Pantoprazole Induces Apoptosis of Leukemic Cells by Inhibiting Expression of P-Glycoprotein/Multidrug Resistance-Associated Protein-1 Through PI3K/AKT/mTOR Signaling

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Abstract This study aims to investigate the effects and mechanism of pantoprazole on multidrug resistant leukemia K562/A02 and K562/ADM cell lines. K562/A02 and K562/ADM cells at logarithmic growth phase were pretreated with different concentration of pantoprazole (0, 50, 100, 200 µg/mL) for 24 h. Flow cytometry was used to measure the cell growth cycle and apoptosis. RT-PCR and Western blot were used to measure the expression of p-PI3K, p-AKT, p-mTOR, P-glycoprotein (P-gp) and multidrug resistance-associated protein-1 (MRP1). Pantoprazole pretreatment significantly increased the ratio of G0/G1 phase but decreased the S phase of K562/A02 and K562/ADM cells in dose-dependent manner ($p < 0.05$). Flow cytometry analysis indicated that pretreatment of leukemic cells with pantoprazole induced apoptosis in a dose-dependent manner. RT-PCR and Western blot analysis indicated that pantoprazole pretreatment inhibited the mRNA and protein expression of p-PI3K, p-Akt, p-mTOR, P-gp and MRP1 in K562/A02 and K562/ADM cells in a dose-dependent manner ($p < 0.05$). Pantoprazole arrested cell cycle and induced apoptosis of multidrug resistant leukemic cells by inhibiting the expression of P-gp and MRP1 through PI3K/Akt/mTOR signaling pathway.

Keywords Pantoprazole · Proton pump inhibitor · Leukemia · PI3K/AKT/mTOR signaling · P-glycoprotein · Multidrug resistance-associated protein-1

Introduction

Multidrug resistance (MDR) refers to the crossed chemotherapy resistance of tumor cells to multiple other anti-tumor drugs having different construct after the tumor cells contact one anti-tumor drug and show resistance. MDR of leukemic cells to chemotherapy is the major reason for therapeutic failure and poor prognosis of leukemia. Therefore, how to reverse the drug-resistance is a hotspot of research. The classical mechanism of MDR proposes that MDR is related with the MDR1 gene productions, P-glycoprotein (P-gp), and multidrug resistance-associated protein-1 (MRP1) [1, 2]. PI3K/Akt/mTOR signaling is one of the important pathways for cellular survival and plays important roles in promoting the cellular growth, proliferation, motility and invasion, and chemotherapy resistance [3]. Recently, more and more studies indicated that PI3K/Akt/mTOR signaling is related with the chemotherapy resistance of leukemia and is considered as a new target for treatment of chemotherapy resistance [4–6]. However, if PI3K/Akt/mTOR is involved in MDR of leukemia and related with P-gp/MRP1 is not clear.

Hypoxia and acidification are two major features of the micro-environment of tumor cells. There exists a gradient between the intracellular and extracellular pH of tumor cells as lower extracellular pH (pH_e) value and higher intracellular pH (pH_i) value, which is related with the survival, growth, invasion and chemotherapeutic resistance of tumor cells [7]. It is verified that proton pump inhibitors

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(PPIs) can be activated in acidic environment and reverse the drug resistance of tumors in different tissues [8]. However, if PPIs modulate the expression of P-gp/MRP1 and reverse the chemotherapy resistance of leukemia through the PI3K/Akt/mTOR signaling is unclear. The present study investigated the effects of PPIs (pantoprazole) and the related mechanism on the apoptosis of K562/A02 and K562/ADM leukemic cell lines.

Materials and Methods

Cell Culture and Pretreatment

The K562/A02 and K562/ADM cells (Basic Medical College, Faculty of Medicine, Huazhong University of Science and Technology) were incubated in 1640 medium (Gibco) supplied with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in 5% CO₂ and 37 °C. Cells at logarithmic phase with rejection rate of trypan blue over 95% were used for subsequent experiments with pretreatment of pantoprazole at different concentration (0, 50, 100 and 200 µg/ml; Shandong Luoxin Pharmaceutical Limited Inc., Shandong, China) for 24 h. Furthermore, the cell culture medium was adjusted to the pH 6.65 which is close to the tumor environment and beneficial for cell proliferation according to the previous study described [7, 9, 10].

Measurement of Cell Growth Cycle with Flow Cytometry

After pre-treatment, the cells were inoculated in 6-well plate at density of 1×10^5 cells/mL and cultured for 24 h. Then the cells were collected, centrifuged at 1000 r/min for 5 min, rinsed twice with PBS after removal of supernatant, incubated in 1 ml 70% ethanol at 4 °C overnight, centrifuged at 1000 r/min for 5 min according to the manufacturer's instruction. After removal of the supernatant and rinsed with PBS twice, the deposit was added 150 µl RNAase inhibitor and 150 µl PI (20 µg/ml) for incubation at room temperature in dark for 30 min. Finally, the solution was subjected to flow cytometry to check the cell growth cycle.

Measurement of Cellular Apoptosis Using Flow Cytometry

After pre-treatment, the cells were inoculated in 96-well plated at density of 1×10^5 /mL and cultured for 24 h. According to the manual of Annexin-V/PI kit (Beijing Suolaibao Scientific Limited Inc, Beijing, China), the apoptosis was measured with flow cytometry. In brief, the cells were collected, centrifuged at 1000 r/min for 5 min,

rinsed twice with PBS after removal of supernatant, incubated in 1 ml 70% ethanol at 4 °C overnight, centrifuged at 1000 r/min for 5 min. After removal of the supernatant and rinsed with PBS twice, mixed with 500 µl Binding Buffer, 5 µl Annexin-V and 5 µl PI for incubation at room temperature in dark for 5–15 min (negative control was set). Then the apoptosis was measured using flow cytometry.

Expression of p-PI3K, p-AKT, p-mTOR, P-gp and MRP1 mRNA Using RT-PCR

After collection, the total RNA was extracted with Trizol (Invitrogen) and used as template to synthesize cDNA with Oligo(dT)-15 as primer. The cDNA was used as template to transcribe RNA with different primers (Table 1) using RT-PCR kit (Invitrogen). The resulting RNAs were subjected to electrophoresis in 2% agarose and the bands were imaged after addition of EB for analysis.

Measurement of p-PI3K, p-AKT, p-mTOR, P-gp and MRP1 Proteins Using Western Blot

After pretreatment, the cells were collected and added 200 µl lysis buffer to extract total proteins. Then the proteins were isolated in SDS-PAGE by electrophoresis and transferred to nitrocellulose membrane. In 5% slim milk solution, the membrane was incubated with corresponding primary antibody (all from Cell Signal) including: GAPDH (1:1000), p-PI3K (1:2000), p-AKT (1:2500), p-mTOR (1:2000), P-gp (1:1000), MRP1 (1:2000) in dark at 4 °C overnight. Then the membrane was incubated with corresponding secondary (all from Pierce, 1:1000) after repetitive rinse with TBST in a shaking plate for 1 h. The protein bands were developed to take picture for analysis by calculating the absorption value.

Statistical Analysis

All data were expressed Mean $\pm$ SD and analyzed with SPSS 12.0. One-way ANOVA and *t* test were used for multi-group comparison and 2-group comparison, respectively. *p* < 0.05 was set as the level of significant difference.

Results

Effects of Pantoprazole on the Cellular Cycle of Drug-Resistant Leukemic Cells

In order to investigate the effect of PPIs on the cellular cycle of K562/A02 and K562/ADM cells, we pretreated these cells with different concentration of pantoprazole (0,

Table 1 Primers and amplification conditions for reverse transcription-polymerase chain reaction

Gene	Primers	Tm	Cycles	Product size (bp)
β -actin	S:CAA ATC AGA GCA GAC AAT A:TTA GAT TCT GTC ATA CTG	59 °C	40	197
p-PI3K	S:ACA TAG TCA GAA GTG AG A: TGC TGA AGC GCT CAC GT	55 °C	40	138
p-AKT	S:ACT TAG GAT GAA TTA TGC A:CAT AAG GCA CGC TAG TA	57 °C	40	149
p-mTOR	S:ACC AGG ATG TCA CTC GG A:GAT CAG ATG ATC GCC AG	61 °C	40	112
P-gp	S:AAT TGT AAT CAC ATG TA A:GTC GAT ACC AGA CTG CG	51.5 °C	40	105
MRP1	S:ATC GCG ACT GAA TTG GA A:CTT ATG ACC CAT AAG TC	53 °C	40	176

50, 100 and 200 $\mu\text{g/ml}$) for 24 h and examined the cellular cycle using flow cytometry. The results indicated that pantoprazole (50 $\mu\text{g/ml}$) had no significant effect on cellular cycle of sensitive K562 cells (Fig. 1a). Therefore, in the following study, we investigated the effects of pantoprazole on the multidrug resistant leukemia K562/A02 and K562/ADM cell lines. The flow cytometry analysis indicated that pantoprazole increased the cell number in G0/G1 phase and decreased the cell number in S phase in a dose-dependent manner and there was significant difference when compared to control group ($p < 0.05$; Fig. 1b), suggesting that pantoprazole arrested the K562/A02 and K562/ADM cells in S phase. In addition, pantoprazole (50 $\mu\text{g/ml}$) alone and pantoprazole (50 $\mu\text{g/ml}$) combined with amycin (1 $\mu\text{g/ml}$) had no significant difference in cellular cycles ($p > 0.05$; Fig. 1a,c).

Effects of Pantoprazole on the Apoptosis of Drug-Resistant Leukemic Cells

In order to clarify the effect of PPIs on the cellular apoptosis of K562/A02 and K562/ADM cells, we pretreated these cells with different concentration of pantoprazole (0, 50, 100 and 200 $\mu\text{g/ml}$) for 24 h and examined the cellular apoptosis using flow cytometry. The results indicated that pantoprazole induced apoptosis in K562/A02 and K562/ADM cells in a dose-dependent manner (Fig. 2). Following the increase of pantoprazole dose, the apoptosis rate increased. The apoptosis rate in K562/A02 and K562/ADM cells treated with 50 $\mu\text{g/mL}$ pantoprazole for 24 h was 12.57 and 17.34%, respectively. When the dose of pantoprazole increased to 200 $\mu\text{g/mL}$, the apoptosis rate was 48.96 and 55.28% for K562/A02 and K562/ADM cells, respectively. There was significant difference when compared to control group ($p = 0.035$ and $p = 0.041$, respectively).

In addition, pantoprazole (50 $\mu\text{g/ml}$) alone had no obvious effect on the apoptosis of K562 cells (Fig. 2a); however, the apoptosis rate of K562 cells and K562/ADM cells treated with pantoprazole (50 $\mu\text{g/ml}$) combined with amycin (1 $\mu\text{g/ml}$) was significantly increased when compared to cells treated with pantoprazole (50 $\mu\text{g/ml}$) alone ($p < 0.05$; Fig. 2a,c).

Effects of Pantoprazole on the PI3K/AKT/mTOR Signaling in Drug-Resistant Leukemic Cells

PI3K/AKT/mTOR is an important pathway in the cellular apoptosis while pantoprazole is a PPI which is found to be able to induce apoptosis of tumor cells [11]. In order to investigate if pantoprazole induce apoptosis of leukemic cells through modulating the PI3K/AKT/mTOR signaling, we examined the expression of mRNAs and proteins of the PI3K/AKT/mTOR signaling in K562/A02 and K562/ADM cells treated with pantoprazole, using RT-PCR and Western blot. The results indicated that pretreatment with 50 $\mu\text{g/mL}$ pantoprazole (24 h) significantly inhibited the expression of p-PI3K, p-Akt and p-mTOR mRNAs and proteins in K562/A02 and K562/ADM cells ($p < 0.05$), and the inhibitory effects was dose-dependent (Figs. 3, 4). When the concentration of pantoprazole increased to 200 $\mu\text{g/mL}$, the inhibitory effect on the expression of p-PI3K, p-Akt and p-mTOR was the most significant. Compared to control group, the mRNA expression of p-PI3K, p-Akt and p-mTOR in K562/A02 and K562/ADM cells was significantly decreased by 44.01 ± 1.78 , 38.16 ± 2.03 and $39.23 \pm 1.92\%$ (all $p < 0.05$) for K562/A02 cells, and 47.69 ± 3.35 , 38.55 ± 3.06 and $41.17 \pm 4.01\%$ (all $p < 0.05$) for K562/ADM cells, respectively. At protein level, the expression of p-PI3K, p-Akt and p-mTOR was significantly decreased by

A K562 **B K562/A02** **C K562/ADM**

1

2

3

4

5

Fig. 1 Cellular cycle of K562 cells (a) and K562/A02 cells (b) and K562/ADM (c) cells pretreated with different concentration of pantoprazole. 1 Control; 2 50 µg/ml of pantoprazole; 3 100 µg/ml of pantoprazole; 4 200 µg/ml of pantoprazole; 5 50 µg/ml of pantoprazole + 1 µg/ml of amycin

73.05 ± 3.27, 56.84 ± 3.56 and 68.04 ± 2.47% (all $p < 0.05$) for K562/A02 cells, and 66.79 ± 3.92, 71.10 ± 2.89 and 67.91 ± 3.95% (all $p < 0.05$) for K562/ADM cells, respectively, when compared to control group.

Fig. 2 Apoptosis of K562 cells (a) and K562/A02 cells (b) and K562/ADM (c) cells pretreated with different concentration of pantoprazole. 1 Control; 2 50 µg/ml of pantoprazole; 3 100 µg/ml

of pantoprazole; 4 200 µg/ml of pantoprazole; 5 50 µg/ml of pantoprazole + 1 µg/ml of amycin

Fig. 3 RT-PCR analysis of the expression of p-PI3K, p-Akt and p-mTOR mRNAs in K562/A02 cells (a) and K562/ADM (b) cells pretreated with different concentration of pantoprazole. *M* marker; *1*

Control; 2 50 µg/ml of pantoprazole; 3 100 µg/ml of pantoprazole; 4 200 µg/ml of pantoprazole

Effects of Pantoprazole on the P-gp/MRP1 Signaling in Drug-Resistant Leukemic Cells

The classical MDR mechanism is proposed to be related with the P-gp/MRP1 signaling while overexpression of P-gp/MRP1 in tumor cells can induce MDR [12]. In order to investigate if pantoprazole could reverse the MDR through P-gp/MRP1 signaling, the present study examined the effects of pantoprazole on the expression of P-gp/MRP1 in K562/A02 and K562/ADM cells in mRNA and protein levels (Figs. 5, 6). The results indicated that pretreatment with 50 µg/mL pantoprazole for 24 h significantly inhibited the expression of P-gp and MRP1 in both mRNA and protein levels ($p < 0.05$), when compared to control group. The inhibitory effect was dose-dependent. When the concentration of pantoprazole increased to 200 µg/mL, the inhibitory effect was the most significant. When compared to control group, 200 µg/mL pantoprazole significantly inhibited the expression of P-gp and MRP1 mRNA by 62.20 ± 3.73 and $48.81 \pm 2.59\%$ ($p < 0.05$) in K562/A02 cells, and 63.26 ± 4.03 and $55.39 \pm 3.28\%$ ($p < 0.05$) in K562/ADM cells. The protein expression of P-gp and MRP1 was significantly

decreased by 200 µg/mL pantoprazole to 60.01 ± 3.22 and $66.17 \pm 3.76\%$ ($p < 0.05$) for K562/A02 cells, and 73.17 ± 4.33 and $54.75 \pm 3.35\%$ ($p < 0.05$) for K562/ADM cells (Figs. 5, 6), when compared to control group.

Discussion

MDR is an important factor resulting in chemotherapy failure and recurrence, and therefore how to reverse the chemotherapy resistance has become one hotspot in current research of tumors. The extracellular pH value of tumor cells is acidulous, which is related with the growth, invasion and chemotherapy resistance of tumor cells [7]. Acidulous environment is favorable for tumor [9, 10] to develop MDR while intervening the pH gradient can reverse the MDR and prevent the growth of tumor. PPIs are alkaline drugs and can be activated in acidic environment to reverse the MDR of tumors [8]. Previous study indicated that pretreatment with PPI significantly enhanced the sensitivity of tumors [13]. Consistently, the present study indicated that PPI pantoprazole arrested the MDR leukemic K562/A02 and K562/ADM cells in S phase and

Fig. 4 Western blot analysis of the expression of p-PI3K, p-Akt and p-mTOR proteins in K562/A02 cells (a) and K562/ADM (b) cells pretreated with different concentration of pantoprazole. 1 Control; 2 50 μ g/ml of pantoprazole; 3 100 μ g/ml of pantoprazole; 4 200 μ g/ml of pantoprazole

Fig. 5 RT-PCR analysis of the expression of P-gp and MRP1 mRNAs in K562/A02 cells (a) and K562/ADM (b) cells pretreated with different concentration of pantoprazole. M marker; 1 Control; 2 50 μ g/ml of pantoprazole; 3 100 μ g/ml of pantoprazole; 4 200 μ g/ml of pantoprazole

Fig. 6 Western blot analysis of the expression of P-gp and MRP1 proteins in K562/A02 cells (a) and K562/ADM (b) cells pretreated with different concentration of pantoprazole. 1 Control; 2 50 µg/ml of pantoprazole; 3 100 µg/ml of pantoprazole; 4 200 µg/ml of pantoprazole

induced apoptosis of K562/A02 and K562/ADM cells in dose-dependent manner, suggesting that pantoprazole may increase the sensitivity of tumor cells to chemotherapy and therefore reverse MDR.

The classical mechanism of MDR is related with MDR1 gene and its production P-gp [14–16]. As a glycoprotein with 1280 amino acids, P-gp can bind hydrophobic anti-tumor drugs through the hydrophobic sites and pump drugs out of cells against the concentration gradient with energy provided by ATP to decrease the intracellular drug concentration and result in resistance. Thus, P-gp can decrease intracellular drug concentration and mediate resistance in chemotherapeutic drugs including adriamycin, mitomycin, vincristine, etc. In addition, P-gp can specifically play anti-apoptotic effect through inhibiting the cysteine aspartase-dependent apoptosis. The prognosis for patients with high expression of P-gp is poor, which can be used as an index for evaluation of prognosis [17]. MRP1 is also related with the mechanism of resistance in chemotherapy [18]. As a member of the ABC transport protein family, MRP1 is expressed at low level in normal tissues but at high level in tumor tissues. Previous study indicated that MRP1 is highly expressed in hematologic tumors and is related with MDR, tumor deterioration, chemotherapy prognosis and whole survival rate [19]. The present study indicated that pantoprazole inhibited the expression of P-gp and MRP1 in MDR leukemic cells, suggesting that PPI may induce apoptosis through inhibiting the expression of P-gp and MRP1 in leukemic cells.

PI3K/Akt/mTOR signaling is one of the important pathways for cellular survival. Recent study indicated that PI3K/Akt/mTOR signaling is related with MDR [20] and is over-activated in hematologic tumors [21]. In consistence,

O’Gorman et al. [22] found that inhibition of PI3K/Akt/mTOR signaling pathway reversed the MDR in HL-60 cells. Jazirehi et al. [23] found that Akt activation was related with the chemotherapeutic resistance in non-Hodgkin’s lymphoma B-cells while inhibition of Akt signaling enhanced the taxinol-induced apoptosis and reversed MDR. These studies suggest that PI3K/Akt/mTOR pathway is involved in the MDR of leukemia. Furthermore, Ma et al. [24] found that activation of PI3K/Akt signaling pathway to induced up-regulation of P-gp and MRP1 produced MDR in leukemic K562 cells, while PI3K/Akt inhibitor LY294002 reversed the MDR. Huang et al. [25] found that the PI3K/Akt signaling was over-activated in chronic granulocytic leukemia while decrease of the signaling pathway reversed the MDR of tumor cells. These studies indicated that P-gp/MRP1 is involved in the modulation of MDR by PI3K/Akt/mTOR. The present study indicated that pantoprazole inhibited the expression of P-gp, MRP1, and PI3K/Akt/mTOR signaling in MDR leukemic cells, further suggesting that pantoprazole may reverse MDR through P-gp/MRP1 and PI3K/Akt/mTOR pathway.

In summary, the present study indicated that pantoprazole inhibited the intracellular PI3K/Akt/mTOR signaling and the expression of P-gp and MRP1 to induce apoptosis of MDR leukemic cells, which may enhance the sensitivity of tumor cells to chemotherapy and reverse the MDR of tumor. These results suggest that PPIs (pantoprazole) can be a hopeful new adjunct for chemotherapy of leukemia.

Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent This article does not contain any studies with human participants.

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