Inhibition of tetramethylpyrazine on P-gp, MRP2, MRP3 and MRP5 in multidrug resistant human hepatocellular carcinoma cells

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Abstract. Some membrane transporters in liver, such as P-glycoprotein, multidrug resistance-associated protein 2 (MRP2), MRP3, and MRP5 can lead to a complex multidrug resistance (MDR) to antineoplastic agents. How to inhibit these proteins is still an issue. Tetramethylpyrazine is a bioactive constituent isolated from the root of *Ligusticum chuanxiong Hort*, a Chinese herb. Recent studies showed that it can enhance the chemosensitivity effects of a drug on human hepatocellular carcinoma cells, acting as a multidrug resistance modulator. In this study, the reversal effect of TMP on MDR was evaluated and its activity mechanism *in vitro* was explored. The IC_{50} value shows that TMP reversed the multidrug resistance of BEL-7402/ADM cells 9.23-fold (P<0.01) at the concentration of 600 μM. The mean fluorescence intensity of ADM in BEL-7402/ADM cells with TMP was found to be 163.78±39.5% (P<0.01) versus in BEL-7402/ADM cells without TMP by flow cytometry and 126.73±28.72% in BEL-7402/ADM cells with TMP versus in BEL-7402/ADM cells without TMP (P<0.01) by high performance liquid chromatography, respectively. It was also found that the mRNA level of multidrug resistant gene MDR1, MRP2, MRP3 and MRP5 and the level of the proteins they encode were decreased after treatment with TMP, indicating that TMP can effectively reverse the MDR in BEL-7402/ADM cells, and its activity mechanism may be correlated with the down-regulation of expression in these transporters.

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Introduction

For many anti-neoplastic agents, multidrug resistance (MDR) is a severe problem in human hepatocellular carcinoma (HCC). Many members of the super-family have been reported for their contribution to MDR, some of which are richly expressed in the liver (1,2).

The permeability glycoprotein (P-gp) encoded by MDR1 gene (ABCB1) is a well-known transporter acting as an efflux pump. The overexpression of P-gp can lower intracellular drug accumulation and decrease the cellular toxicity of chemotherapeutics, such as adriamycin, daunorubicin, epirubicin, mitoxantrone, bisantrene, vincristine, vinblastine, etoposide, and paclitaxel (2-4).

Other important ATP-binding cassette transporters located in the liver include multidrug resistance-associated protein 2 (MRP2), MRP 3, and MRP5 encoded by ABCC2, ABCC3 and ABCC5, respectively.

MRP2, a major transporter of bilirubin glucuronides and other organic anions from liver into bile, functions as a pump effluxing cisplatin (different from MRP1), vincristine, anthracyclines, adriamycin, methotrexate (MTX), etoposide, epirubicin, and mitoxantrone (5,6). MRP3, located in the basolateral membrane of the hepatocyte, may allow efflux of organic anions from the liver into the blood when the secretion into bile is blocked, effluxing etoposide, MTX, and teniposide (7,8). MRP5 can be resistance to thiopurines (e.g. 6-mercaptopurine and thioguanine), but no noticeable resistance to other anticancer drugs tested (e.g. anthracyclines, vinca alkaloids, podophyllotoxins, or MTX). The transfected cells tend to accumulate less 6-mercaptopurine and PMEA [9-(2-phosphonylmethoxyethyl) adenine, i.e. adefovir] and extrude increased amount of 6-thioinosinemonophosphate and PMEA from the cells. MRP5, therefore, appears to be a nucleotide analogue pump (9,10).

Tetramethylpyrazine (i.e. ligustrazine, TMP) is a bioactive constituent isolated from the root of *Ligusticum chuanxiong Hort*, a Chinese herb (*Chuanxiong*), which is often used as an antioxidant in cardiovascular diseases (11,12). Previous studies showed that such a calcium channel blocker can enhance the chemosensitivity effects of drug on human

hepatocellular carcinoma BEL-7402 cells, acting as an MDR modulator (13,14). However, it is still unknown if it has effect on other transporters. In this study, the reversal effect of TMP on MDR in multidrug resistant human hepatocellular carcinoma BEL-7402/ADM was investigated and relationships among TMP and MDR1, MRP2, MRP3 and MRP5 were explored.

Materials and methods

Cell lines and cell culture. The parental human hepatocellular carcinoma cells BEL-7402 and ADM-selected drug-resistant cells BEL-7402/ADM were cultured in our laboratory. All cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37˚C in humidified atmosphere of 5% CO_2 and 95% air. BEL-7402/ADM were cultured in the above-mentioned medium with the addition of 2,000 nM ADM until at least 2 weeks before the experiment.

Drug and agents. TMP was purchased from Changchun Guoao Pharmaceutical Company (China), verapamil (VRP) was purchased from Shanghai Hefeng Pharmaceutical Company (China), adriamycin (ADM) from Zhejiang Haizheng Pharmaceutical Co., Ltd. (China), P-gp and ß-actin monoclonal antibodies from Sigma-Aldrich Chemical Company (USA), while multidrug resistance-associated protein 2 (MRP2), multidrug resistance-associated protein 3 (MRP3) and multidrug resistance-associated protein 5 (MRP5) monoclonal antibodies from Santa Cruz Biotechnology, Inc. (USA). Trypsin, RPMI-1640 and Trizol Reagent were purchased from Gibco Invitrogen (USA), FBS and phosphate buffer solution (PBS) was purchased from Hangzhou Sijiqing Company (China), HRP-conjugated goat anti-mouse IgG from Beijing Biosynthesis Biotechnology Co., Ltd. (China).

Measurement of resistance and reversal by methylthiazoletetrazolium (MTT) assay. To assess cell resistance and drug reversal effect, a measurement of cells proliferation by methylthiazoletetrazolium (MTT) assay was conducted as described previously (15). BEL-7402/ADM cells were preconditioned with TMP (400 and 600 μM) or VRP (5 and 10 μM) for 24 h, respectively, then treated with 0.3, 0.6, 1.2, 2.4, 4.8 μM of ADM in 96-well plate for 72 h, respectively. Verapamil was conducted as positive control. Then the medium was discarded and the cells were washed with PBS. After 30 μl MTT solution was added for 2 h, 100 μl dimethyl sulphoxide (DMSO) was used to dissolve formazan crystals. The absorbance at 540 nm was recorded. Negative control (without drug) was set as 100% of cell survival. IC_{50} value, resistance index and reversal index were calculated as described previously. The resistance index (RI) is calculated as RI = (IC Resistance group)/(IC Parental group) x100%. The reversal index is calculated as RI = (IC Resistance group)/(IC TMP/VRP group) x100% (16).

Intracellular accumulation of adriamycin (ADM) by flow cytometry (FCM). To assess the steady accumulation of ADM, with/without TMP or VRP as a precondition for 3 h, BEL-7402 cells and BEL-7402/ADM were incubated with 8,000 nM ADM for 2 h at 37˚C, washed 3 times with ice-cold PBS.

Table I. Primer sequences.

Primers	Sequences of primers (5'-3')	Size (bp)
MDR1	CAT TGG TGT GGT GAG TCA GG CTC TCT CTC CAA CCA GGG TG	176
MRP2	CGA TAC TCT GTG GCA CTT TCC TCA TCA ACT TCC CAG ACA TCC	277
MRP3	TGT ATG TGG GTC AAA GTG CG CCA GCC TCA GGG AAG TGT T	108
MRP5	ACT CGA CCG TTG GAA TGC C GGG TGC TGG TGT TTG GAA GT	179
ß-actin	GTT GCG TTA CAC CCT TTC TTG CTG CTG TCA CCT TCA CCG TT	155

The fluorescence intensity of intracellular ADM was recorded by FCM with an excitation wavelength *(λex)* of 480 nm and emission wavelength *(λem)* of 575 nm (17,18).

Intracellular accumulation of ADM by high performance liquid chromatography (HPLC). The accumulation of ADM in cells was also quantified by the HPLC method according to Cox *et al* with a minor modification (19). Internal standard solution (50 μl) (alprazolam, 4 μg/ml) was added to 1,000 μl samples, after the mix for 2 sec, then centrifuged at 4,000 rpm, 2.8 ml supernatant was evaporated at 45˚C for 2 min under a gentle stream of nitrogen. The resulted residue was reconstituted in 100 μl mobile phase, 50 μl of the standard/sample was injected onto the high performance liquid chromatography. HPLC was performed using a Shimadzu LC-20AD HPLC system consisted of a UV/VIS detector (SDP-20AV), an autosampler (SIL-20A) and an analytical column (Gimini C18, 4.6x250 mm, ID 5 μm). The mobile phase consisted of acetonitrile-water with KH_2PO_4 (pH 3.0 adjusted with H_3PO_4) (75:25, v/v), with a flow rate of 0.8 ml·min^{-1}. The wavelength of ADM detected was 233 nm (20,21).

Expression levels of MDR1, MRP2, MRP3 and MRP5 mRNA by real-time reverse-transcription-PCR analysis. Total RNA was isolated from BEL-7402 and BEL-7402/ADM in RNA clean solution by Trizole Reagent according to the manufacturer's protocol. All primer pairs (Table I), and their appropriate fluorescent hybridization probes, were designed and produced by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., China. The mRNA levels of MDR1, MRP2, MRP3 and MRP5 were measured by real-time RT-PCR and quantitated by SLAN real-time PCR detection system (Shanghai Hongshi Medical Science Co., Ltd., China). In addition, the mRNA level of the internal control gene ß-actin was measured and used to normalize the mRNA levels of the drug resistance genes. The relative expression levels of the resistance genes were calculated using the comparative Ct value as descirbed previously, and relative amount of target is $2^{-\Delta\Delta Ct}$ value. The $\Delta\Delta Ct$ value in this formula is defined as the ΔCt value of the target gene for
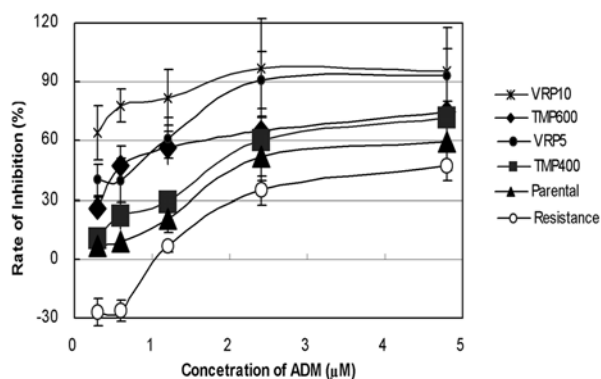
Figure 1. The inhibitory effect of ADM on BEL-7402/ADM cells and BEL-7402 cells preconditioned by TMP. A range from 0.3 to 4.8 μM of ADM was added into BEL-7402/ADM cells with TMP (400 and 600 μM) or VRP (5 and 10 μM) for 72 h. VRP was conducted as a positive control. Proliferation of cells was measured using the MTT assay, and the inhibition rate (%) was calculated. Data are expressed as mean ± SD (n=3).
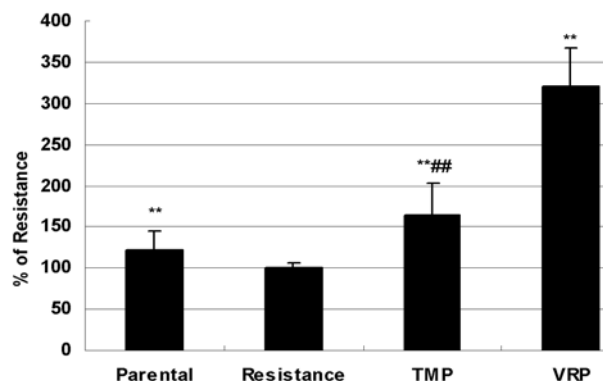

Figure 2. Intracellular accumulation of ADM by FCM. BEL-7402 (Parental group) and BEL-7402/ADM cells (Resistance group) were treated with ADM, all the samples were preconditioned with TMP (600 μM) or VRP (10 μM) for 24 h, then treated with ADM 8,000 nM for 3 h. Data are expressed as mean ± SD (n=3). [**]P<0.01 vs. Resistance; [##]P<0.01 vs. VRP.

Table II. Reversal effect of TMP on BEL-7402/ADM cells.

Group	IC$_{50}$ value	Resistant index	Reversal index
Parental	3.13±0.56[a]		
Resistance	4.8±0.85	1.53	
TMP400	2.01±0.15[a]		1.59
VRP5	0.96±0.45[a]		5.00
TMP600	0.52±0.16[a,b,c]		9.23
VRP10	0.18±0.04[a]		120.00

Data presented are mean ± SD value, n=3. [a]P<0.01 vs. Resistance; [b]P>0.05 vs. VRP5; [c]P<0.01 vs. VRP10.

a particular sample subtract the ΔCt value of that target gene of ß-actin [$\Delta\Delta$Ct=ΔCt (target) -ΔCt (ß-actin)] (22-24).

Expressions of MDR1, MRP2, MRP3 and MRP5 proteins by Western blot analysis (WB). BEL-7402 and BEL-7402/ADM cells were harvested, the total protein concentration was determined using bovine serum albumin as a standard. An aliquot of protein (150 μg or 10 μg) was loaded in each lane to detect the expressions of P-gp, MRP2, MRP3 and MRP5, electrophoresed on 7.5% SDS-polyacrylamide gel, and transferred to a 0.2 μm pore nitrocellulose (NC) membrane (Beyotime Institute of Biotechnology, China). For immuno-blotting, the membranes were blocked with 5% defatted powdered milk in 20 μM Tris-HCl, 135 mM NaCl, 0.1% Tween-20 buffer at 4°C for 2 h. The blots were incubated with the monoclonal antibodies for 2 h, then with peroxidase-conjugated sheep affinity-purified antibody to mouse Ig G as a secondary antibody for 40 min, and washed for 5 times with 0.1% Tris buffered saline Tween-20 (TBS-T). All washing and incubation steps were performed at ambient temperature. Proteins were detected and ß-actin as a reference. Blots were then exposed to a computer scanner and detected by ImageJ 1.38x (NIH, USA).

Statistical analysis. All experiments were performed at least 3 times. Results are presented as the mean ± SD. Statistical analysis was calculated using the Student's t-test or one-way analysis of variance (oneway ANOVA). P<0.05 was considered significant.

Results

Resistance and reversal results by MTT. Results from the experiment showed that the IC$_{50}$ value of ADM for BEL-7402/ADM cells was 1.53 (P<0.01) times than that of ADM for BEL-7402 cells. The reversal was 1.59-fold (P<0.01) by TMP at the concentration of 400 μM and 9.23-fold (P<0.01) at the concentration of 600 μM, respectively, similar to VRP, indicating that the reversal effects of regulators took place in a dose-dependent manner (Fig. 1 and Table II).

Intracellular accumulation of ADM by FCM. The mean fluorescence intensity of ADM in the Parental group, Resistance +TMP group and Resistance +VRP group were 121.83±23.2% (P<0.01), 163.78±39.5% (P<0.01) and 320.1±47.18% (P<0.01) versus Resistance group by flow cytometry, respectively (Fig. 2).

Intracellular accumulation of ADM by HPLC. The peak area ratio of ADM and internal standard alprazolam was obtained and calculated by HPLC. The calibration curve was obtained as follows with the content ranging from 10 ng·ml^{-1} to 2,000 ng·ml^{-1}. The limit of detection (LOD) was 5 ng·ml^{-1}, and RSD was 0.407.

$$C=47.73A+23.62 \text{ (correlation factor: 0.9998)}$$

Where C refers to the concentration of ADM, A refers to the peak area ratio of ADM and alprazolam. The mean con-centration of ADM in the Parental group, Resistance group, Resistance +TMP group and Resistance +VRP group were 1,421.356, 909.32, 1,152.37, and 1,194.13, respectively. Compared with the ADM level of the Resistance group, that of Parental group, Resistance +TMP group and Resistance
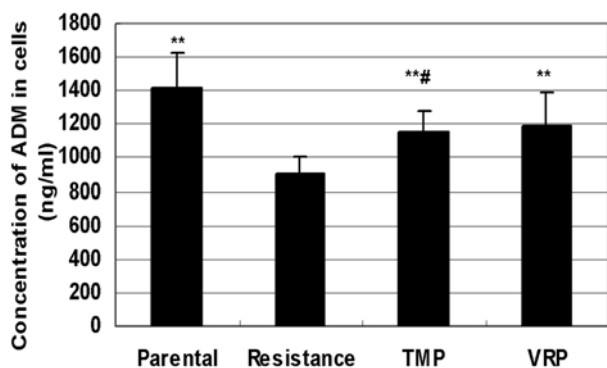
Figure 3. Intracellular accumulation of ADM by high performance liquid chromatography (HPLC). The mobile phase consisted of acetonitrile-water with KH_2PO_4 (pH 3.0 adjusted with H_3PO_4) (75:25, v/v). The injection volume was 50 μl and the flow rate was 0.8 ml·min^{-1}. The wavelength for detection of ADM was 233 nm, alprazolam was used as an internal standard. The calibration curve was C=47.73A+23.62 (r=0.999 8) with the content range of 10 ng·ml^{-1} to 2,000 ng·ml^{-1}. The experiments were performed 3 times. Data presented are mean ± SD value. $^{**}P<0.01$ vs. Resistance; $^{#}P<0.05$ vs. VRP.
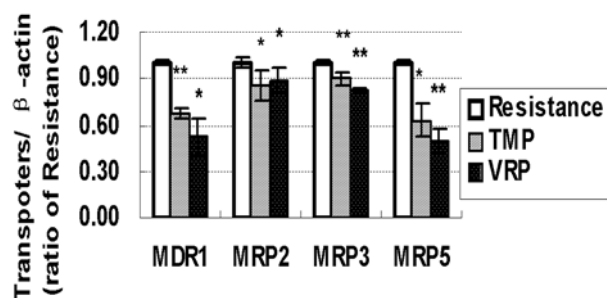

Figure 4. mRNA expression of transporters in BEL-7402/ADM cells. The data presented are the mean ± SD from at least 3 independent experiments. $^{*}P<0.05$ vs. Resistance; $^{**}P<0.01$ vs. Resistance.
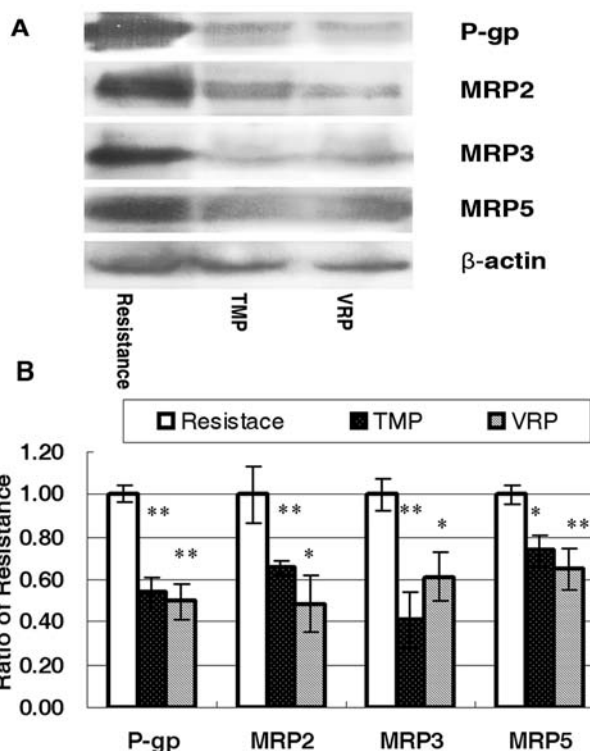

Figure 5. Expressions of MDR1, MRP2, MRP3 and MRP5 proteins in BEL-7402/ADM cells by WB. BEL-7402/ADM cells were treated with TMP or VRP (positive control) for 24 h. Proteins were extracted and subjected to Western blotting to determine P-gp, MRP2, MRP3 and MRP5 expression. Data presented are means ± SD values, n=3. $^{**}P<0.01$ vs. Resistance.

+VRP group are 156.31±25.12% (P<0.01), 126.73±28.72% (P<0.01) and 131.32±47.34% (P<0.01), respectively (Fig. 3).

Expressions of MDR1, MRP2, MRP3 and MRP5 mRNA by real-time PCR. The expressions of MDR1, MRP2, MRP3 and MRP5 mRNA in resistant BEL-7402/ADM increased 78 times than that in parental BEL-7402 (Data not shown). Both TMP and VRP can reverse this phenotype remarkably (P<0.05) (Fig. 4).

Expressions of MDR1, MRP2, MRP3 and MRP5 proteins by WB. After treated with TMP or VRP in BEL-7402/ADM cells, Western blot results indicated that expressions of MDR1, MRP2, MRP3, and MRP5 proteins decreased significantly compared with the Resistance group (P<0.05), in line with their mRNA counterparts (Fig. 5) (22).

Discussion

A great number of factors is documented to be associated with human carcinomas (1,25,26). MDR is a severe problem to be addressed in resistant HCC. Many membrane transporters are reported to lead to MDR, some of which share the characteristic of being richly expressed in the liver.

P-gp encoded by MDR1 gene (ABCB1) is a classical transporter acting as an efflux pump. The overexpression of P-gp can decrease intracellular drug accumulation and reduce the cellular toxicity of chemotherapeutics (1,27-29). The other ATP-binding cassette transporters in liver are multidrug resistance-associated proteins (MRP2, MRP3, MRP5) encoded by ABCCs. MRP2 is a major transporter of bilirubin glucuronides and other organic anions from liver into bile. MRP3 may efflux organic anions from the liver into the blood when obstale of secreting into bile occurs. MRP5 can be resistant to thiopurines (e.g., 6-mercaptopurine and thioguanine), but is not notable resistant to other anticancer drugs tested (e.g., anthracyclines, vinca alkaloids, podophyllotoxins, or MTX).

It is unclear if all the proteins mentioned above are reversed together by the same kind of agent. Tetramethylpyrazine (TMP) is a bioactive constituent isolated from the root of a Chinese herb, *Chuanxiong*. Recent studies have shown that *Chuanxiong* can enhance the chemosensitivity effects of drug on resistant HCC, acting as an MDR modulator to limit the influx of P-gp substrate into the cells. However, it is still unknown if it has any effect on other transporters. In this study, the reversal experiments of TMP on MDR in multidrug-resistant human hepatocellular carcinoma BEL-7402/ADM were conducted and investigated, and it was found that MDR1, MRP2, MRP3 and MRP5 are

highly expressed in resistant liver cancer cells and play an important role and jointly contribute to resistance (24) and can be reversed by TMP dose-dependently.

We also found that TMP and another calcium channel blocker, VRP (30) play a similar role on MDR1 (31,32), MRP2, MRP3 and MRP5, and concluded that calcium may play an important role in MDR of MDR1, MRP2, MRP3 and MRP5 which can be reversed by certain calcium channel blockers that deserves further study.

The concentration of TMP (600 μM) in this study is too high to be used in human beings, but it might offer a novel way for finding new chemosensitizers from natural products. In conclusion, this study shows that MDR1, MRP2, MRP3, and MRP5 are contributing to MDR mechanism in resistant HCC, and can be reversed by TMP dose-dependently.

Acknowledgements

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