Down-regulation of the P-glycoprotein relevant for multidrug resistance by intracellular acidification through the crosstalk of MAPK signaling pathways

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Abstract

In our previous study, we have found that the tumor multidrug resistance mediated by P-glycoprotein could be reversed by sustained intracellular acidification through down-regulating the multidrug resistance gene 1 mRNA and P-glycoprotein expression. However, the molecular events linking the intracellular acidification and the regulation of P-glycoprotein remain unclear. In the present study, the molecular pathways involved in the regulation of P-glycoprotein expression by the intracellular acidification were investigated. We found that the P-glycoprotein expression was down-regulated by the intracellular acidification through inhibition of p38 mitogen-activated protein kinase (MAPK) and the activation of c-Jun N-terminal kinase (JNK) in the resistant K562/DOX cells. In the sensitive K562 and HL60 cell lines, the changes of the p38 MAPK expression after the acidification are not as obvious as that of K562/DOX cells, but the activation of extracellular signal-regulated kinase (ERK) is also observed, which indicates that the down-regulation of p38 MAPK by the intracellular acidification might be the resistant cell line specific. Blockade of ERK and JNK signaling by the inhibitors or RNA interference increased p38MAPK activities suggesting that cross-talk within MAPKs is also important for this response. Our study provides the first direct evidence that the reversal of P-glycoprotein-mediated multidrug resistance by intracellular acidification is mediated by the crosstalk of MAPK signaling pathways.

1. Introduction

Multidrug resistance, by which cells resist many structurally and functionally unrelated drugs, is a major problem in chemotherapy of cancer. The MDR phenotype is most often due to over-expression of drug efflux pumps in the plasma membrane of cancer cells (Baker and El-Osta, 2004). P-gp, a 170 kDa transmembrane glycoprotein encoded by the MDR1 gene, is the best characterized drug efflux pump, which pumps out a variety of anticancer agents from the cell in energy-dependent manners (Bodor et al., 2005). A wide range of anticancer drugs has been described to be substrates for P-glycoprotein. Over-expression of P-gp has been shown to confer MDR in cultured cells and has also been implicated in the clinical MDR (Anuchapreeda et al., 2006; Arvelo et al., 1995). In addition, P-gp over-expression correlates with poor prognosis for a number of human cancers (Bellarosa et al., 2009; Cao et al., 2007; Chan et al., 2004). It is believed that inhibition of P-gp function or its expression may reverse P-gp-mediated MDR phenotype and improve the effectiveness of chemotherapy. The mechanisms underlying MDR reversal have been increasingly studied over the past few years, since it seems likely that they will be responsible for varying responses to drug therapy in the population. However,
the mechanisms have not been fully clarified and need further study. (Eichelbaum et al., 2004; Gutmann et al., 2008).

The malignant cells show a strong tendency towards an alkaline deviation of the entire homeostasis when they are resistant to therapeutic intervention, and they can live and multiply at pH levels from 7.46 to 7.6 and even higher. Recent studies demonstrated that cancer microenvironment, such as intra- and extra-cellular pH, can have an impact on the Pgp expression and activity and by this may affect the cytotoxic efficacy of drugs which are substrates of this transporter (Pereke et al., 2002). Even though these studies gave some hints on the mechanisms by which the intracellular pH may affect the Pgp activity, the signal cascade is still unclear and needs further elucidation. The Na\(^+/\)H\(^-\) exchanger 1 (NHE1) is a ubiquitously expressed integral membrane protein which can regulate intracellular pH (pHi) by removing a proton in exchange for an extracellular sodium ion. In our previous study, we experimentally induced sustained intracellular acidification in K562 and K562/DOX cell lines with selective NHE1 inhibitor cariporide and the "high K\(^+\)" buffer method to decrease the intracellular pH. The data indicated that the tumor MDR mediated by P-gp could be reversed by sustained intracellular acidification through down-regulating the P-gp expression and activity, and there is a regulative link between the pHi and P-gp in K562/DOX cells (Lu et al., 2008).

It has been reported that MAPK signaling pathways were sensitive to the change of the extra- or intra-cellular pH in several cell types, although the mode of the regulation appears to be highly variable and cell type-dependent. The MAPK pathways are activated by various kinds of stimuli, including those provided by growth factors, different types of stress or inflammatory cytokines, which can result in cell proliferation, differentiation, development, inflammation or apoptosis. The principal components of the MAPK comprise three subfamilies: a growth factor-responsive pathway, including ERK1/2, and two stress-responsive pathways, including JNK/SAPK and p38 MAPK. The activities of the MAPKs are regulated by two events, phosphorylation and dephosphorylation (Chang and Karin, 2001). It was reported that inhibition of the p38-MAPK pathway down-regulated P-gp expression level and diminished the cellular multidrug resistance (Sauvant et al., 2008).

It has recently been shown that MAPKs pathways, although alternative possible mechanisms may exist, if so, the underlying signal pathway might be exploited for overcoming P-gp-mediated MDR.

Previously we have demonstrated that the MDR mediated by P-gp could be reversed by intracellular acidification through down-regulating the expression of MDR1 mRNA and P-gp, and decreasing the activity of P-gp in K562/DOX cells. In the present study, we employed three kinds of tumor cell lines: K562/DOX, K562 and HL-60 cells to investigate the role of MAPK signaling pathways, as well as the potential transcriptional factors, in the reversal of MDR by intracellular acidification, and in order to find out a better therapeutic outcome.

2. Materials and methods

2.1. Materials and chemicals

RPMI 1640 media were purchased from Gibco-BRL Life Technologies, Inc. (Burlington, ON, Canada). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). Human monoclonal antibody against P-gp, rabbit polyclonal antibody against GAPDH and horseradish peroxidase-conjugated anti-rabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence reagent plus (ECL) reagents were from Santa Cruz Biotechnology (Santa Cruz, CA). 2’,7’-Bis (2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxyethyl (BCECF-AM), doxorubicin cycloheximide (CHX), 4’,4’-disothiocyanostilbene-2,2’-disulfonic acid (DIDS) and cariporide were purchased from Sigma (Shanghai, China).

2.2. Cell culture

The MDR cell line K562/DOX was derived from K562 cells by selection in increased concentrations of doxorubicin. The derivative cell line was shown to have MDR phenotype by its elevated expression of P-gp, wide cross-resistance and defect in intracellular drug accumulations. K562 and HL-60 cells were cultured in RPMI 1640 with 10% (v/v) FBS, penicillin (50 units/mL), streptomycin (50 μg/mL) and l-glutamine (2 mmol/L) at 37 °C in humidified air with 5% CO\(_2\). Medium for K562/DOX cells were further supplemented with doxorubicin 2 μmol/L. Prior to use in experiments, K562/DOX cells were cultured in drug-free medium for two weeks.

2.3. Small interfering RNA transfection

For small interfering RNA-mediated gene silencing, small nucleotide RNAs were chemically synthesized by Qiagen. Cells were transfected with small interfering RNA using lipo2000 transfection reagent in a 6-well plate for 12 h. Gene silencing was assayed 48 h post-transfection by Western blot analysis of the total cell lysates. RNA rescue experiments were performed by transiently cotransfecting cells with small interfering RNA (siRNA) and MAPK overexpression vectors (flag-tagged vector (pCMV)). Nonsilencing siRNA and flag-tagged (empty) vector were cotransfected as a control.

2.4. Measurement of pHi

The pHi of cells was assessed by flow cytometry using the pH-sensitive fluorescent probe BCECF-AM (Ozkam and Mutharasas, 2002). We did not observe any reduction of intracellular BCECF fluorescence intensities in the cell lines, nor was loss of BCECF during the time course of the experiment in the two cell lines (data not shown). Cell suspensions in serum-free RPMI 1640 were washed and labeled with BCECF-AM. The labeled cells were analyzed with an excitation wavelength of 488 nm, and the ratio of the fluorescence at 530 nm and 640 nm was plotted vs. pH. In order
to obtain the calibration curve, a linear regression within the pH range 6.2–7.4 was obtained.

2.5. Induction of intracellular acidification

Cell suspensions in serum-free RPMI 1640 medium were washed and resuspended (1 × 10⁶ cells/mL) with cariporide 3 mg/L in serum-free RPMI 1640, or inhibitor of bicarbonate transport 100 μmol/L, or the “high K⁺” buffer containing HCl/K⁺ ionophore nigercin 5 μmol/L to induce the pH to 7.2, 7.0 and 6.8 for 1 h and 3 h as described previously (Lu et al., 2008).

2.6. Real-time RT-PCR

Total RNAs were isolated from cells treated with cariporide 3 mg/L or “high K⁺” buffer using TRIzol reagent (Invitrogen, San Diego, CA). Real-time PCR was done with reverse transcription product in a MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA) by using SYBR Green PCR Super mix (Bio-Rad). Human MDR1 primers were 5′-TGC TCA GAC AGG ATG TGA GTTG-3′ (forward) and 5′-TTA CAG CAA GCCCTG AACCTAT-3′ (reverse; 120 bp); Human GAPDH primers used as an internal control were 5′-GAA GGT GAA GGT CCG AGT-3′ (forward) and 5′-GGA GAT GGT GAT GGG ATT TC-3′ (reverse; 226 bp).

2.7. Western blotting

Proteins isolated from cell lines treated with cariporide 3 mg/L or the “high K⁺” buffer were resolved by 6% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked for 1 h and then incubated first with primary antibodies for 2 h and then horseradish peroxidase-conjugated secondary antibodies 1 h, respectively. Specific proteins were visualized with enhanced chemiluminescence detection reagent and determined by densitometric analysis with a Lynx video densitometer (Biochemical Vision).

2.8. Colony assay

K562/DOX, K562 and HL-60 cells were incubated for 24 h in RPMI 1640 medium supplemented with 20% FBS. 10 μM Cariporide was added at the initiation of cultures on exposure to 10 μM doxorubicin. After extensive washing, 1 × 10⁵ cells were plated in 0.8% methylcellulose in RPMI 1640 with 20% FBS and 50 ng/mL GM-CSF. Duplicate cultures were incubated in 35-mm petri dishes for 7 days at 37 °C in a humidified atmosphere of 5% CO₂ in air. Colonies were microscopically evaluated on day 7. A blast colony was defined as a cluster of 40 or more cells.

2.9. Caspase-3 activity assay

Activity of caspase-3 was detected using a colorimetric caspase-3 cellular activity assay kit (Beyotime, Haimen, Jiangsu, PRC). Preparation of cell extracts and analysis of caspase-3 activity was performed according to the manufacturer’s protocol. The amount of hydrolyzed substrate was measured at an optical density of 405 nm. Relative caspase activity was calculated as a ratio of emission of treated cells to untreated cells.

2.10. Data analysis

Statistical analyses were made using GraphPad Prism (San Diego, USA). Significance was assumed for P values less than 0.05.

3. Results

3.1. Down-regulation of P-gp by intracellular acidification is independent of its protein stability

The decreased MDR1 mRNA and P-gp expressions have been observed in our previous study after sustained intracellular acidification in K562 and K562/DOX cells (Lu et al., 2008). In K562/DOX cells, both Cariporide and high K⁺ buffer down-regulated MDR1
mRNA and protein levels in a time- and pH$_i$-dependent manner. After 48 h recovery in normal culture condition, the MDR1 mRNA and protein levels returned to the similar value of the primary levels. Moreover, we also investigate the influence of bicarbonate transporter inhibitor in P-gp regulation. According to our results shown in Fig. 1A and B, in K562/DOX cells, bicarbonate transporter inhibitor DIDS (100 μmol/L) down-regulated MDR1 mRNA expression ($P<0.05$) in a time dependent manner and protein levels in day 2 ($P<0.05$). These data indicated that the sustained intracellular acidification decreased P-gp at the mRNA and protein levels, and this process was reversible. However, the mRNA and protein expressions of MDR1 were undetectable in K562 and HL-60 cells (Lu et al., 2008).

Protein turnover assay was used to determine whether decreased P-gp level after sustained intracellular acidification was regulated by protein stability. As shown in Fig. 1C and D, 10 μM Cycloheximide (CHX) was used to inhibit protein synthesis and was present throughout the Cariporide incubation. In K562/DOX cells cultured with 10 μM Cariporide for 24 h, half-life of P-gp was 20.1 h, which was slightly reduced compared with untreated K562/DOX cells ($P>0.05$). According to these results, protein stability may not significantly influence the P-gp level regulated by intracellular acidification in K562/DOX cells.

### 3.2. Decreased pH$_i$ induces the inhibition of p38 MAPK, but the activation of ERK1/2 and JNK signaling pathways in K562/DOX cells

It has been reported that the regulation of pH$_i$ can induce the activation of several mitogen-activated protein kinase (MAPK) pathways in a variety of cancer cells. In this study, we determined the effect of intracellular acidification on ERK1/2, p38 MAPK and JNK pathways in K562, HL-60 and K562/DOX cells. As shown in Fig. 2B, high pH$_i$ buffer decreased the phosphorylation of p38 MAPK in K562/DOX cells in a pH$_i$ and time-dependent fashion, and phosphorylation of p38 MAPK was maximally inhibited at 60 min after regulating pH$_i$ to 6.8 ($P<0.05$). In contrast, there was no obvious change of p38 activity in K562 and HL-60 cells (Fig. 2A and C, $P>0.05$, Supplementary Fig. A1). After stimulation with high K$^+$ buffer, the phosphorylation of
ERK1/2 increased in the HL-60 and K562/DOX while decreased in K562 cells (Fig. 2, Supplementary Fig. A1). Moreover, the phosphorylation of JNK increased in K562 and K562/DOX cells without obvious change in HL-60 cells (Fig. 2, Supplementary Fig. A1).

Then we considered the phosphorylation of these three kinases after intracellular acidification using cariporide, a specific NHE1 inhibitor, which has been proved to regulate both K562 and K562/DOX cells to pH 7.0 according to our previous results. As shown in Fig. 3A, phosphorylation of p38 in K562/DOX cells decreased in a time-dependent manner, and remained decreased at 48 h (P < 0.05). However, in K562 and HL-60 cells, there was no significant change in p38 phosphorylation (Fig. 3B and C, P > 0.05). This result clearly showed that intracellular acidification was capable of decreasing p38 MAPK activity in multidrug resistant K562/DOX cells. However, no obvious difference was observed in ERK1/2 and JNK activation among the indicated cell lines induced by cariporide (data not shown).

### 3.3. Down-regulation of Pgp expression by intracellular acidification was mediated by p38 MAPK and JNK pathway

Based on the above data, we hypothesized that some MAPK pathway(s) would be implicated in the pH$_1$-induced P-gp down-regulation in K562/DOX cells. First, p38 inhibitor SB203580 (20 μM) and p38 siRNA were used to examine whether p38 MAPK pathway would be involved in the P-gp down-regulation after intracellular acidification in K562/DOX cells. It was confirmed that both specific inhibitor and RNAi were capable of sufficiently inhibiting p38 activation in K562/DOX cells and did not influence the viability of the cells (data not shown). As shown in Figs. 4 and 5, pre-incubation with SB203580 or RNAi of p38 kinase co-inhibited MDR1 mRNA (Fig. 4A and D, P < 0.05) and protein level (Fig. 5A and D, P < 0.05) in a pH$_1$-dependent manner after treatment with cariporide.

JNK pathway has been reported to play a critical role in the regulation of p-glycoprotein (Liu et al., 2007; Zhou et al., 2006).
To investigate the role of JNK in this process, specific JNK inhibitor SP600125 (20 μM) and RNA interference was used. The cells pretreated with SP600125 or transfected with siRNA sequence showed higher levels of both MDR1 mRNA (Fig. 4B, \( P < 0.05 \); Fig. 4E, \( P < 0.05 \)) and P-gp (Fig. 5B and E; \( P < 0.05 \)) compared with the cells treated with high K⁺ buffer or cariporide alone. However inhibition of JNK could not thoroughly reverse the effect of cariporide. All these results showed that JNK pathway at least played a part role in the down-regulation of P-gp after pH decreased.

We then investigated the effect of ERK1/2 kinase on the regulation of P-gp expression after intracellular acidification using both ERK1/2 inhibitor PD98059 and RNA interference. As shown in Fig. 4C and F, after inhibition of ERK1/2 pathway, MDR1 mRNA expression in K562/DOX cells decreased compared to the control cells, which was accordant to some other research groups (Katayama, 2007). But the same down-regulation effect could not be seen in P-gp protein expression (Fig. 5C and F, \( P > 0.05 \)), which indicates that some other pathway(s) may be involved in and crosstalk with ERK pathway in regulating P-gp level induced by intracellular acidification.

In order to identify the efficiency of RNA interference, we also perform experiments to rescue MAPK knockdown. In the present experiments, K562/DOX cells were transiently co-transfected with siRNA sequence and flag-tagged overexpression vectors targeting MAPK mRNA. As a result, P-gp mRNA and protein expressions were fully rescued in K562/DOX cells gene-silenced for endogenous MAP kinase, and re-expressing MAPK mRNA (Figs. 4 and 5).

3.4. The crosstalk between ERK/JNK and P38 MAPK induced by intracellular acidification

We have already shown that decreased pH, induced the activation of ERK1/2, JNK and the inactivation of P38 kinase signaling pathways, and both JNK and p38 MAPK are definitely required for intracellular acidification induced P-gp down-regulation. We therefore examined the crosstalk of these three MAPK pathways
by western blot analysis. Figs. 6A and 7A showed that suppression of JNK and ERK1/2 activities significantly enhanced p38 phosphorylation ($P<0.05$). The data indicates that JNK and ERK1/2 activation suppresses p38 activity in intracellular acidification induced P-gp down-regulation. Moreover, activation of JNK and ERK1/2 could also be suppressed by p38 MAPK (Figs. 6B and C and 7B and C) ($P<0.05$), indicating the crosstalk among the three kinases.

3.5. Intracellular acidification induced overexpression of AP-1 components c-jun and c-fos in K562/DOX cells

The promoter of the mdr1 gene was reported previously to contain a negative binding site of the heterodimeric transcription factor activator protein (AP-1; notably the c-Jun/c-Fos dimer) (Mealey and Bentjen, 2003; Zhou et al., 2006). To test whether intracellular acidification down-regulates P-glycoprotein through enhanced AP-1 binding to the mdr1 promoter, we examined protein levels of the AP-1 component proteins c-jun and c-fos in K562/DOX cells. Using western blot analysis, we observed that increased levels of both c-jun ($P<0.05$) and c-fos ($P<0.05$) transcripts in resistance K562/DOX cells after treatment with high K+ buffer (Fig. 8).

3.6. Intracellular acidification decreased colony-forming units in K562/DOX cells

According to our previous result, intracellular acidification induced down-regulation of P-glycoprotein could enhance the intracellular drug accumulation in the MDR K562/DOX cells. So in this study, Colony assay was used to assess the effect of intracellular acidification on HL-60, K562 and K562/DOX cells. Cells were treated with or without 10 μM Cariporide on exposure to 10 μM doxorubicin. Significant decreases of colony-forming units (CFUs) were observed in K562/DOX cells (Fig. 9A and B, 68.67%, $P=0.0101$). Our studies demonstrate that decreasing P-gp expression after intracellular acidification significantly reduced CFUs of K562/DOX cells dependent of cells’ responses to doxorubicin. However, treatment with or without 10 μM Cariporide on exposure to 10 μM
doxorubicin didn’t lead to significant changes of caspase-3 activity (Fig. 9C), indicating that the cell viability was not affected.

4. Discussion

The role of ATP-binding cassette (ABC)-superfamily multidrug efflux pumps in resistance to anticancer drugs has been known for over 30 years (Gottesman and Ling, 2006). P-glycoprotein (ABCB1) is one of the most important and well characterized members in this family. High expression of P-gp has been observed prior to chemotherapy treatment in many different tumor types, including kidney, colon, liver, breast and ovarian cancers. In hematologic malignancies, such as leukemias, lymphomas and multiple myelomas, the low levels of P-gp expression observed initially are markedly increased after chemotherapy treatment and relapse. According to our previous result that tumor MDR mediated by P-gp could be reversed by sustained intracellular acidification, we investigated the potential mechanisms involved. Understanding the mechanisms of intracellular acidification mediated MDR reversal and the relevant signaling pathway regulation may lead to novel and informed therapeutic strategies that can target cancers including leukemias as well as solid tumors.

Regulation of pH starts with changes in the expression or activity of several plasma membrane molecules such as pumps and transporters that facilitate H+ efflux to maintain the alkaline pHi and the acidic pHt in tumor cells (Huber et al., 2010). In order to clarify the internal correlation between pHi and P-gp in MDR leukemia cells, we experimentally induced sustained intracellular acidification with “high K+” buffer method to induce the short-time acidification (3 min to one hour) and with selective NHE1 inhibitor Cariporide to investigate the long-term acidification influence, which will more objectively reflect the physiologic conditions. Furthermore, Bicarbonate transport has been previously shown to participate in tumor development induced by various stress factors. In the present study, besides NHE1 inhibitor and “high K+” buffer, we also investigate the influence of bicarbonate transporter inhibitor in P-gp regulation. According to our results showed in Fig. 1, bicarbonate transporter inhibitor DIDS decreased MDR1 mRNA expression (P<0.05) in a time dependent manner and protein levels in day 2 (P<0.05). Although the effect of DIDS on pgp level was not significant in day 1, this result was in some extent accord agreed with the “high K+” buffer method and Cariporide.

Many previous studies have reported several mechanisms that reverse P-gp mediated drug resistance via some MAPK pathways (Katayama, 2007; Liu et al., 2007; Zhou et al., 2006). The best characterized Map kinase signaling cascade in acute and chronic human leukemias is the Raf/Mek/Erk signaling cascade. In our study, intracellular acidification by both high K+ buffer and cariporide was able to activate Erk1/2 protein in a time and pHi-dependent manner. But constitutive activation of this kinase was not observed which was consist with other reports (Platanias, 2003). Through suppression of ERK pathway signaling, intracellular acidification induced down-regulation of P-gp level was reversed. On the other hand, the regulatory effect of the JNK and p38 Map kinase pathways vary, depending on the specific cellular type and possibly the distinct isoforms involved. Importantly, the JNK and p38 pathways also mediate signals responsible for sensitivity or resistance to the effects of various pharmacologic and biologic agents currently

Fig. 6. Crosstalk of MAPKs with inhibitors. Effect of specific MAPKs inhibitors on p38 MAPK (A), ERK1/2 (B) and JNK (C) activities in K562/DOX cells was shown. Left panel shows representative western blots for MAPK phosphorylation. Right panel is the quantification of MAPK phosphorylation. The values are mean ± SD of 3 independent experiments. *p < 0.05, significantly different from the cells treated only with high K+ buffer.
Fig. 7. Crosstalk of MAPKs with RNA interference. Effect of MAPKs RNA interferences on p38 MAPK (A), ERK1/2 (B) and JNK (C) activities in K562/DOX cells was shown. Left panel shows representative western blots for MAPK phosphorylation. Right panel is the quantification of MAPK phosphorylation. The values are mean ± SD of 3 independent experiments. *p < 0.05, significantly different from the cells treated only with high K+ buffer.

Fig. 8. Analysis of c-Jun and c-Fos protein levels after intracellular acidification. The cells were treated with high K+ buffer for 3 h. The cell lysates were prepared and analyzed for c-Fos (A) and c-Jun (B) protein levels by Western blotting. The lower panel is the quantification of protein expression. The values are mean ± SD of 3 independent experiments. *p < 0.05, significantly different from the cells untreated.
in use for the treatment of various hematologic malignancies. As
shown in our present study, p38 and JNK pathways were impor-
tant in the control of sensitivity or resistance signals that regulated
resistance status of K562/DOX cells when the intracellular pH
changed.

Some other groups have also reported the effect of acidosis on
Pgp activity and expression in cancer cells via p38 and Erk1/2 MAPK
pathways (Lotz et al., 2007; Riemann et al., 2011; Sauvant et al.,
2008; Thews et al., 2011). Sauvant et al. showed that extracellular
acidity (pH 6.6) activated p38 and ERK1/2 and thereby induced
daunorubicin resistance via a pronounced activation of pgp in vitro.
Intracellular acidification also induced daunorubicin resistance via
activation of pgp, which was mediated by activation of p38 alone
(Sauvant et al., 2008). Lotz et al. (2007) reported that extracel-
lar acidosis can increase the pgp-mediated drug efflux. Above
studies all focus on the solid tumor such as prostate cancer and
colon cancer. But in hematological malignancies, such as leukemia,
lymphomas and multiple myelomas, low levels of Pgp expression
observed initially are markedly increased after chemotherapy
treatment and relapse. All these data indicate that Pgp overex-
pression is an important clinical mechanism in the diversity of
resistance mechanisms in leukemia.

Biochemical pathways always operate in conjunction with each
other and their interplay decides the final outcome. The existence
of cross-talk among MAPK pathways have been proved in several
studies (Shen et al., 2003). Inactivation of p38 MAPK, which was
essential in P-gp down-regulation and reversal of anticancer drug
resistance, could be induced by ERK1/2 activation. So according
to above results, ERK1/2 may crosstalk with p38 MAPK pathway
in this process. Figs. 6 and 7 provide evidence suggesting that
ERK pathway stimulation modulates p38 signaling. A direct evi-
dence about the negative feedback effect of ERK on p38 is that
p38 signaling increased after inhibition of ERK pathway signaling
(Fig. 6A and 7A). The existence of this link is substantiated by the
finding that inhibition of p38 signaling increases ERK1/2 phosphor-
ylation (Fig. 6B and 7B), suggesting that the site of crosstalk control
occurs up-stream of ERK1/2 in the ERK cascade.

Depending on the stimuli and the strength and duration of
JNK activation, the cellular response has diverse outcomes, which
ranges from the increased survival to reversal of drug resistance
and induction of apoptosis (Bode and Dong, 2007; Nateri et al.,
2005; Weston and Davis, 2002). Cross-talk between JNK and p38
MAPK has also been widely demonstrated (Stepniak et al., 2006).
Increased activation of JNK on p38 activation has also been
observed in mouse models (Perdiguero et al., 2007). Here, along
with above reports, we also found that JNK suppressed p38 activity
after intracellular acidification, suggesting a potential role of JNK
in reversal of drug resistance in K562/DOX cells.

Despite many years of research, the molecular mechanisms that
control MDR1 expression remain unclear. Some studies have been
presented previously for the regulation of P-glycoprotein/mdr1.
For example, cross-coupled nuclear factor-nb/p65 and c-Fos tran-
scription factors have been reported to negatively regulate the
promoter activity of mdr1. On the other hand, the heat-shock tran-
scription factors HSF1, the Y-box binding protein YB1, and the
Sp1 transcription factor have been shown to positively regulate P-
glycoprotein/mdr1 expression (Zhou et al., 2006). According to our
present study, we found the involvement of AP-1 during intracel-
lar acidification. It has been proven that the promoter region
of MDR1 contains a binding site for AP-1. We also found in our present
study that K562/DOX cell lines constitutively overexpressed the
component AP-1 proteins c-jun and c-fos after intracellular acidifi-
cation, and intracellular acidification treatment of K562/DOX cells
was shown to activate c-Jun N-terminal kinase, suggesting a possi-
able role for AP-1 in the signal pathway leading to MDR. In the future
study, whether intracellular acidification could influence the AP-1
binding activity of the mdr1 gene in the MDR cells, using EMSA
assay, should be investigated.

5. Conclusion

In conclusion, according to above results, intracellular acidifi-
cation was capable of the reversal of multidrug resistance in
leukemia cells via crosstalk of MAPKs pathways, based on already
described interactions (Supplementary Fig. A2). From the perspec-
tive of clinical application, clinical trials to evaluate the combined
use of MAPK inhibitors with intracellular acidification treatment for
hematologic neoplasia would be appropriate, and its importance in
regulating multidrug resistance has made it a potential target for designing new therapeutics against cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.06.016.

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