnet.com/bpg/gerinfo/240
FREQUENCY	PUBLICATION ETHICS
Thrice Monthly	https://www.wjgnet.com/bpg/GerInfo/288
<b>EDITORS-IN-CHIEF</b>	PUBLICATION MISCONDUCT
Dennis A Bloomfield, Sandro Vento, Bao-Gan Peng	https://www.wjgnet.com/bpg/gerinfo/208
EDITORIAL BOARD MEMBERS	ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/2307-8960/editorialboard.htm	https://www.wjgnet.com/bpg/gerinfo/242
PUBLICATION DATE	STEPS FOR SUBMITTING MANUSCRIPTS
June 26, 2021	https://www.wjgnet.com/bpg/GerInfo/239
COPYRIGHT	ONLINE SUBMISSION
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World J Clin Cases 2021 June 26; 9(18): 4542-4552

DOI: 10.12998/wjcc.v9.i18.4542

ISSN 2307-8960 (online)

ORIGINAL ARTICLE

# **Basic Study** Tetramethylpyrazine inhibits proliferation of colon cancer cells in vitro

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Author contributions: Li H, Hou YX and Yang Y contributed equally to this work; Chen SB and Liu DX designed the research; Li H and Yang Y analyzed the data and drafted the manuscript; Hou YX revised the manuscript critically for important intellectual content and contributed to data analysis; He QQ, Gao TH, Zhao XF, and Huo ZB helped draft the manuscript; all authors read and approved the final manuscript.

# Institutional review board

statement: Our study was not involving human and animal subjects.

Conflict-of-interest statement: The authors declare no conflict of interest related to this manuscript.

Data sharing statement: No additional data are available.

Open-Access: This article is an

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# Abstract

# BACKGROUND

Colon cancer is one of the most common malignancies worldwide, and chemotherapy is a widely used strategy in colon cancer clinical therapy. However, chemotherapy resistance is a major cause of disease recurrence and progression in colon cancer, and thus novel drugs for treatment are urgently needed. Tetramethylpyrazine (TMP), a component of the traditional Chinese medicine Chuanxiong Hort, has been proven to exhibit a beneficial effect in tumors.

# AIM

To investigate the potential anticancer activity of TMP in colon cancer and its underlying mechanisms.

# **METHODS**

Colon cancer cells were incubated with different concentrations of TMP. Cell viability was evaluated by crystal violet staining assay and cell counting kit-8 assay, and cell apoptosis and cell cycle were assessed by flow cytometry.

# RESULTS

TMP significantly inhibited the proliferation of colon cancer cells in a dose- and time-dependent manner. In addition, flow cytometry revealed that TMP induced cell cycle arrest at the G0/G1 phase. TMP treatment caused early stage apoptosis in SW480 cells, whereas it caused late stage apoptosis in HCT116 cells.

# CONCLUSION

Our studies demonstrated that TMP inhibits the proliferation of colon cancer cells



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Manuscript source: Unsolicited manuscript

Specialty type: Gastroenterology and hepatology

Country/Territory of origin: China

# Peer-review report's scientific quality classification

Grade A (Excellent): 0 Grade B (Very good): 0 Grade C (Good): C Grade D (Fair): 0 Grade E (Poor): 0

Received: January 14, 2021 Peer-review started: January 14, 2021 First decision: March 15, 2021 Revised: March 27, 2021 Accepted: April 22, 2021 Article in press: April 22, 2021 Published online: June 26, 2021

P-Reviewer: Reiff T S-Editor: Lin M L-Editor: Wang TQ P-Editor: Li JH



in a dose- and time-dependent manner by inducing apoptosis and arresting the cell cycle at the G0/G1 phase. Our findings suggest that TMP might serve as a potential novel therapeutic drug in the treatment of human colon cancer.

Key Words: Tetramethylpyrazine; Colon cancer; Apoptosis; Cell proliferation; Chemotherapy; Cell cycle

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**Core Tip:** Colon cancer is one of the most common malignancies worldwide, and chemotherapy is a widely used strategy in colon cancer clinical therapy. However, many patients eventually relapse and develop drug resistance and chemotherapy resistance. This is one of the main causes of chemotherapeutic failure. Tetramethylpyrazine (TMP) has been proven to exhibit a beneficial effect in many types of malignant tumors. Here, we find out that TMP can induce apoptosis and inhibits proliferation of colon cancer cells, suggesting that TMP might serve as a potential novel therapeutic drug in the treatment of human colon cancer.

Citation: Li H, Hou YX, Yang Y, He QQ, Gao TH, Zhao XF, Huo ZB, Chen SB, Liu DX. Tetramethylpyrazine inhibits proliferation of colon cancer cells in vitro. World J Clin Cases 2021; 9(18): 4542-4552

URL: https://www.wjgnet.com/2307-8960/full/v9/i18/4542.htm DOI: https://dx.doi.org/10.12998/wjcc.v9.i18.4542

# INTRODUCTION

Colon cancer is one of the most commonly diagnosed cancers and its incidence is growing in many countries. The incidence of colon cancer is high in developed countries, reflecting a prevalence of risk factors, including an unhealthy diet and obesity[1]. Although patients with early stage colon cancer can be treated by surgical resection, due to a lack of characteristic clinical manifestations and colon cancer screening, many patients are at an advanced stage at the time of diagnosis<sup>[2]</sup>. Chemotherapy is a commonly used strategy for colon cancer treatment, as many patients have regional or distant spread at the time of diagnosis. However, many patients eventually relapse and develop drug resistance and chemotherapy resistance. This is one of the main causes of chemotherapeutic failure. Therefore, clinical treatment of colon cancer is still a great challenge, and searching for novel drugs for colon cancer treatment with high selectivity and low toxicity has become a recent focus of attention.

Ligusticum chuanxiong, a Chinese herb, has a long history in traditional Chinese medicine[3]. Tetramethylpyrazine (2,3,5,6-tetramethylpyrazine, TMP) is an alkaloid monomer that exists in the roots or stems of Ligusticum chuanxiong[4]. TMP has been widely used for the treatment of neurovascular and cardiovascular disorders, such as acute ischemic stroke, angina pectoris, and atherosclerosis[5-7]. The underlying mechanisms involve inhibition of platelet aggregation, suppression of apoptosis, and scavenging peroxyl radicals, superoxide, and hydroxyl radicals[8]. Recent studies have demonstrated that TMP has potent inhibitory effects on a number of types of tumors, such as lung, breast, ovarian carcinoma, gastric carcinoma, osteosarcoma, leukemia, and hepatocellular carcinoma, through affecting the proliferation and apoptosis of tumor cells[9-13]. However, the effects of TMP on colon cancer have not been investigated. In the present study, we evaluated the effects of TMP on the viability, cell cycle distribution, and apoptosis of colon cancer cell lines.

# MATERIALS AND METHODS

Cell culture

Six human colon cancer cell lines, DLD1, HCT116, LOVO, LS1747, SW480, and SW620,



were purchased from the Cell Resource Center of the Institute of Basic Medicine, Chinese Academy of Medical Sciences. All cell lines were cultured in RPMI 1640 medium (Gibco, United States) with 10% fetal bovine serum (FBS, HyClone, United States) in a cell incubator with an atmosphere of 50 mL/L CO<sub>2</sub> at 37 °C.

# Chemicals and reagents

TMP was purchased from Sigma-Aldrich (United States) and dissolved in DMSO (Sigma-Aldrich, United States). Cell counting kit-8 (CCK-8) was purchased from Med Chem Express (United States). The Annexin V-FITC/ propidium iodide (PI) kit and PI were purchased from BD (United States) and Sigma, respectively.

# Morphological observation and crystal violet staining

Cells were seeded in 6-well plates (1 × 10<sup>5</sup> cells/well) and incubated in RPMI 1640 supplemented with 10% FBS for 24 h. Cells were treated with TMP at a final concentration of 0  $\mu$ g/mL, 300  $\mu$ g/mL, 600  $\mu$ g/mL, 900  $\mu$ g/mL, 1200  $\mu$ g/mL, or 1500  $\mu$ g/mL for 24 h, 48 h, or 72 h. The morphology of the cells was examined under an inverted light microscope.

Cell viability was evaluated by crystal violet staining assay. Cells were seeded in 6well plates (5 × 10<sup>4</sup> cells/well) and incubated in RPMI 1640 supplemented with 10% FBS for 24 h. The cells were then incubated with TMP at various concentrations for 48 h; cells incubated with 0  $\mu$ g/mL TMP served as a control. The treated cells were washed with PBS and then mixed with crystal violet solution and incubated within 10 min. The morphology of the cells was examined under an inverted light microscope. The experiment was performed in triplicate.

# Cell proliferation assay

CCK-8 assay was used to detect cell proliferation following the manufacturer's instructions. SW480 and HCT116 cells were seeded in 96-well microtiter plates at a density of  $5 \times 10^3$  cells/well. After 24 h, the medium was replaced with RPMI 1640 containing different concentrations of TMP (0 µg/mL, 300 µg/mL, 600 µg/mL, 900 µg/mL, 1200 µg/mL, or 1500 µg/mL) and cells were cultured for 24 h, 48 h, or 72 h. At the indicated time points, 10 µL of CCK-8 kit solution was added to the cells, and cells were incubated for an additional 2 h. Absorbance was measured at 450 nm. Six duplicate wells were run for each treatment. All experiments were performed at least three times. The cell viability index was calculated according to the formula: Experimental OD value/control OD value × 100%.

# Cell cycle and apoptosis assays

SW480 and HCT116 cells were treated with TMP at a final concentration of  $0 \mu g/mL$ , 300  $\mu g/mL$ , 600  $\mu g/mL$ , 900  $\mu g/mL$ , or 1200  $\mu g/mL$  for 24 h. The cells were centrifuged, fixed in 70% ethanol for at least 24 h, washed with PBS, and treated with RNase A. Cells were then incubated with 300  $\mu$ l of PI (50  $\mu g/mL$ ) at room temperature for 15 min. The cell cycle distribution was examined by flow cytometry. The experiment was conducted in triplicate.

For apoptosis assays, SW480 and HCT116 cells were treated with TMP at a final concentration of 0  $\mu$ g/mL, 300  $\mu$ g/mL, 600  $\mu$ g/mL, 900  $\mu$ g/mL, or 1200  $\mu$ g/mL for 24 h. Cells were then stained using the reagents in the Annexin V-FITC/PI kit for 30 min at 0 °C in the dark. The percentage of apoptotic cells was determined by flow cytometry. The experiment was conducted in triplicate.

## Statistical analysis

All the experiments were repeated three times independently and each sample was analyzed in triplicate. Data from all experiments are presented as the mean  $\pm$  SD. Oneway ANOVA with the least-significant difference tests was used to determine the significance of difference. Student's *t*-test was used for comparing two treatments. Graph Pad Prism 6 software (Graph Pad Software, United States) was used to make statistical charts. FlowJo 7.0 (BD, United States) was used to process and analyze the flow cytometer data, and Modfit (Verity Software House, United States) software was used to analyze the cell cycle distribution. *P* < 0.05 was considered statistically significant.

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# RESULTS

# TMP suppresses the proliferation of colon cancer cells

The chemical structure of TMP is presented in Figure 1. To investigate the effect of TMP on the proliferation of colon cancer cells, six colon cancer cell lines, DLD1, HCT116, LOVO, LS1747, SW480, and SW620, were used for experiments. The colon cancer cell lines were exposed to different concentrations of TMP for 48 h. As shown in Figure 2A, the results showed that TMP suppressed the cell viability in a dose-dependent manner, especially in SW480 and HCT116 cells. Logistic regression analysis was used to calculate the concentration of TMP that resulted in 50% inhibition ( $IC_{50}$ ) of cell proliferation through CCK-8 assay. The results showed that the  $IC_{50}$  values in SW480 and HCT116 cells were the lowest among all cell lines (Figure 2B). Therefore, the SW480 and HCT116 colon cancer cell lines were selected for further investigation.

# TMP inhibits colon cancer cell proliferation in a dose- and time-dependent manner

To identify the optimum drug level of TMP in colon cancer cells, we treated colon cancer cell lines SW480 and HCT116 with various concentrations of TMP for 24 h, 48 h, and 72 h. The morphological changes of the SW480 and HCT116 cells were examined under an inverted light microscope. SW480 and HCT116 cell morphology became distorted, round, and fragmented after TMP treatment, especially when the concentration of TMP reached 600  $\mu$ g/mL (Figure 3A). The results of crystal violet staining of SW480 and HCT116 cells treated with TMP for 48 h were consistent with the microphotograph results (Figure 3B). CCK-8 assays showed that the proliferation and viability of SW480 and HCT116 cells gradually decreased with increasing TMP concentration and prolonged exposure time (Figure 3C). Together, these results show that TMP significantly inhibits colon cancer cell proliferation in a dose- and time-dependent manner.

# TMP inhibits colon cancer cell proliferation by inhibiting S phase synthesis

Cell cycle arrest is always accompanied with inhibition of cell proliferation. Based on the proliferation inhibition of SW480 and HCT116 cells treated with TMP, we next tested the cell cycle distribution. Flow cytometry analysis showed that TMP treatment for 24 h increased the G0/G1 phase cell proportion, whereas it decreased the proportion of cells in S phase (Figure 4A). Moreover, a significant increase in the percentage of G1 phase was detected in cells treated with TMP at 600  $\mu$ g/mL for 24 h (Figure 4B). These data suggest that TMP treatment inhibits the proliferation of colon cancer cells by inducing a G0/G1 phase arrest.

# TMP induces apoptosis of colon cancer cells

TMP has been reported to induce apoptosis in some cancer cells[14-17]. To determine whether TMP similarly induces colon cancer cell apoptosis, we performed Annexin-V/PI double staining and analyzed cell apoptosis. We treated SW480 and HCT116 cells with different concentrations of TMP for 24 h, and flow cytometry analysis was performed to measure the apoptosis rates. As shown in Figure 5A, SW480 and HCT116 cells treated with TMP showed a marked increase in apoptosis rate, especially when the concentration of TMP reached 600  $\mu$ g/mL. TMP significantly induced apoptosis of colon cancer cells in a dose-dependent manner. As shown in Figure 5B, we found further evidence that SW480 cells treated with TMP showed increased cell numbers in early apoptosis (Annexin + / PI -, lower right quadrant in Figure 5A), whereas the HCT116 cells showed late apoptosis (Annexin + / PI +, upper right quadrant in Figure 5A).

# DISCUSSION

Colon cancer is one of the most commonly diagnosed cancers worldwide and one of the most fatal malignancies in terms of estimated new cases and estimated deaths[18]. Although surgical resection is the main curative therapy for early stage colon cancer, chemotherapy still has an important role, as many patients have regional or distant spread at the time of diagnosis[19]. However, many patients have died from chemotherapy resistance, disease progression, and recurrence. Progress in improving overall survival has been relatively slow[20]. Hence, searching for effective chemotherapy drugs and overcoming drug resistance is a looming problem.

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#### Li H et al. TMP inhibits colon cancer cell proliferation



Figure 1 Chemical structure of tetramethylpyrazine with a molecular weight of 136.19 g/mol.

Over the last few decades, plants have been shown to contain a variety of antitumor components, and many plant compounds, such as vincristine, paclitaxel, topside, and topotecan, have been used in the clinic to treat cancer<sup>[21]</sup>. TMP is an alkaloid monomer that exists in the roots or stems of a Chinese herbal medicine plant and is considered to be safe due to its long history of use in Chinese traditional medicine. Recent studies confirmed its anti-tumor effects in lung, breast, ovarian carcinoma, osteosarcoma, gastric carcinoma, leukemia, and hepatocellular carcinoma [22,23]. However, the anti-tumor activity of TMP against colon cancer cells has not been investigated.

In the present study, we examined the potential effects of TMP on six colon cancer cell lines, DLD1, HCT116, LOVO, LS1747, SW480, and SW620. We found that TMP significantly suppressed colon cancer cell viability in a dose-dependent manner, especially in SW480 and HCT116 cells. The IC<sub>50</sub> values in SW480 and HCT116 cells were the lowest, and therefore, the SW480 and HCT116 colon cancer cell lines were selected for further investigation.

To examine the effect of TMP in regulating colon cancer cell proliferation, we initially examined the dose-response effect of TMP on cell viability. After treatment with a concentration gradient of TMP, the morphology of SW480 and HCT116 cells became distorted, round, and fragmented, especially when the concentration of TMP reached 600  $\mu$ g/mL. CCK-8 assays demonstrated that TMP markedly inhibited colon cancer cell proliferation in a dose- and time-dependent manner. Our results are consistent with those of previous reports on the effect of TMP on the proliferation of cancer cells<sup>[24]</sup> and demonstrate that TMP significantly inhibits the proliferation of colon cancer cells in a dose- and time-dependent manner.

Cell cycle arrest in cancer cells is often accompanied with inhibition of cell proliferation[25]. Uncontrolled cell proliferation is an important biological characteristic that distinguishes tumor cells from ordinary somatic cells[26]. The loss of cell cycle control plays a critical role in tumor development and proliferation[27]. Ji et al [28] showed that TMP inhibited the proliferation of human gastric cancer cell line SGC-7901 and caused G1 phase cell cycle arrest. We performed flow cytometry to examine the effect of TMP on the cell cycle and found that TMP induced a significant cell cycle arrest in the G0/G1 phase, with a decrease in S phase cells. Moreover, there was a significant increase in the percentage of the G0/G1 phase in cells treated with TMP at 600  $\mu$ g/mL for 24 h. These data suggest that TMP treatment could inhibited the proliferation of colon cancer cells by inducing a G0/G1 cell cycle arrest.

Apoptosis and proliferation are always closely related. Several studies have shown that TMP can induce tumor cell apoptosis[29-31]. We used flow cytometry to examine the effect of TMP on apoptosis and found that HCT116 and SW480 cells treated with TMP showed a marked increase in the proportion of apoptotic cells, especially with TMP at 600  $\mu$ g/mL. These data suggest that TMP significantly induced apoptosis of colon cancer cells in a dose-dependent manner. Further analysis revealed that TMP treatment caused early stage apoptosis in SW480 cells, whereas it caused late stage apoptosis in HCT116 cells.

# CONCLUSION

In conclusion, our study demonstrated that TMP inhibited the proliferation of colon



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Figure 2 Tetramethylpyrazine suppresses the proliferation of colon cancer cells. A: Tetramethylpyrazine significantly suppressed the cell viability in a dose-dependent manner, especially in SW480 and HCT116 cells; B: The 50% inhibition values in colon cancer cell lines. SW480 and HCT116 cells had the lowest 50% inhibition values. TMP: Tetramethylpyrazine.

cancer cells in a dose- and time-dependent manner by inducing apoptosis and arresting the cell cycle at the G0/G1 phase. The results of the present study suggest that TMP may be a novel drug candidate for the treatment of colon cancer patients. Further studies of the mechanisms of TMP in human colon cancer are needed.

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Figure 3 Tetramethylpyrazine inhibits the growth of colon cancer cells in a dose- and time-dependent manner. A: Cell morphology of SW480 and HCT116 cells treated with different concentrations of tetramethylpyrazine (TMP) observed by inverted light microscopy; B: Crystal violet staining assay for evaluating the SW480 and HCT116 cell viability; C: Cell Counting Kit-8 assay showed that the proliferation and viability of HCT116 and SW480 cells gradually decreased with increasing TMP concentration and prolonged action time. TMP: Tetramethylpyrazine.

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Figure 4 Tetramethylpyrazine inhibits colon cancer cell proliferation by inhibiting S phase synthesis. A: Tetramethylpyrazine (TMP) treatment increased the proportion of cells in the G0/G1 phase, whereas it decreased the proportion of cells in the S phase; B: The percentage of G1 phase cells significantly increased in cells treated with TMP at 600 µg/mL for 24 h. TMP: Tetramethylpyrazine.



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Figure 5 Tetramethylpyrazine induces apoptosis of colon cancer cells. A: SW480 and HCT116 cells were induced to have a marked increase in the proportion of apoptosis rate, especially when the concentration of tetramethylpyrazine reached 600 µg/mL. B: The SW480 cells showed early apoptosis, whereas the HCT116 cells showed late apoptosis.

# **ARTICLE HIGHLIGHTS**

# Research background

Colon cancer is one of the most common malignancies worldwide, and chemotherapy is a widely used strategy in clinical therapy for colon cancer. However, chemotherapy resistance is a major cause of recurrence and disease progression in colon cancer, and thus novel drugs for treatment are urgently needed. Tetramethylpyrazine (TMP), a component of the traditional Chinese medicine Chuanxiong Hort, has been proven to exhibit a beneficial effect in a number of types of malignant tumors.

# **Research motivation**

To investigate the potential anticancer activity of TMP in colon cancer and its underlying mechanisms.



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# Research objectives

To investigate the potential anticancer activity of TMP in colon cancer and its underlying mechanisms.

# Research methods

Colon cancer cells were incubated with different concentrations of TMP. Cell viability was evaluated by crystal violet staining assay and Cell Counting Kit-8 assay, and cell apoptosis and cell cycle were assessed by flow cytometry.

## Research results

TMP significantly inhibited the proliferation of colon cancer cells in a dose- and timedependent manner. In addition, flow cytometry revealed that TMP induced cell cycle arrest at the G0/G1 phase.

# Research conclusions

Our study demonstrated that TMP inhibits the proliferation of colon cancer cells in a dose- and time-dependent manner by inducing apoptosis and arresting the cell cycle at the G0/G1 phase.

# Research perspectives

Further studies of the mechanisms of TMP in human colon cancer are needed.

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