Department of Hematology, West China Hospital, Sichuan University, Chengdu, China

The MEK1/2 inhibitor U0126 reverses imatinib resistance through down-regulating activation of Lyn/ERK signaling pathway in imatinib –resistant K562R leukemia cells

R. Shi*, J. Lin*, Y. Guo, Y.P. Gong

Received October 19, 2013, accepted November 22, 2013

Yuping Gong, Ph.D, Department of Hematology, West China Hospital, Sichuan University 37# Guo Xue Xiang, Chengdu, Sichuan Province, China 610041
gongyuping@hotmail.com
* *These authors equally contributed to this work.*

Pharmazie 69: 346–352 (2014) *doi: 10.1691/ph.2014.3881*

Chronic myelogenous leukemia (CML) is triggered by the constitutively activated BCR-ABL oncoprotein and multiple downstream signaling pathways, including the Raf/MEK/ERK, Akt/mTOR, SRC, and STAT5 pathways. The BCR-ABL tyrosine kinase inhibitor imatinib is the standard treatment for CML. However, the development of imatinib resistance has become a new challenge for CML treatment. Here, we investigated the expression levels of the signaling pathways to explore the cause of imatinib resistance and seek new reversing drugs. Our results showed that abnormal activation of the BCR-ABL-independent Lyn/ERK signaling pathway was involved in imatinib-resistance of K562R cells. Furthermore, p-Lyn and p-ERK were up-regulated after treatment with imatinib alone. However, U0126, a MEK1/2 inhibitor, could counteract the up-regulation induced by imatinib, and the combination of imatinib and U0126 could overcome the resistance to imatinib in K562R cells. In conclusion, our studies suggest that the combination of imatinib and an inhibitor of the ERK signaling pathway may be effective in imatinib-resistant CML patients.

1. Introduction

Imatinib (IM), which specifically targets the BCR-ABL oncoprotein, is the first targeted molecular drug applied in clinical practice and has achieved significant success in the treatment of CML. Imatinib competitively binds to the ATP docking site of tyrosine kinase proteins. Subsequently, this compound inhibits the auto-phosphorylation of tyrosine kinases and the phosphorylation of their substrates, and it blocks abnormal signal transduction, thereby exerting anti-tumor effects (Quintas-Cardama et al. 2009). Imatinib is currently used as a frontline therapy for CML. However, it is reported that approximately 15–20% of patients exhibit primary resistance, and 7–15% of patients develop secondary resistance (Bixby et al. 2009). Thus, the mechanisms of imatinib-resistance have been extensively investigated.

The most common clinical imatinib-resistance mechanism is mutation and amplification of the *bcr-abl* gene (Branford et al. 2002). In addition, there are some *bcr-abl* independent mechanisms, including the overexpression of P-glycoprotein (P-gp) (Mahon et al. 2003), abnormal activation of relevant survival signaling pathways ((Mahon et al. 2003; Coppo et al. 2006; Aceves-Luquero et al. 2009) and abnormal epigenetic modification (Fiskus et al. 2006; Lee et al. 2007; Wu et al. 2008). The SRC family of tyrosine kinases (SFKs) is a group of structurally similar non-receptor tyrosine kinases, including the members Lyn, Fyn, Lck, Hch, Fgr, Blk, Yrk, Yes, and c-src. Among these kinases, Lyn is closely associated with the BCR-ABL protein. This protein can enhance the activity of BCR-ABL by SH2-containing tyrosine phosphotase-1 (SHP-1) and is also directly or indirectly activated by BCR-ABL (Warmuth et al. 1997). Activated Lyn kinase can directly phosphorylate and activate STAT5, subsequently activating BCL-XL, CyclinD1, and c-myc and inducing proliferation and the anti-apoptosis of hematopoietic stem cells independent of growth factors (Kieslinger et al. 2000). Lyn kinase can activate the PI3K/Akt signaling pathway, which can phosphorylate downstream cell growth proteins, such as GSK-3β, MDM-2, ASK1, Bim and BAD. Lyn kinase can also phosphorylate the TSC1/TSC2 complex and activate the mTOR signaling pathway, which plays an important role in the proliferation, anti-apoptosis, and angiogenesis of tumor cells (Skorski et al. 1997; Warmuth et al. 2003). Importantly, Lyn kinase can phosphorylate Tyr177 on BCR-ABL and allow the Grb-2 adapter protein to bind with BCR-ABL, leading to the activation of the RAS protein and the downstream Ras/Raf/MEK/ERK signaling pathway, which eventually phosphorylates and activates ERK1 and ERK2, and further activates multiple downstream substrates (Warmuth et al. 1997; Wilson et al. 2002). There is a growing body of preclinical and clinical evidence suggesting that abnormal activation of BCR-ABL-independent Lyn is involved in the imatinib-resistant mechanism in CML (Wilson

Abbreviations: CML, Chronic myelogenous leukemia; IM, imatinib; P-gp, P-glycoprotein; EFS, The event-free survival; OS, Overall survival; SFKs, SRC family of tyrosine kinases; SHP-1, SH2-containing tyrosine phosphotase-1; MAPKs, Mitogen activated protein kinases; TKI, Tyrosine kinase inhibitor; HRP, Horseradish peroxidase; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide; FBS, Fetal bovine serum; OD, Optical density; IC_{50}, value is 50% inhibition of cell growth; RF, Relative resistance factor; I, Interaction index; TKD, Tyrosine kinase domain.

et al. 2002; Meyn MA et al. 2006). However, there are few reports regarding the MEK/ERK signaling involved in the resistance to imatinib (Nambu et al. 2010).
Imatinib resistance has become a new challenge for CML, and other investigational therapies should be developed. Perhaps the combination of TKIs with inhibitors that are specific for BCR-ABL-independent targets or downstream BCR-ABL substrates will provide a synergistic effect or even overcome resistance.
K562 cells, a classical representative of CML cell lines, have been widely used in the study of CML. Imatinib resistant K562R cells, established by Mahon in Victor Segalen University, do not have *bcr-abl* gene amplification, kinase domain mutations or P-gp over-expression (Mahon et al. 2000). Our previous study found that p-mTOR was up-regulated in K562R compared with K562 cells, and the mTOR inhibitor rapamycin could not inhibit the up-regulation of the mTOR protein and reverse imatinib resistance (Xing et al. 2011). In this study, we further explored the imatinib resistant mechanism in K562R cells, particularly the BCR-ABL downstream signaling pathways including Akt/mTOR, Raf/MEK/ERK, STAT5 and Lyn, in search of a new strategy for reversing imatinib-resistance.

2. Investigations and results

2.1. The aberrant activation of the BCR-ABL-independent Lyn/ERK pathway is involved in imatinib resistance in K562R cells

To study imatinib resistance in K562R cells, we treated K562R and K562 cells with a series of imatinib concentrations for 72 h, and the growth rates and IC_{50} values as measured by MTT assays are shown in Fig. 1A. The results showed that imatinib had cytotoxic effects on both cell lines, with IC_{50} values for K562R and K562 cells of 1.505 ± 0.459 μM and 0.159 ± 0.032 μM, respectively. K562R cells were more resistant to imatinib compared with K562 cells, and the relative resistance factor (RF) for the K562R: K562 cells was 9.465. All of the results reflect the mean of at least three different experiments ± s.d.
The protein expressions of p-cAbl, p-Lyn, p-STAT5, the ERK pathway (p-MEK, p-ERK) and the PI3K/AKT/mTOR pathway (p-AKT, p-mTOR, p-4EBP1) in K562R and K562 cells were examined by Western Blot analysis (Fig. 1B). The results demonstrated that p-cAbl and p-STAT5 were highly expressed in the two cell lines without significant differences, while p-Lyn, p-MEK, and p-ERK expression was higher in K562R than in K562 cells. In addition, in the mTOR pathway, the p-Akt level was low, but the levels of the downstream proteins p-mTOR and p-4EBP1 were high in both cell lines, particularly in the K562R cells. All of these results indicate that BCR-ABL and STAT5 are not involved in K562R cell drug resistance; however, the levels of p-Lyn, p-MEK and p-ERK were significantly higher in these cells than in K562 cells, which may be the key point for imatinib resistance in K562R cells. Meanwhile, the up-regulation of p-mTOR and p-4EBP1 resulted from an activated ERK pathway but not upstream AKT. Therefore, activation of the Lyn/ERK pathway is likely an important cause of drug resistance in K562R cells.
We further examined the expression levels of signaling pathway in K562R cells after treatment with imatinib. The results showed that imatinib decreased the expression levels of p-Abl and p-MEK but did not suppress the p-Lyn, p-ERK, p-mTOR and p-4EBP1 expression levels, and p-Lyn and p-ERK were up-regulated (Fig. 1C). These results demonstrated that the activation of p-Lyn, p-ERK, p-mTOR and p-4EBP1 in K562R cells was independent of BCR-ABL.

2.2. The MEK1/2 inhibitor U0126 reversed imatinib resistance in K562R cells

We treated K562 and K562R cells with the indicated concentrations of U0126 for 72 h and analyzed the proliferation rate using MTT assays. The results showed that U0126 could inhibit the growth of the two cell lines in a dose-dependent manner, and the IC_{50} values of the K562 and K562R cells were 85.824 ± 4.474 and 34.235 ± 5.658 μM, respectively, indicating that the K562R cells are more sensitive to U0126 compared with the K562 cells ($P < 0.05$) (Fig. 2A).
Furthermore, the cells were incubated with a series of concentrations ranging from 0.001 to 20 μM for imatinib plus 0, 5, 10, 15, and 20 μM U0126 for 72 h, and the proliferation rates were examined by MTT assays. The results showed that U0126 enhanced the anti-proliferative effects of imatinib on K562 and K562R cells in a concentration-dependent manner (Figs. 2B and 2C), and the IC_{50} values for imatinib combined with 5, 10, 15, and 20 μM U0126 were 0.159 ± 0.032, 0.136 ± 0.035, 0.086 ± 0.024, 0.086 ± 0.024 and 0.003 ± 0.002 μM, respectively, in K562 cells. Moreover, in K562R cells, the IC_{50} values for imatinib in combination with 0, 5, 10, and 15 μM U0126 were 0.159 ± 0.032, 0.386 ± 0.051, 0.134 ± 0.051, and 0.024 ± 0.017 μM; however, we could not calculate the IC_{50} value for imatinib when combined with 20 μM U0126 because the cell growth inhibition rate had reached 59.42% even when imatinib was given at the lowest concentration of 0.001 μM, at which imatinib alone had no effect on cell viability.
The interaction index (I) was used to analyze the anti-tumor effects of the imatinib and U0126 combination. The results in Fig. 2D demonstrate that all I values were < 1 when imatinib was combined with U0126 (5, 10, 15, and 20 μM), indicating a synergism between the two anti-leukemia drugs. In K562 cells, imatinib plus 15 μM U0126 displayed the greatest synergic effects, with an I value of 0.23, while in K562R cells, the combination of imatinib and 10 μM U0126 was the best, with an I value of 0.38. The K562R cell line displayed an IC_{50} value of 0.134 ± 0.051 μM, which was lower than for imatinib alone (1.505 ± 0.459 μM) in K562R cells and even lower than that for imatinib alone (0.159 ± 0.032 μM) in the drug-sensitive K562 cells; these results indicated that U0126 completely reversed imatinib resistance in K562R cells.
Flow cytometric detection of apoptosis indicated that 0.2 μM imatinib, 10 μM U0126 and the combination of the two drugs induced apoptosis in K562 and K562R cells after 48 h but not after 24 h (Figs. 3B and 3A). In addition, the apoptosis rate with the combination of two drugs was higher than that with imatinib alone; however, no difference with U0126 alone was observed.

2.3. Mechanism of the U0126-mediated reversal of imatinib resistance in K562R cells

RT-PCR was used to detect the level of *lyn* and *erk1,2* gene expression in K562 and K562R cells after treatment with 0.2 μM imatinib, 10 μM U0126 or the combination of the two for 48 h. The results showed that the expression of the *lyn* and *erk1,2* genes was not significantly different between the two cell lines, and neither drug alone or in combination affected their expression (Fig. 4A).
We further examined the protein levels of BCR-ABL, p-Lyn, ERK signaling and mTOR signaling in K562 and K562R cells after treated with 0.2 μM imatinib, 10 μM U0126 alone or the combination of the two for 48 h by Western Blot (Fig. 4B). The results showed that imatinib reduced the expression of p-ABL in the two cell lines, whereas no effects were apparent with U0126; however, the effects of the combination of the two drugs were synergistic and significantly reduced the expression of p-

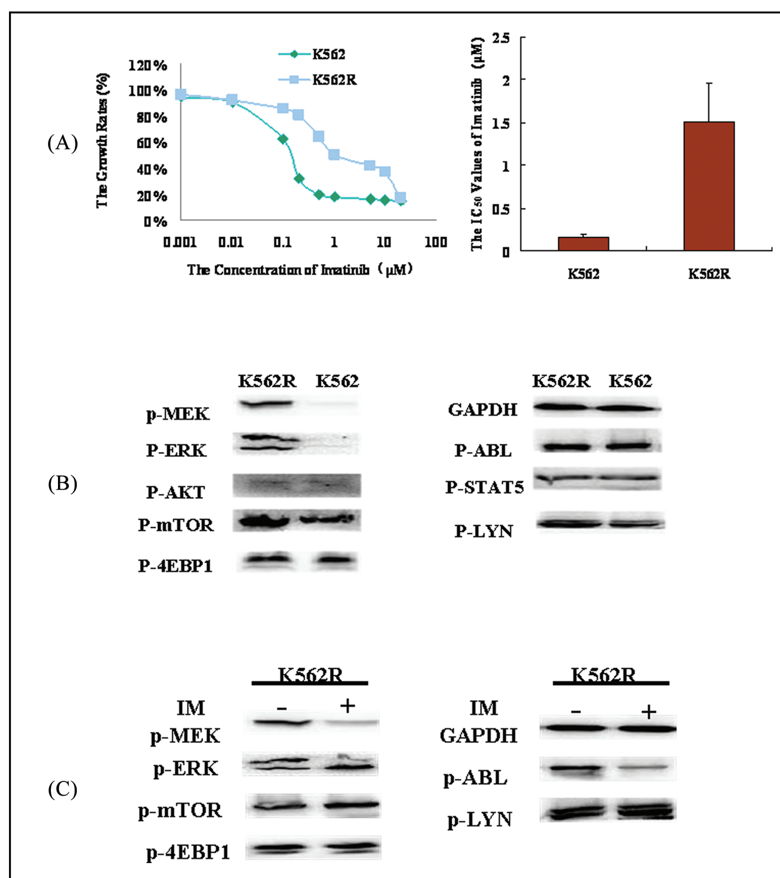

Fig. 1: Mechanism of imatinib resistance in imatinib-resistant K562R cells. The proliferation of the imatinib-sensitive K562 cells and imatinib–resistant K562R cells was examined by MTT assay after incubation with imatinib for 72 h. The results showed that imatinib had cytotoxic effects on both cell lines. K562R cells were more resistant to imatinib than K562 cells, and the IC_{50} values for the K562R and K562 cells were 1.505 ± 0.459 and 0.159 ± 0.032 μM, respectively (A). The level of p-cAbl and the downstream proteins p-Lyn and p-STAT5, the ERK signaling pathway (p-MEK, p-ERK) and the PI3K/AKT/mTOR signaling pathway (p-AKT, p-mTOR, p-4EBP1) were examined by Western Blot analysis. Compared with K562 cells, the expression of p-Abl and p-STAT5 was higher in K562R cells, while the expression of p-Akt was low but similar in both cell lines. Surprisingly, p-Lyn, p-ERK, p-mTOR and p-4EBP1 levels were higher in K562R cells than in K562 cells (B). Treatment with imatinib for 24 h decreased the expression of p-Abl and p-MEK, whereas p-Lyn, p-ERK, p-mTOR and p-4EBP1 were up-regulated in K562R cells (C). All of the results are presented as the mean ± s.d. of at least three independent experiments.

ABL. Neither imatinib nor U0126, alone or in combination, had effects on p-STAT in the two cell lines. Moreover, p-Lyn was up-regulated in K562R cells by imatinib but down-regulated by U0126 alone or in combination with imatinib. Likewise, p-MEK was down-regulated by imatinib but up-regulated by U0126, and the combination of the two drugs reduced the MEK level. In addition, p-ERK was up-regulated by imatinib but down-regulated by U0126 in K562R cells, and the combination of imatinib and U0126 counteracted the up-regulation induced by imatinib. In K562 cells, p-ERK expression was low; thus, we did not observe apparent changes after treatment with the drugs. In the Akt/mTOR signaling pathway, both U0126 alone and in combination with imatinib up-regulated p-Akt expression. p-mTOR was up-regulated by imatinib but down-regulated by U0126 in the two cell lines; combination of the two drugs could eliminate the p-mTOR up-regulation induced by imatinib and further inhibit p-4EBP1 expression.

These results suggest that U0126 enhances the cytotoxic effects of imatinib on K562R cells by inhibiting p-cAbl, p-Lyn, p-ERK, p-mTOR and p-4EBP1. In addition, imatinib inhibited the up-regulation of p-MEK induced by U0126 to better inhibit the growth of K562R cells.

3. Discussion

Our study demonstrated that the expression of p-cAbl, p-STAT5 and p-Akt was similar in the two cell lines, but the expression of p-Lyn, p-MEK, p-ERk, p-mTOR and p-4EBP1 was higher in K562R cells, suggesting that these proteins may be relevant to drug resistance in K562R cells. In addition, the activation of p-mTOR and p-4EBP1 was independent of upstream p-Akt because p-Akt expression was low in the two cell lines. In fact, it has been reported that the PI3K/AKT pathway is closely correlated with the ERK pathway, and there is cross-talk between the two signaling pathways. Both pathways could be activated by Ras, while Akt could inhibit Raf-1, and ERK could activate mTOR (Skorski et al. 1997). Thus, we thought that the high expression of p-mTOR and p-4EBP1 in K562R cells was activated by the ERK pathway. To confirm whether up-regulation of these proteins was correlated with BCR-ABL, we analyzed their expression after K562R cells were treated with imatinib. The results showed that imatinib inhibited the expression of p-Abl but had no effect on the level of p-Lyn, p-ERK, p-mTOR and p-4EBP1, while p-Lyn and p-ERk were up-regulated. The p-Lyn and p-ERk trend remained fairly consistent. As mentioned above, Lyn could phosphorylate BCR-ABL on Tyr177, thereby leading to the association of the adapter protein Grb-2 with BCR-ABL at this locus, resulting in activation of Ras and its downstream-Raf/MEK/ERK signaling pathway. Thus, phosphorylation of Tyr177 is essential to the activation of the pathway (Wilson et al. 2002). Therefore, we hypothesized that abnormally activated Lyn caused the up-regulation of p-ERK in K562R cells relative to K562 cells. We also observed a difference between ERK1 and ERK2 in the two cell lines: ERK2 is expressed at a lower level in K562 cells, and after treating

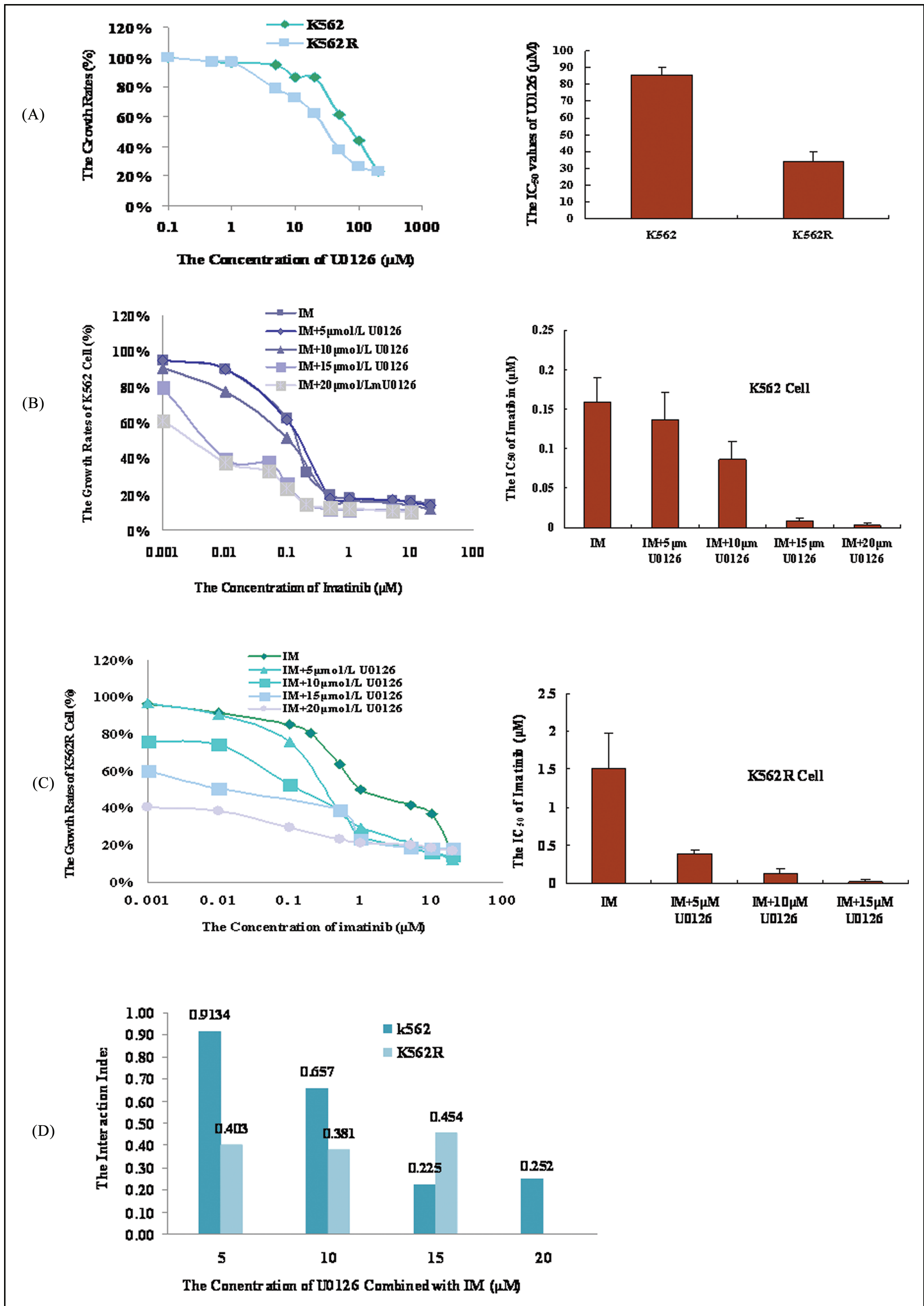

Fig. 2: Cytotoxic effect of the MEK inhibitor U0126 alone or combination with imatinib on K562 and K562R cells. A. The proliferation of cells was examined by MTT assay after incubation with U0126 for 72 h, and the growth rates were exhibited. The IC_{50} values of U0126 in K562 and K562R cells were 85.824 ± 4.474 and 34.235 ± 5.658 μM, respectively (A). K562 (B) and K562R (C) cells were treated with 5, 10, 15, and 20 μM U0126 combined with a series of imatinib concentrations ranging from 0.001 to 20 μM for 72 h, and the proliferation rates and IC_{50} values were analyzed by MTT assays. The interaction index (I) was used to quantitatively depict drug combination effects, and the results showed that U0126 significantly enhanced the anti-leukemia effects of imatinib in the two cell lines (D). The results are presented as the mean ± s.d of I from at least three independent experiments.

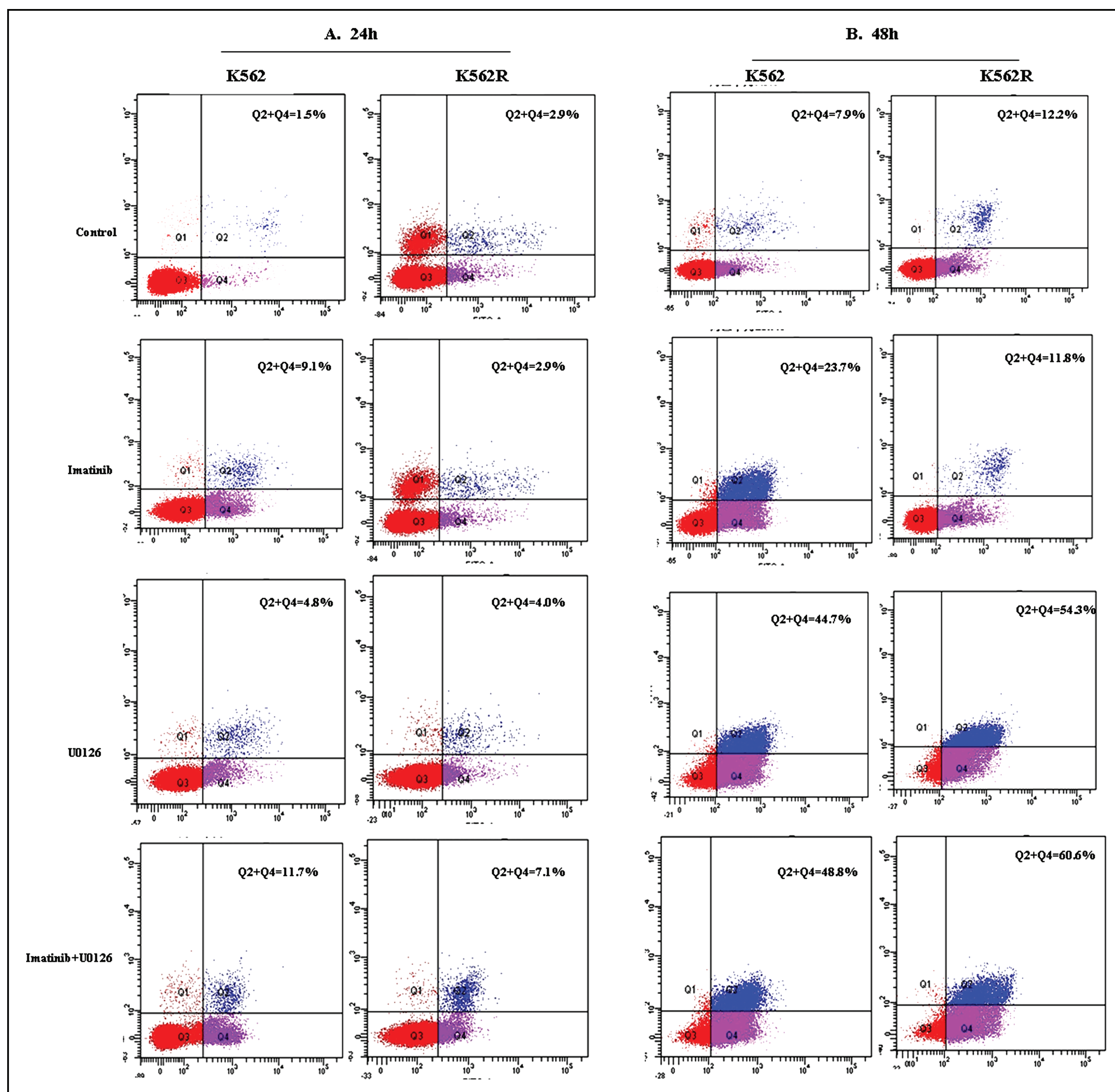

Fig. 3: Flow cytometric detection of the apoptosis rates of K562 and K562R cells. K562 and K562R cells were incubated with 0.2 μM imatinib, 10 μM U0126, or the combination of the two drugs for 24 (A) and 48 h (B). The results indicated that the drugs induced apoptosis in K562 and K562R cells after 48 but not 24 h, and the apoptosis rate measured with the combination of two drugs was higher than for imatinib alone; however, there was no difference with U0126 alone.

K562R cells with imatinib, ERK1 is down-regulated and ERK2 demonstrates the opposite result (Fig. 1C). This phenomenon confirmed that ERK2, rather than ERK1, is involved in drug resistance in CML and that ERK1 could suppress ERK2 activation, as previously reported (Pang et al. 1995).

Our studies showed that BCR-ABL independent Lyn/ERK pathway activation is involved in drug resistance in K562R cells. Activation of this pathway ultimately leads to ERK1/2 activation, thus affecting its downstream targets. There are more than 160 targets downstream of ERK playing important roles in tumorigenesis and resistance. Compared with K562 cells, ERK1/2 expression is significantly increased in K562R cells. Thus, we speculate that an ERK1/2 inhibitor might reverse the resistance of K562R cells. However, there is no ERK1/2 inhibitor at present; thus, we chose an upstream MEK inhibitor instead. Fortunately, MEK only targets and activate ERK1/2. Thus, MEK1/2 inhibitors can be used to suppress ERK1/2. In light of these facts, we next tested the anti-proliferation effects of the MEK1/2 inhibitor U0126 in the K562R cell line.

MTT results demonstrated that K562R cells are more sensitive to U0126 than K562 cells. The results above have shown that the expression of p-MEK and p-ERK was different in two cell lines; there was higher expression in K562R cells compared with K562 cells, and this expression was independent of the BCR-ABL pathway. The over-expression of MEK/ERK in K562R cells played a key role in promoting cell growth; thus, the MER1/2 inhibitor could strongly suppress K562R cells proliferation. In contrast, ERK did not appear to be important in K562 cells because multiple BCR-ABL-dependent pathways (not only MEK/ERK) controlled cell proliferation, and the direct inhibition of BCR-ABL by imatinib resulted in better effects than those of U0126.

The combination of U0126 with imatinib showed significant synergistic anti-leukemia effects in the two cell lines, particularly in K562R cells. In the K562R cells, 10 μM U0126 combined with imatinib completely reversed imatinib resistance, and the IC_{50} value was 0.134 ± 0.051 μM, which was lower than the IC_{50} value (0.159 ± 0.032 μM) of imatinib alone in K562 cells. The apoptosis rates as measured by flow cytometry demonstrated that U0126 induced apoptosis in the two cell lines, and the combination of two drugs had better effects than imatinib alone; however, there was no difference compared with

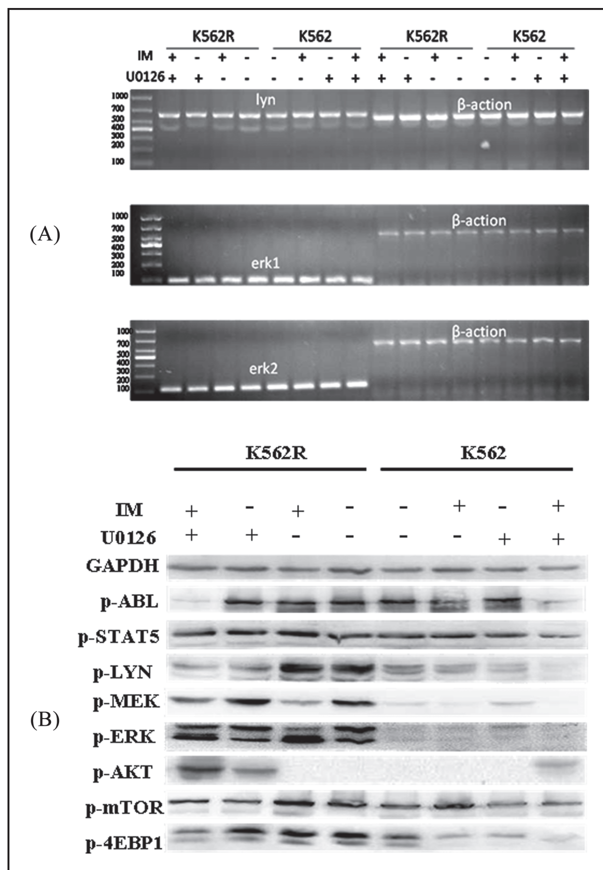

Fig. 4: Mechanism of the U0126-mediated reversal of the imatinib resistance of K562R. K562R cells were treated with 0.2 μM imatinib, 10 μM U0126 or the combination of the two for 48 h. The expression level of the lyn and erk1,2 genes, as examined by the RT-PCR assay, was unchanged after treatment (A). The protein levels of BCR-ABL, p-Lyn, ERK signaling pathway and mTOR signaling pathway were determined by Western Blot. Imatinib reduced the expression of p-ABL and MEK but increased the p-Lyn and p-ERK levels. While U0126 alone down-regulated the expression of p-Lyn and p-ERK, but increased the MEK level. The combination of the two drugs counteracted the up-regulation of p-Lyn and p-ERK induced by imatinib and the up-regulation of p-MEK induced by U0126, thus synergistically inhibiting the growth of K562R cells (B).

the effects of U0126 alone, indicating that the pro-apoptotic effect was not the main cause of their synergism. Therefore, we further explored the critical synergistic mechanism.

In K562R cells, Western Blot analysis of BCR-ABL and its downstream signaling pathways showed that imatinib increased the p-Lyn, p-ERK, and p-mTOR levels, that U0126 decreased the expression of these proteins, that the combination of two drugs overcame the over-expression of Lyn/ERK in K562R cells and that drug resistance resulted from up-regulated proteins induced by imatinib. U0126 inhibited p-ERK by inhibiting p-MEK activity, resulting in the inhibition of p-mTOR and its downstream target 4EBP1. It remains unclear why U0126 decreased p-Lyn expression. It is possible that U0126 is not specific, that other targets exist, or that there is a direct relationship between ERK and Lyn. Lyn increases BCR-ABL activity, and in turn, the down-regulation of p-Lyn inhibits BCR-ABL (Tanis et al. 2003). Moreover, we found that U0126 distinctly increased p-MEK expression, which might result from negative feedback from ERK: U0126 inhibits p-ERK, and p-MEK is activated by feedback (Pouyssegur et al. 2002). Imatinib subtly inhibited p-MEK in K562R cells; thus, the combination of imatinib and U0126 mutually suppressed proteins that play essential roles in cell proliferation to achieve an anti-leukemia effect.

Taken together, our study found that U0126 significantly enhanced the anti-leukemia effects of imatinib in imatinib-sensitive K562 cells and imatinib-resistant K562R cells and completely reversed K562R resistance. These findings suggest that the combination of a MEK1/2 inhibitor with imatinib is a potential therapy for CML patients with drug resistance and thus requires further study.

4. Experimental

4.1. Cell culture

K562 and imatinib-resistance K562R cells (provided by Mahon in Victor Segalen University, France) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 u/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 incubator at 37 °C.

4.2. Reagents and antibodies

The MEK1/2 inhibitor U0126 was purchased from Beyotime, imatinib was from Novartis. The rabbit monoclonal antibodies to (p)-cAbl, (p)-Akt, (p)-mTOR, (p)-4EBP1, (p)-cRAF, (p)-MEK, (p)-ERK, GAPDH and goat anti-rabbit horseradish peroxidase (HRP) conjugate were all purchased from Cell Signaling Technology, the rabbit monoclonal antibodies to (p)-Lyn and (p)-STAT5 were purchased from Abcam. Superscript reverse transcriptase was purchased from TaKaRa Biotechnology, Dalian, China and TaKaRa RNA PCR Kit was purchased TaKaRa, Shiga, Japan.

4.3. MTT assay

Cell proliferation was examined by MTT assay, as previously described (Guo et al. 2012). After treated with imatinib, U0126, or the combination for the indicated concentrations for 72 h, the optical density (OD) absorbance value of each well was read on a Minireader at 590 nm. The cell growth rate was displayed as a percentage: [OD(drug)-OD(blank)]/ [OD (control)-OD(blank)] × 100%. IC_{50} value was the concentration at 50% inhibition of cell growth. The relative resistance factor RF = IC_{50} value of K562R / IC_{50} value of K562 cells. The synergistic cytotoxicity was determined by the interaction index (I). I = 1 indicates an additive effect, I < 1 indicates synergy, and I > 1 indicates antagonism between two drugs (Berenbaum 1989). Every sample was analyzed in triplicate in three independent experiments.

4.4. Western Blot analysis

Cells were collected by centrifugation and washed in PBS. Then, protein expression was examined as previously described (Guo et al. 2012). GAPDH was used as an endogenous control to standardize the amount of the sample protein.

4.5. Flow cytometry

Apoptosis rates were determined by Annexin V-FITC and PI staining as previously described (Guo et al. 2012).

4.6. RT-PCR assay

Total RNA was extracted from 10^6 cells according to the manufacturer's instructions (Tiangen Biotech, Beijing, China), and the mRNA expression of *lyn*, *erk1* and *erk* was examined as previously described (Guo et al. 2012). PCR products (10 μl) were analyzed by electrophoresis in a 2% (w/v) agarose gel, photographed and quantified by densitometric scanning. The relative expression of each gene was analyzed in comparison with β-actin, an internal control.

4.7. Statistical evaluation

The data are presented as mean values from three separate experiments ± s.d. Data were statistically analyzed by Student's t-test after one-way analysis of variance at a level of significance of $P < 0.05$ *vs.* control samples.

Acknowledgements: This study was supported by grants from Doctoral Fund of Ministry of Education (No. 20120181110008), Foundation of the Science & Technology Department of Sichuan Province (No. 2013SZ0025). Conflicts of interest declared: none.

References

Aceves-Luquero CI, Agarwal A, Callejas-Valera JL, Arias-González L, Esparís-Ogando A, del Peso Ovalle L, Bellón-Echeverria I, de la Cruz-Morcillo MA, Galán Moya EM, Moreno Gimeno I, Gómez JC, Deininger

MW, Pandiella A, Sánchez Prieto R (2009) ERK2, but not ERK1, mediates acquired and "de novo" resistance to imatinib mesylate: implication for CML therapy. PLoS One 4: e6124.

Berenbaum MC (1989) What is synergy? Pharmacol Rev 41: 93–141.

Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, Herrmann R, Lynch KP, Hughes TP (2002) High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood 99: 3472–3475.

Bixby D, Talpaz M (2009) Mechanisms of resistance to tyrosine kinase inhibitors in chronic myeloid leukemia and recent therapeutic strategies to overcome resistance. Hematology Am Soc Hematol Educ Program 461–476.

Coppo P, Flamant S, De Mas V, Jarrier P, Guillier M, Bonnet ML, Lacout C, Guilhot F, Vainchenker W, Turhan AG (2006) BCR-ABL activates STAT3 via JAK and MEK pathways in human cells. Br J Haematol 134: 171–179.

Fiskus W, Pranpat M, Bali P, Balasis M, Kumaraswamy S, Boyapalle S, Rocha K, Wu J, Giles F, Manley PW, Atadja P, Bhalla K (2006) Combined effects of novel tyrosine kinase inhibitor AMN107 and histone deacetylase inhibitor LBH589 against Bcr-Abl-expressing human leukemia cells. Blood 108: 645–652.

Guo Y, Shan Q, Gong Y, Lin J, Yang X, Zhou R (2012) Oridonin in combination with imatinib exerts synergetic anti-leukemia effect in Ph + acute lymphoblastic leukemia cells *in vitro* by down-regulating LYN/mTOR signaling pathway. Cancer Biol Ther 13: 1244–1254.

Kieslinger M, Woldman I, Moriggl R, Hofmann J, Marine JC, Ihle JN, Beug H, Decker T (2000) Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes Dev 14: 232–244.

Lee SM, Bae JH, Kim MJ, Lee HS, Lee MK, Chung BS, Kim DW, Kang CD, Kim SH (2007) Bcr-Abl-independent imatinib-resistant K562 cells show aberrant protein acetylation and increased sensitivity to histone deacetylase inhibitors. J Pharmacol Exp Ther 322: 1084–1092.

Mahon FX, Belloc F, Lagarde V, Chollet C, Moreau-Gaudry F, Reiffers J, Goldman JM, Melo JV (2003) MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. Blood 101: 2368–2373.

Mahon FX, Deininger MW, Schultheis B, Chabrol J, Reiffers J, Goldman JM, Melo JV;1; (2000) Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. Blood 96: 1070–1079.

Meyn MA 3rd, Wilson MB, Abdi FA, Fahey N, Schiavone AP, Wu J, Hochrein JM, Engen JR, Smithgall TE (2006) Src family kinases phosphorylate the Bcr-Abl SH3-SH2 region and modulate Bcr-Abl transforming activity. J Biol Chem 281: 30907–30916.

Nambu T, Araki N, Nakagawa A, Kuniyasu A, Kawaguchi T, Hamada A, Saito H (2010) Contribution of BCR-ABL-independent activation of ERK1/2 to acquired imatinib resistance in K562 chronic myeloid leukemia cells. Cancer Sci 101: 137–142.

Pang L, Sawada T, Decker SJ, Saltiel AR (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J Biol Chem 270: 13585–13588.

Pouyssegur J, Volmat V, Lenormand P (2002) Fidelity and spatio-temporal control in MAP kinase (ERKs) signaling. Biochem Pharmacol 64: 755–763.

Quintas-Cardama A, Cortes J (2009) Molecular biology of bcr-abl1-positive chronic myeloid leukemia. Blood 113: 1619–1630.

Skorski T, Bellacosa A, Nieborowska-Skorska M, Majewski M, Martinez R, Choi JK, Trotta R, Wlodarski P, Perrotti D, Chan TO, Wasik MA, Tsichlis PN, Calabretta B (1997) Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. EMBO J 16: 6151–6161.

Tanis KQ, Veach D, Duewel HS, Bornmann WG, Koleske AJ (2003) Two distinct phosphorylation pathways have additive effects on Abl family kinase activation. Mol Cell Biol 23: 3884–3896.

Warmuth M, Bergmann M, Priess A, Häuslmann K, Emmerich B, Hallek M (1997) The Src family kinase Hck interacts with Bcr-Abl by a kinase-independent mechanism and phosphorylates the Grb2-binding site of Bcr. J Biol Chem 272: 33260–33270.

Wu J, Meng F, Kong LY, Peng Z, Ying Y, Bornmann WG, Darnay BG, Lamothe B, Sun H, Talpaz M, Donato NJ (2008) Association between imatinib-resistant BCR-ABL mutation-negative leukemia and persistent activation of LYN kinase. J Natl Cancer Inst 100: 926–939.

Wilson MB, Schreiner SJ, Choi HJ, Kamens J, Smithgall TE (2002) Selective pyrrolo-pyrimidine inhibitors reveal a necessary role for Src family kinases in Bcr-Abl signal transduction and oncogenesis. Oncogene 21: 8075–8088.

Warmuth M, Simon N, Mitina O, Mathes R, Fabbro D, Manley PW, Buchdunger E, Forster K, Moarefi I, Hallek M (2003) Dual-specific Src and Abl kinase inhibitors, PP1 and CGP76030, inhibit growth and survival of cells expressing imatinib mesylate-resistant Bcr-Abl kinases. Blood 101: 664–672.

Xing H, Liu T, Meng W, Gong Y (2011) An investigation of reversal of imatinib resistance in the Bcr-Abl positive imatinib-resistant cell line K562r by dasatinib, nilotinib, rapamycin and bortezomib. Int J Lab Hematol 33: 176–181.