EFFECT OF PLUMBAGIN ON GROWTH INHIBITION AND APOPTOSIS OF IMATINIB-RESISTANT CHRONIC MYELOID LEUKEMIA

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ABSTRACT

Development of a new inhibitor of *BCR-ABL* tyrosine kinase is necessary for the treatment of chronic myeloid leukemia (CML) because of increasing resistance and tolerance to Imatinid efforts. Herein, we reported Plumbagin can significantly inhibit the growth of CML. The results revealed that Plumbagin inhitbited TCCY and TCCY/T315I cells with IC_{50} values 3 μM and 2.1 μM, respectively. Plumbagin also showed anti-proliferative effects on both the wide type Ba/F3 and the *BCR-ABL*-transfected Ba/F3 cells with a range of IC_{50} from 3.2 to 3.8 μM. In addition, Plumbagin induced the apoptosis of CML cells. That would provide a new and potential drug as a chemotherapy medication in the treatment of Imatinid -resistant CML.

Keywords: Apoptosis; BCR-ABL/T315I; CML; Imatinib-resistance; Inhibition; Plumbagin.

1. Introduction

Chronic myeloid leukemia (CML) is a stem cell disease in which the *BCR-ABL* tyrosine kinase plays a key role in the growth of abnormal cells. Inhibition of *BCR-ABL* activity by small molecules is considered as a potential approach in the treatmenbt of CML. Currently, the tyrosine kinase inhibitor (TKI) - Imatinib mesylate (IM) has emerged as a chemotherpy medication in the treatment of CML patients. Unfortunately, There are 95% of CML patients who developped IM-resistance. IM-resistance involves in *BCR-ABL* protein mutation, especially a replacement of threonine to isoleucine at position of 315 (T315I)) that creates a significant clinical problem (Hu Y et al., 2006; Kimura S et al., 2014). IM inhibits the phosphorylation of tyrosine in wild type (WT) BCR-ABL whereas does not act on the mutant BCR-ABL (T315I) (Gorre ME et al., 2001). Many potential TKIs such as dasatinib and nilotinib have been used to against IM-resistant cells. However, these moleculesdid not effect on the IM-resistance causing by T315I mutation in CML patients (Chen R et al., 2015). Nevertheless, development of a new TKI would provide an alternative chemotherapy medication in the treatment of drug-resistant CLM. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a natural naphthoquinone which is isolated from the root of *Plumbago zeylanica* L. Plumbagin has been demonstrated to have anti-tumour effect and induce apoptosis in various types of cancers (Hafeez BB et al., 2013; Liu X et al., 2015). In this study, we have investigated the potential effect of plumbagin against IM-

resistant BCR-ABL/T315I in CML cells.

2. Materials and Methods

2.1. Cell lines, culture conditions

Experiments were conducted by using human leukemia cell lines: TCCY and TCCY-T315I. The cells were grown in RPMI 1640 medium (Sigma-Aldrich, Ho Chi Minh, Vietnam) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Sigma-Aldrich, Ho Chi Minh, Vietnam) in a humidified incubator of 5% CO_2 at 37^oC.

The parental Ba/F3 cells was cultured in RPMI 1640 medium supplemented with 1 ng/ml interleukin-3 (IL-3, R&D Systems)

2.2. Construction of plasmids

Full-length human P210 BCR-ABL E255K cDNA (kindly provided by Dr. Charsle Sawyers U.C.L.A, USA), cloned into pMSCVpuro vector (Clontech, Laboratories, Inc, USA) at EcoRI sites, was re-cloned into the pcDNA3.1(+) vector. The pcDNA3.1–BCR-ABL/WT, pcDNA3.1–BCR-ABL/T315I and pcDNA3.1–BCR-ABL/Y253H vectors were generated by using the PrimeSTAR Mutagenesis Basal kit (Takara, Tokyo, Japan) according to manufacturer's instructions.

All constructs were chemically analyzed and confirmed by DNA sequencing.

2.3. Generation of Ba/F3 cells expressing BCR-ABL WT/T315I/Y253H

Ba/F3 cells stable expressing *BCR-ABL/WT, BCR-ABL/T315I or BCR-ABL/Y253H* were generated by using plasmid pcDNA-BCR-ABL/WT vectors, respectively. These plasmids were transfected by using Lipofectamine 2000 (Invitrogen, Ho Chi Minh, Vietnam) according to the manufacturer's instructions. These cells were selected in the presence of 0.8 mg/ml G418 for 2 weeks to establish Ba/F3-BCR-ABL/WT (T315I/Y253H). Ba/F3 transfectants cells were maintained in RPMI 1640 medium containing 10% FBS in the absence of rmIL-3.

2.4. Cell proliferation assays

Cell proliferation was determined by trypan blue dye exclusion test as described previously (Ly BT et al, 2013).

2.5. Reagents

Plumbagin was generously gifted by Dr. Quach Ngo Diem Phuong (academy?). Plumbagin was dissolved in dimethylsulfoxide (DMSO) (Sigma Aldrich, Ho Chi Minh, Vietnam). The Control cells were cultured with the same concentration of carrier DMSO as used in the highest dose of reagents. The concentration of DMSO was kept under 0.1% throughout all the experiments to avoid its cytotoxicity.

2.6. Determination of apoptosis

TCCY and TCCY-T315I cells were treated with 5μM plumbagin for 8 hours. The apoptotic cell was then evaluated by 7-amino-actinomysin (7-AAD) (BD PharMingen) and analyzed by FACS Calibur (Becton, Dickinson). Collected data were analyzed by FlowJo software (Tree Star)

2.7. Real-time reverse transcription-PCR (RT-PCR) analysis

Total RNA was extracted from untreated cells or plumbagin-treated cells using the TRIzol method (Invitrogen). Reverse transcription was performed by Transcriptor First Strand cDNA Synthesis Kit (Roche Molecular Diagnostics). Quantitative real-time PCR (TaqMan; Roche Molecular Diagnostics) was used to measure the expression of *BCR-ABL*. PCR reaction and primers used in this study were exactly the same as reported elsewhere (Luthra R et al., 2004). Data were expressed (calculated or expressed?) relative to the housekeeping gene *ABL*.

2.8. Statistical analysis

All data were expressed as the mean ± standard deviation. Statistical analyses were done using Student's *t-test*, in which $p < 0.05$

was the minimum requirement for a statistically significant difference.

3. Results and Discussion

3.1. Plumbagin inhibited the growth of IM-resistant cells

To test the inhibitory effect of Plumbagin on the growth of CML cell lines, TCCY or TCCY/T315I cells were incubated either with the carrier DMSO alone (control) or with different concentrations of reagents for 72 hours. Cell proliferations were evaluated by using the trypan blue exclusion test. Interestingly, both the parental TCCY and the strongly IM-resistant TCCY/T315I cells were sensitive to Plumbagin in a dose-dependent manner (Fig. 1). We subsequently investigated the overcoming IM-resistant effect of EGCG in Ba/F3 cells stably expressing BCR-ABL/T315I. Notably; parental Ba/F3 cells do not express BCR-ABL. Ba/F3 cells stably expressing BCR-ABL are grown dependent on BCR-ABL signalling. Ba/F3 cells expressing BCR-ABL/WT were as sensitive to Plumbagin as those stably expressing mutant BCR-ABL as shown in Fig. 1. We further examined the tumour-suppressive effect of Plumbagin with BCR-ABL/Y253H cells – another abandon mutation of BCR-ABL. Cellular growth was significantly suppressed by Plumbagin treatment in both BCR-ABL/Y253H cells (Fig. 1). These results suggest that Plumbagin suppresses the growth of CML cells.

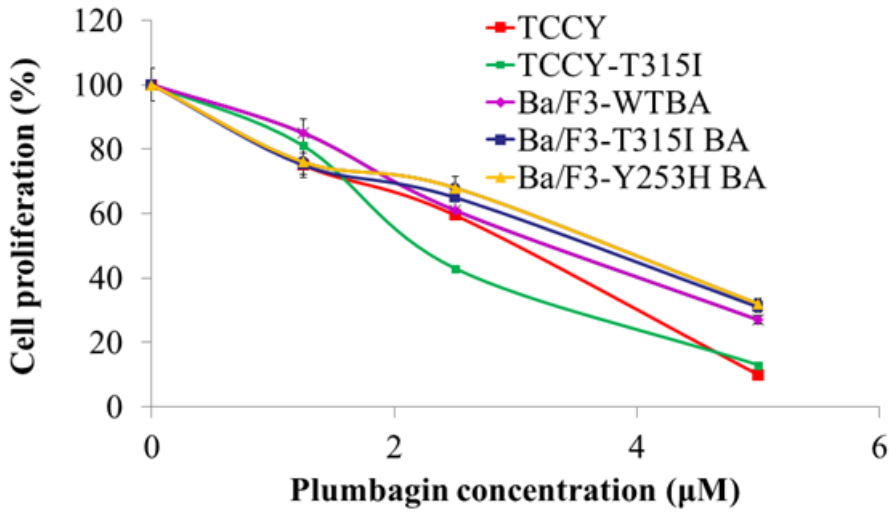

Figure 1. Plumbagin inhibited cell survival of IM-resistance CML cells

TCCY, TCCY/T315I, Ba/F3–BCR-ABL/WT, Ba/F3–BCR-ABL/T315I and Ba/F3–BCR-ABL/Y253H cells at a density of 1 x 10^5 cells/ml were treated with indicated concentration of Plumbagin or DMSO alone as control for 72 hours. The number of alive cells was counted after trypan blue exclusion test. Data were calculated as the percentage of the control values.

3.2. Plumbagin suppressed transcription of BCR-ABL

To investigate whether *BCR-ABL* is a downstream signalling target of Plumbagin, we tested the *BCR-ABL* mRNA in TCCY and TCCY/T315I cells treated with Plumbagin using real-time RT-PCR analysis. As shown in Fig. 2, we found that *BCR-ABL* in either Plumbagin-treated TCCY ($p<0.01$) or TCCY/T315I ($p<0.001$) cells were down-regulated at mRNA levels. These data indicated that *BCR-ABL* may be as a downstream signalling target of Plumbagin in CML.

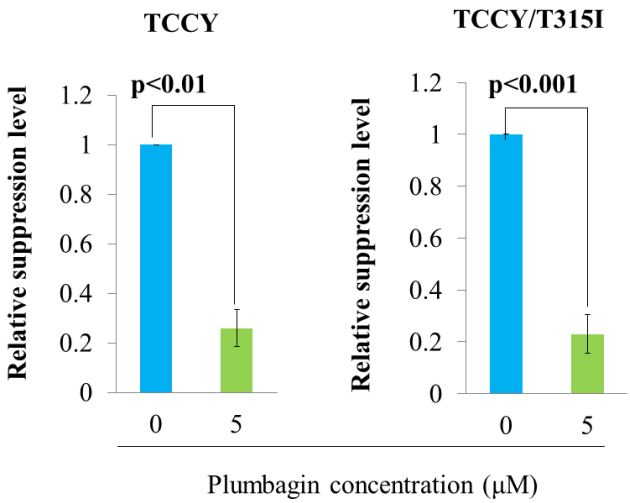

Figure 2. Expression level of *BCR-ABL* in response to Plumbagin treatments

TCCY and TCCY/T315I cells at a density of 1 x 10^5 cells/ml were treated with Plumbagin (5 μM) or DMSO alone as control for 8 hours. Relative mRNA level of *BCR-ABL* were determined by RT-PCR.

3.3. Plumbagin induced apoptosis in imatinib resistant cells

Plumbagin has been shown to induce apoptosis of cancer cells (Kawiak A et al., 2012; Pan ST et al., 2015) but this has not been characterized in CML cells. Herein, Plumbagin-induced apoptosis of TCCY and TCCY/T315I cells were observed by using 7-AAD staining-based FACS analysis. As Fig. 3 showed, in TCCY and TCCY-T315I cells, following incubation for 8 h, approximately 36.9 % cells underwent apoptosis when exposed to 5 μM Plumbagin. These results showed that Plumbagin induces apoptosis of CML cells.

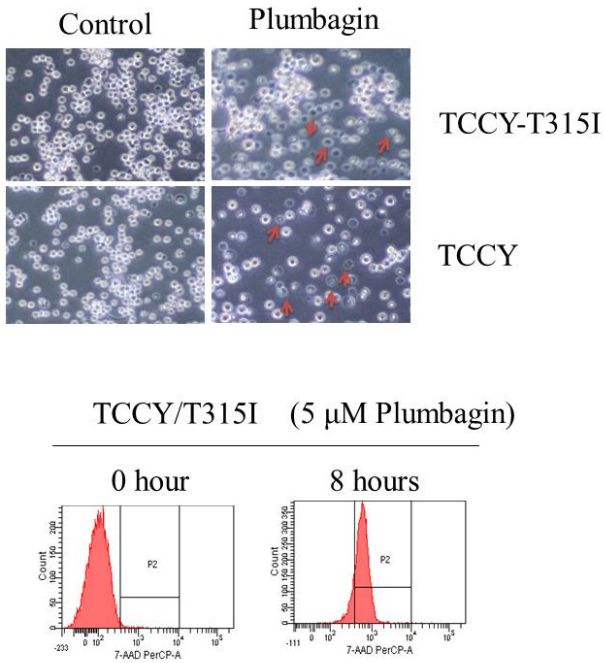

Figure 3. Plumbagin induced apoptosis in TCCY and TCCY-T315I cells

TCCY and TCCY/T315I cells at a density of 1 x 10^5 cells/ml were treated with 5 µM Plumbagin or DMSO alone as control for 8 hours. (A) Morphology of TCCY or TCCY-T315I cells observed under an inverted microscope 8 h after treatment with 5 µM Plumbagin or without Plumbagin (control). Magnification, x10. (B) Total cell lysates of TCCY-T315I after treatment with 5 µM Plumbagin or without Plumbagin for 8 h were stained with 7-AAD and analyzed by FACS Calibur. Collected data were analyzed by FlowJo software.

4. Conclusion

The results showed Plumbagin can not only inhibit the growth of both wide type and IM-resistant CML cells but also induce apoptosis of these cells. Interestingly, Plumbagin may act as a potential natural inhibitor of *BCR-ABL* tyrosin kinase that allows us to continue studying insight into the molecular mechanism of interaction between Plumbagin and *BCR-ABL* or mutant *BCR-ABL*. Resistance against TKIs is a problem in the treatment of CML. Therefore, Plumbagin will be a potential drug to overcome resistance and improve anti-CML therapy■

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