ABSTRACT

The purpose of this study was to detect the changes of P-Glycoprotein (P-GP) expression in rat brain microvascular endothelial cell line RBE4 after the action of Tetramethylpyrazine (TMP) on Carbamazepine (CBZ), so as to clarify the potential mechanism of TMP combined with CBZ in the treatment of intractable epilepsy drug resistance. The RBE4 cell line was utilized for in vitro analysis. Cells were divided into control, CBZ, and CBZ-TMP groups. The expression of P-GP was assessed using Western blot and reverse transcription polymerase chain reaction (RT-PCR). Intracellular concentration of CBZ was measured through high-performance liquid chromatography (HPLC). The differential expression of mRNA was evaluated by RNA sequencing. The intracellular concentration of CBZ in the CBZ-TMP group was significantly higher than that in the other groups. The expression of P-GP in the CBZ group was significantly higher than that in the control group, while in the CBZ&TMP group, it was significantly lower than that in the other groups. Comparative analysis also revealed some differentially expressed genes. Compared with the CBZ group, FAM106A, SLC3A2, TENM2, etc. were upregulated most significantly in the CBZ&TMP group. ZBTB10, WDR7, STARD13, etc. were downregulated most significantly. Results suggest that TMP increases the intracellular concentration of CBZ, downregulates the expression of P-GP increased by CBZ, and modulates related cellular metabolism and signaling pathways, thus reversing the drug resistance mechanism of intractable epilepsy.

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Introduction

Epilepsy is a chronic neurological disorder characterized by sudden, repetitive and transient abnormal discharge of brain neurons. It is one of the most prevalent neurological diseases worldwide, with an estimated 50 million people suffering from epilepsy and around 5 million new cases each year (1). Currently, drug therapy remains the main treatment approach for epilepsy, such as first-line antiepileptic drug CBZ. It is effective in controlling abnormal neuronal discharge and relieving epilepsy symptoms by blocking sodium channels (2).

Although most patients can control their condition after treatment, approximately 30% of epileptic patients will develop intractable epilepsy, a condition that may be caused by drug resistance, which is related to various factors such as drug transporters, drug target sensitivity, epilepsy type, and heredity (3-5). Studying the mechanism of drug resistance to antiepileptic drugs is of great significance for guiding the clinical medication of intractable epilepsy. A key factor in drug resistance is the multidrug transporter P-GP, encoded by the multi-drug resistance (MDR) gene. P-GP facilitates antiepileptic drug excretion from cells, thus reducing intracellular drug concentration and significantly impacting its efficacy and brain concentration (6-9). In addition, Gissmann T et al. demonstrated that the progesterone X receptor (PXR) from the nuclear receptor superfamily has the capability to regulate the expression of P-GP. Specifically, PXR binds to the promoter region of the MDR gene, thereby activating the transcription of P-GP mRNA and resulting in the upregulation of P-GP expression. CBZ, an inducer of PXR, exhibits the ability to effectively enhance the expression level of P-GP by inducing PXR (10-12).

Tetramethylpyrazine (TMP) is one of the key active components found in traditional Chinese medicine Chuanxiong, which possesses various physiological functions, such as antioxidant, anti-inflammatory, anti-apoptotic, regulation of angiogenesis, and neuroprotective properties (13,14). Studies have shown that TMP can reduce the content of intracellular P-GP, thus reversing the multidrug resistance mediated by P-GP, while also increasing the...
in intracellular concentration of other drugs and playing a synergistic role when combined with them (15,16). In this study, we established an RBE4 cell model and used RT-PCR, Western blot, HPLC and RNA sequencing to explore the effect of TMP on the expression of P-GP increased by CBZ and to explain the mechanism of TMP combined with CBZ to reverse drug resistance in intractable epilepsy.

Materials and Methods

Cell culture
RBE4 cells, a type of immortalized rat brain microvessel endothelial cells, were cultured using a mixture of minimum essential medium and Ham's F10 in a 1:1 ratio supplemented with various compounds, including 300 µg/mL of neomycin, 10% FBS, 1 ng/mL of β-FGF, 100 U/mL of penicillin G, 0.25 µg/mL of amphotericin B, 100 µg/mL of streptomycin, 25 mM of sodium bicarbonate, and 25 mM of HEPES. This mixture is referred to as the cell culture medium. The cells were maintained and grown in a humidified atmosphere containing 5% CO₂ at 37°C.

Grouping and drug treatment
TMP and Carbamazepine (CBZ) were procured from MedChemExpress (Monmouth Junction, NJ, USA). The cells were randomly divided into three experimental groups: the control group, the CBZ group, and the CBZ&TMP group. The control group received no medication treatment, while the CBZ group was treated with 200 µM CBZ for 6 hours, 12 hours, Day 1, Day 2, Day 3, Day 4, Day 5, Day 6 and Day 7. The CBZ&TMP group was simultaneously treated with 200 µM CBZ and 300 µg/mL TMP, with the same treatment durations as the CBZ group.

Quantification of P-GP mRNA by RNA isolation and qPCR
The RNA was isolated by chloroform-isopropanol-ethanol protocol from tissue and cells using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). SYBR Green for quantitative polymerase chain reaction (qPCR) was used to perform real-time reverse transcription (RT) qPCR using 1 g RNA input. Primers were synthesized as follows: rat P-GP forward primer: 5’-AGCGACAGCGATCTG-GAGGA-3’, and reverse primer: 5’-GACGTCACTTG-TGAGCCGG-3’; GAPDH forward: 5’-CAAGAACGG-TGGTGAAAGCAG-3’ and GAPDH reverse: 5’-CAAAAG-GTGGAAAGATGCGAG-3’. mRNA quantification was normalized to the housekeeping gene GAPDH and represented as fold change (2–ΔΔCt).

Analysis of differentially expressed genes by mRNA sequencing
For the library construction process, a total of 1 µg of RNA was extracted from the specified RBE4 cell lines using TRIZOL reagent. Ribo-Zero rRNA Removal Kits (Illumina, San Diego, CA, USA) were then used according to the manufacturer's instructions to remove the rRNAs from the RNA samples. Subsequently, the rRNA-depleted RNAs were used to construct RNA sequencing libraries with the TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA), following the manufacturer's protocol. Each extracted RNA sample was used for the preparation of the RNA sequencing library, cDNA synthesis, and PCR amplification, with a selection of approximately 150 bp PCR amplicons size of products. The constructed RNA-Seq libraries were quality-controlled and quantified using the BioAnalyzer 2100 system (Agilent Technologies, Inc., Santa Clara, CA, USA). Finally, the library sequencing process was performed on an Illumina Hiseq instrument with 150 bp paired-end reads.

Statistical analysis
Statistical analysis in this study was conducted using SPSS 24.0 software (IBM, located in Armonk, NY, USA). To assess distinctions between the two groups, the Student's t-test was employed. For comparisons involving multiple groups, the One-way ANOVA test was utilized, followed by a Post Hoc Test (Least Significant Difference). A significance level of p<0.05 was adopted to determine statistical significance.

Results

Tetramethylpyrazine elevated the intracellular level of CBZ in RBE4 cells
To understand the effect of TMP on the intracellular level of CBZ in RBE4 cells, we detected the intracellular concentration of CBZ in RBE4 cells with or without TMP treatment. After HPLC detection, we found that in the CBZ&TMP group, the intracellular concentration of CBZ was significantly higher than in the CBZ group after 24 hours and remained constant after the fifth day (Figure 1). This result suggests that TMP elevates the intracellular level of CBZ in RBE4 cells.

Tetramethylpyrazine reversed the CBZ-elevated expression of P-GP in RBE4 cells
To evaluate the effect of TMP and CBZ on the expression level of P-GP in the RBE4 cells, we treated the RBE4...
significant downregulation were SLITRK1, GYPE, PPP4R3C, OCA2, PRDM13, MAOB, TRO, CSMD1, COX7B2, and GPX7 (Table 1). In comparison to the CBZ group, the CBZ&TMP group showed upregulation of 699 genes and downregulation of 645 genes (Figure 5). The ten genes that displayed the most significant upregulation were FAM106A, SLC3A2, TENM2, MUC4, RAD51, VIP, ZNF516, PPP2R5A, DNAH11, and COMMD8. Conversely, the ten genes that exhibited the most significant downregulation were ZBTB10, WDR7, STARD13, GABRA2, RAB4A, SCRG1, TRIM39, ZNF155, HOXD3, and PSMD6 (Table 1). The thermogram analysis revealed notable differences between the CBZ group and the CBZ&TMP group.

To assess the biological functionality of differen-
tially expressed mRNAs between the CBZ group and the CBZ&TMP group, we conducted GO and KEGG enrichment analyses on the mRNA data. In GO enrichment analysis, the significant difference of mRNA in the CBZ&TMP group is mainly related to binding, catalytic activity, and transporter activity in molecular function. In the cellular component, it is mainly related to the cell, cell part, organelle, and organelle part. In biological processes, it is mainly related to cellular component organization or biogenesis, biological regulation, regulation of biological processes, metabolic processes, response to stimuli, and so on. The KEGG enrichment analysis was conducted with a condition of q-value < 0.05. Significant differences in mRNA enrichment were observed in several metabolic pathways, including pyruvate metabolism, citrate cycle (TCA cycle), fructose and mannose metabolism, DNA replication, glycosaminoglycan biosynthesis-heparan sulfate/heparin, Th1 and Th2 cell differentiation, and glycolysis/gluconeogenesis in the CBZ&TMP group.

Discussion

TMP is a traditional Chinese medicine active ingredient that exhibits a universally effective therapeutic effect for multiple systemic diseases. Studies have indicated its extensive application in clinical cardiovascular and cerebrovascular diseases, liver injury diseases, and cancer in China, with positive evaluations of its efficacy (17-20). Tao et al.’s study revealed that TMP can reduce epileptic seizures and improve efficacy when used in combination with conventional antiepileptic drugs (21). Its mechanism of action may involve multiple targets, pathways, and bidirectional regulation, making it a drug with immense research potential.

This study represents the first investigation into the potential mechanisms underlying the combined use of TMP and CBZ in intractable epilepsy and its impact on drug resistance. The experimental drug employed in this study was CBZ, and the findings revealed that TMP is capable of augmenting the concentration of CBZ within RBE4 cells, thus providing further evidence for the synergistic effect between TMP and antiepileptic drugs. This synergistic effect may be attributed to the reduction of P-GP concentration by TMP, which weakens the role of P-GP protein in promoting drug efflux from cells and finally leads to an increase in CBZ concentration in cells. Further investigations revealed that TMP can reverse the upregulation of P-GP mRNA and P-GP induced by CBZ, maintaining their expression at sustained low levels, similar to the inhibitory effect of P-GP inhibitors. Yinxu Zhang, et al.’s study also demonstrated that TMP can inhibit P-GP ATPase activity and suppress P-GP expression (22). Furthermore, enrichment analysis of mRNA from different groups revealed significant enrichment of several metabolic pathways, such as acetate metabolism, fructose and mannose metabolism, and the TCA cycle, upon the addition of TMP. This suggests that the reversal of CBZ resistance by TMP may be associated with cellular energy metabolism pathways. The reversing effect of TMP may be associated with its antioxidant activity, CenJuan, Hao Hong, Yafang Zhang, et al.’s study indicated that reactive oxygen species (ROS) in cells can induce overexpression of P-GP, whereas TMP can significantly inhibit the generation of ROS (23-25).

This study also has some limitations. Firstly, we conducted in vitro experiments using REB4 cells, which cannot fully replicate the complex microenvironment of tissues and organs. Therefore, further in vivo experiments and clinical observations are needed to complement the results obtained from in vitro cell experiments in order to obtain more accurate and comprehensive research conclusions. Secondly, in RNA sequencing and enrichment ana-

![Figure 5. CBZ&TMP vs. CBZ: Differential expression analysis and functional analysis of mRNAs of RNA-seq. (A) Hierarchical clustering analysis (heatmap) for differently expressed genes pValue<0.05&|logFC|>1. (B) The KEGG analysis (q-value < 0.05) of the top 20 differently expressed genes. (C) Gene ontology(GO)term enrichment analysis.](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>Regulation</th>
<th>Gene Id</th>
</tr>
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<tbody>
<tr>
<td>CBZ group vs. control group</td>
<td>Up</td>
<td>PIK3CG, GOLGA85, LMX1B, CD200, POU4F2, ARHGEF16, NKD2, SLC18A1, DLX5, MLIP</td>
</tr>
<tr>
<td></td>
<td>Down</td>
<td>SLITRK1, GYPE, PPP4R3C, OCA2, PRDM13, MAOB, TRO, CSMD1, COX7B2, GPX7</td>
</tr>
<tr>
<td>CBZ&amp;TMP group vs. CBZ group</td>
<td>Up</td>
<td>FAM106A, SLC3A2, TENM2, MUC4, RAD51, VIP, ZNF516, PPP2R5A, DNAH11, COMMD8</td>
</tr>
<tr>
<td></td>
<td>Down</td>
<td>ZBTB10, WDR7, STARD13, GABRA2, RAB4A, SCRG1, TRIM39, ZNF155, HOXD3, PSMD6</td>
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Table 1. The top ten most upregulated genes and the top ten most downregulated genes in each group of mRNA sequencing data.
lysis, we did not deeply analyze the regulatory changes of various genes and signaling pathways. In future research, we can further explain the detailed drug resistance mechanism of TMP combined with CBZ in regulating cellular metabolism and signaling pathways from the gene level.

Our study has confirmed that the combination of TMP and CBZ can reduce the expression of P-GP, thereby reversing drug resistance in epilepsy. This provides a solid experimental foundation and theoretical basis for the clinical application of traditional Chinese medicine in combination with antiepileptic drugs for the treatment of intractable epilepsy, offering a new approach to the use of antiepileptic drug combinations and the management of intractable epilepsy. Furthermore, TMPs have demonstrated neuroprotective and antidepressant effects in treating comorbidities of epilepsy, such as improving cognitive impairment and regulating anxiety and depression levels, thus presenting broad application prospects (26). However, there is still a need for sufficient clinical research data on the clinical dosage, efficacy, and prognosis of TMP, which necessitates further investigation and analysis in future studies.

Conflict of interest statement
The authors declare that they have no conflict of interest.

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Data availability statement (DAS)
The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

References