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The Combination of Genistein and Imatinib has an Increased Effect on Cell Proliferation Inhibition in Philadelphia Positive Leukemia Cell Lines

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ABSTRACT

Background: This study investigated the possible role of Genistein as a combination with Imatinib in controlling leukemia cell line proliferation.
Methods: Three cell lines, K562, Kcl22, and CCRF, were cultured and analyzed for MTT, LDH, apoptosis, and cycle cell gene expression in the presence of different dosages of Imatinib and Genistein in combination or separately.
Results: Data has shown a decrease in proliferation and an increase in apoptosis activity during combination treatment. LDH assay has shown no additional toxicity due to Genistein consumption in combination therapy. Analysis of the expression of responsible genes for cell cycle demonstrated both G1 (p53, p21 upregulation) and G2 (cdc25c downregulation) inhibitory effect in combination treatment.
Conclusion: Altogether, this study suggests thatthe combination treatment of Imatinib and Genistein for leukemia cells resistant to Imatinib can increase treatment efficiency.

Keywords: Cell cycle, Genistein, Imatinib, leukemia, Philadelphia chromosome

INTRODUCTION:

Leukemia, neoplasia of hematopoietic cells,has various types with several degrees of malignancy and prognosis (1). Among the prognostic characteristics which have a significant role in the therapeutical plan, alterations in oncogenes have great importance (2). Gene translocations, such as AML_1-ETO, PML-RARA, BCR-ABL (known as Philadelphia chromosome), are among such alterations and common in leukemia (3).

Philadelphia chromosome is a mutual translocation caused bythe fusion of breakpoint cluster (BCR) gene from chromosome 22 and ABL_1 gene(Abelson Tyrosine Kinase) of chromosome 9 (3). The translocation will lead to constitutive activation of the ABL_1 oncogene with its tyrosine kinase activity (4). Depending on the part of the BCR breaking point participating in fusion with ABL, three different isoforms of P190, P210, P230 can be created. ABL on chromosome 9 has 12 exons, witha breaking point always between exon 1 and 2. The fusion gene always contains the ABL1 gene started from the second exon (5).When ABL kinase, located in the nucleus, combines with the BCR gene, it moves to the cytoplasm. It activates some signaling pathways which cause cell proliferation enhancement and skip from apoptosis (6).

Formerly known as STI571Gleevec, Imatinibmesylate is used as a tyrosine kinase inhibitor by inhibiting phosphate transportation on a substrate that stops phosphorylation in BCR-ABL1 (7, 8). On the other hand, mechanismssuch as gene fusion overexpression, structural alteration in BCR-ABL interfering with drug interaction, alterations in ATP binding domain, chromosomal translocation other than BCR-ABL (like SRC), and expression of proteins which efflux Imatinib from the cell can cause Imatinib resistance (9).

Flavonoids have many different characteristics, such as anti-atherosclerosis, anti-inflammatory, anti-thrombosis, anti-tumorogenesis, anti-viral, and anti-osteoporosis to remove free radicals in cancer (10, 11). They are subgrouped into four classes (12). Genistein belongs to the Flavone class of Flavonoids (12).

Genistein can naturally be found in soya, green tea, onion, etc., and has an anti-cancer effect by inhibiting Topoisomerase II, angiogenesis, tyrosine kinase activity, oncogenic product activity, and prostaglandin synthesis (13). One of the other features of this herbal combination is the inhibitory effect of some tyrosine kinases, including epidermal growth factor, ATM, PI3K, and CRKL, which are also downstream of signaling by BCR-ABL recombinant gene (14, 15). Genisteinaffects 253 JBV bladder cancer cell lines. It causes cell cycle arrest in the G2-M stage through downregulation of cdc25c, cdk-1, and cyclin B1 expression (16). Moreover, it causes apoptosis by inducing DNA destruction, enhancing digested caspase 3, and downregulation of Bcl2 accompanied by Bax upregulation (17).

It is well known that Genistein is an agent for cell growth inhibition in the G2-M stage and also has some tyrosine kinase inhibition characteristics. Thus, in this study, it is proposed that the combination of Imatinib,with an inhibitory effect on the G1-S stage, and Genistein, with an inhibitory effect on G2-M,can have an inhibitory effect on leukemia cells, especially on Philadelphia positive cells.

Material and methods

Cell lines

K562 as a Philadelphia positive CML cell line, KCL22 as a Philadelphia positive ALL cell line, and CCRF-CEM as a Philadelphia negative ALL cell line were provided bythe PastureInstitute of Iran.The cell lines were cultured using RPMI 1640 containing 10% FBS in 37 ºC and a 5% CO2 incubator.

Imatinib and Genistein treatment

The cells were treated with Imatinibmesylate (Novartis, Basel, Switzerland) and Genistein (Sigma, USA) as a single or combination treatment with different dosages of 25, 50, 75, 100, 150, and 200 uM of Imatinib and Genistein. In the combination treatment, the cells were treated with a constant dosage of 100 uMImatinib which was used as a maximum dosage in many articles (18-20) in combination withdifferent dosagesof 25, 50, 75, 100, 150, 200 uM of Genistein. The treatment durationswere 24 and 48 hours. Then the cells were pre-

pared for further experiments as mentioned below.

MTT assay

A number of 10^4 cells were seeded in each well of 96 well plates and incubated at 37 ºC for 24 hours. Samples were considered triplicate. Then, the cells were treated with different dosages of Imatinib and interested genes. The amount of 10 µl of MTT solution (Roche, USA, 11465007001) in a concentration of 5 mg/ml was added to each well in different periods (24, 48 hours). The procedure was followed accordingly to the manual protocol. The absorbance was recorded at 550-570-590-600 nm by ELISA reader (BioTek, USA, Gen5 power wave xs2).

LDH assay

Cell toxicity was studied using Lactate Dehydrogenase (LDH) toxicity assay (Sigma, USA). A number of 10^4 cells were seeded in 96 well platesincubated at 37 ºC and 5% CO2 for 24 hours. As mentioned before, the cellswere treated with different dosages of Imatinib and Genistein for 24 and 48 hours and then prepared for LDH assay based on the manual protocol. The absorbance was recorded at 490-500 nm by ELISA reader (BioTek, USA, Gen5 power wave xs2).

Apoptosis

A number of 1.5 x 104 cells were seeded in 96 well plates and incubatedat 37 ºC and 5% CO2 for 24 hours. According to the manual protocol, the cells were treated with different dosages of Imatinib and Genistein for 24 hours and prepared for caspase assay. The absorbance was recorded at 450-500 nm by ELISA reader (BioTek, USA, Gen5 power wave xs2).

RNA extraction and RT-PCR

RNA was extractedfrom 106treated cells using a high pure RNA isolation kit (Roche, USA, 11828665001) based on the manual protocol. The quantity and quality of extracted RNA were confirmed by Nanodrop (USA, Termo model 2000) and 1.5% agarosegel electrophoresis. According to the manual protocol, cDNA synthesis was made from 1-2 µg of the extracted RNA using an easy cDNA synthesis kit (Pars Tous, Iran).

Real-time PCR was performed using 2µl of cDNA and 1µM of each primer (Table 1) added to primer mix (1µM) and syber green master mix (Takara, Japan) using RT-PCR instrument (Rotor gene Q, Qiagen, USA). The program for reaction started by denaturation stepat 95

Table 1: Primers used for quantitative RT-PCR

Name of Gene	Primer	Sequence	Size of product
P53	Forward	5'-TCT CCC CAG CCA AAG AAG AAA-3'	105
	Reverse	5'-TTC CAA GGC CTC ATT CAG CTC-3›	
P21	Forward	5'-CAA AGG CCC GCT CTA CAT CTT-3›	171
	Reverse	5'-AGG AAC CTC TCA TTC AAC CGC-3›	
CDC2	Forward	5'-GCA CCC ATG TCA AAA ACT TGG-3›	105
	Reverse	5'-GGA TGA TTC AGT GCC ATT TTG C-3›	
CDC25C	Forward	5'-TTT TTC CAA GGT ATG TGC GCT G -3'	102
	Reverse	e 5'-TGG AAC TTC CCC GAC AGT AAG G-3›	

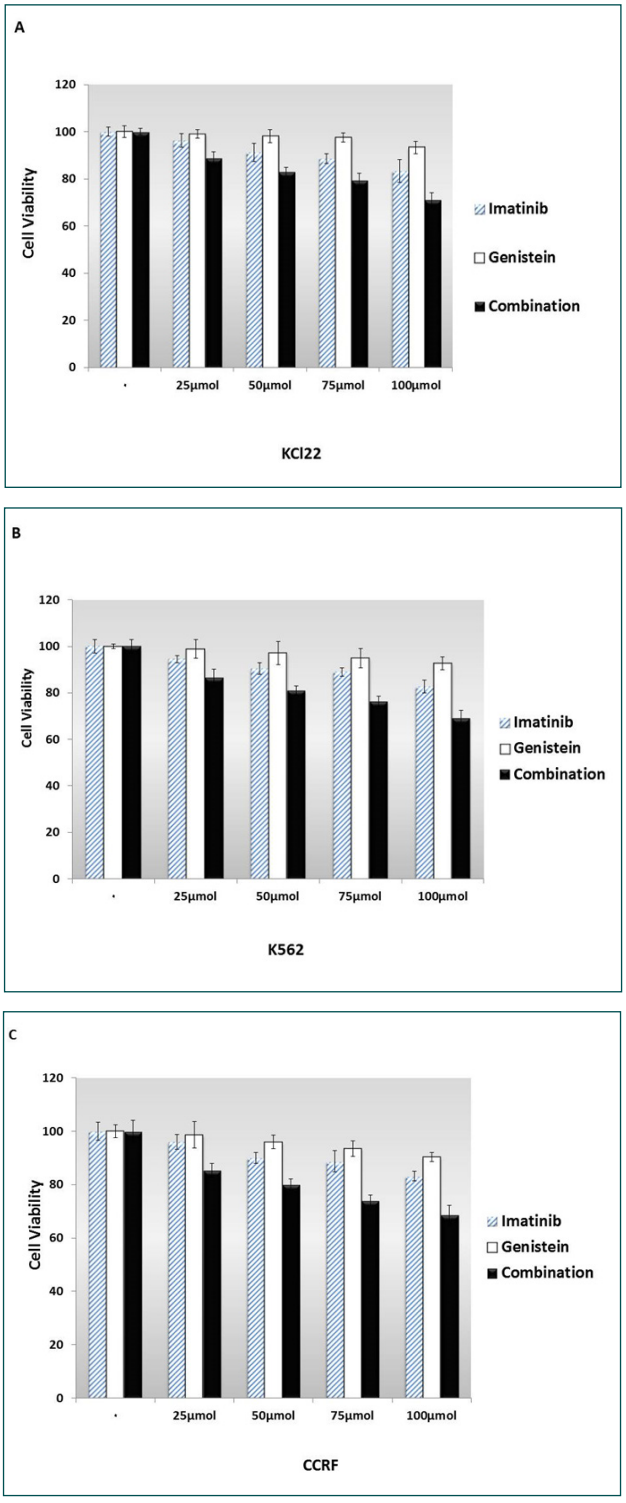

Figure.1. MTT assay of Imatinib, Genistein, and combination therapy as mentioned in the materials and methods. Combination therapy has more effect in the suppression of cell proliferation in a dosage-dependent manner (totally $p<0.05$ in all different dosages showing a significant effect of Genistein when added to the Imatinib in A) Kcl22, B) K562, and C) CCRF cell lines.

ºC for 10minutes followed by 35 cycles of initiation at 95ºC for 10 seconds, annealing at 56 ºC for 15 seconds, elongation at 72 ºC for 20 seconds, and a single final step at 58 ºC for 90 seconds. At the end of the program, the melting curve was checked and data analyzed by CT calculation using REST software (Qiagen, USA). Untreated cells were used as the negative control. Cells treated with an average dosage of Imatinib were used as the positive control. All primers were designed using primer3 (version 0.4.0) online program (https://bioinfo.ut.ee/primer3-0.4.0/) and were confirmed by primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast) to avoid the nonspecific product. Concerning p53 with different isoforms, the melting curve has shown only one clear peak corresponding to the selected product, confirmed by 1.5% agarose gel electrophoresis.

Statistical analysis

Data for the gene amplification and relative fold change was analyzed by Rotor-Gene Q and RESTsoftware (Qiagen, USA), respectively. All data were produced as triplicates and analyzed for the association using SPSS (Statistical Package for the Social Sciences) software (IBM, Chicago, USA), calculating the p-valueand employing the Student›s t-test.

RESULTS:

MTT

The effect of Imatinib on all three cell lines has shown that it causes a decrease in cell viability in a dosage-dependent manner compared with untreated cells (Figure 1). The same results were observed in Genistein treatment in all three cell lines with slight differences. Combination therapy with 100 uM of Imatinib with different dosages of Genistein has shown more effective decrement in cell viability, in which data were significant in all three cell lines during treatment with the highest dosage of Imatinib and Genistein in the combination therapy ($p<0.05$, $p<0.01$, $p<0.01$ for KCL22, K562, CCRF, respectively).

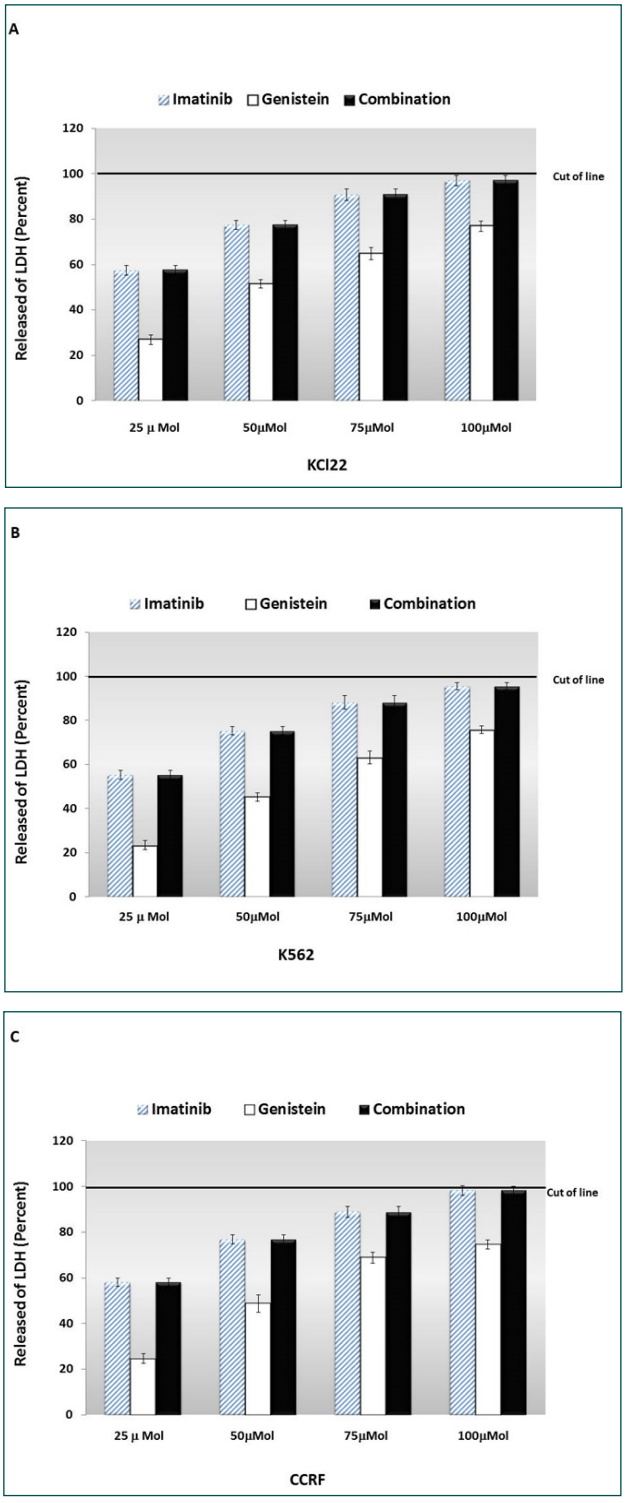

Figure.2. LDH assay of Imatinib, Genistein, and combination therapy as mentioned in the materials and methods. Control cells with maximum release of LDH were determined as cut-off lines (100). Data has shown no additional toxicity of Genistein in combination therapy in all three cell lines; A) Kcl22, B) K562, and C) CCRF.

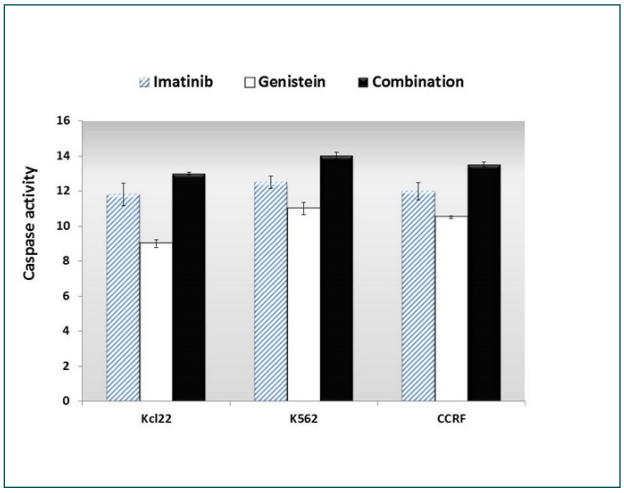

Figure.3. Apoptotic activity was observed by caspase activity. As it is shown, the combination has a dramatic effect on apoptosis in all three cell lines.

LDH

Asillustrated in Figure 2, Imatinib and Genistein toxicity was compared in all three cell lines. The criticalpoint was that adding Genistein to Imatinib treatment in all three cell lines did not increase the toxicity of Imatinib.

Caspase3

Based on Figure 3, the caspase activity increased more during the combination therapy in all three cell lines thansingle treatment with Imatinib. This was more significant in K562 and CCRF ($p<0.01$, $p<0.001$ respectively) cell lines demonstrating that the combination therapy is effective in cell lines without Philadelphia chromosome, suggesting different mechanisms for Genistein as a combination for apoptosis and cell growth suppression other than the possible ABL1 anti-tyrosine kinase inhibitory role.

Real-Time PCR

In the KCL22 cell line, treatment with Imatinib and Genistein and combination treatment has demonstrated-increased p53, p21, and cdc2 gene expression followed by cdc25c downregulation (Figure4A). Combination therapy has shown to be higher in p53 and p21 gene expression compared to Imatinib single treatment ($p<0.01$, $p=0.01$ respectively), while downregulation in cdc25c

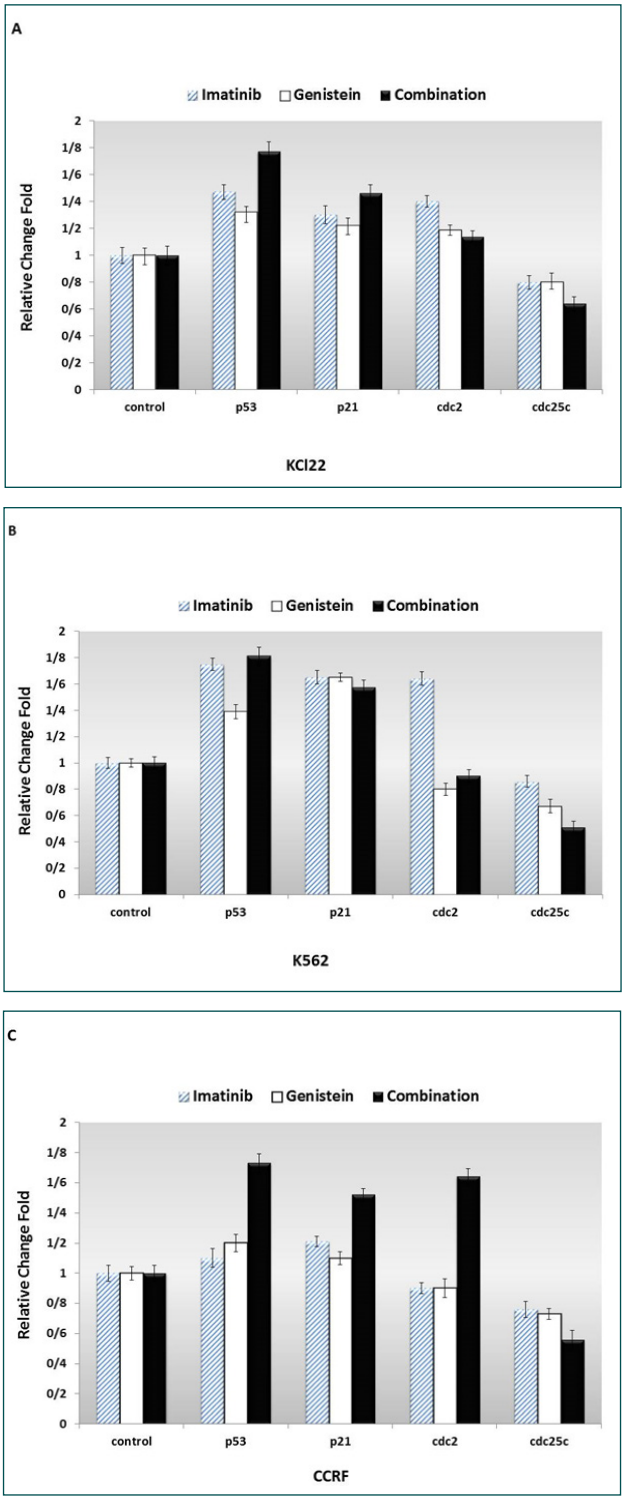

Figure.4. Expression of genes with essential roles in cell cycle control using quantitative RTPCR in A) Kcl22, B) K562, and C) CCRF cell lines.

expression was also more significant in combination treatment (p=0.01).

In K562 cell lines, while single and combination therapy caused p53 and p21 overexpression, cdc2 overexpression was only observed in Imatinib single treatment, but downregulation was shown in Genistein and combination therapy. Genistein seems tohave thedominant effect on combination therapy. Cdc25c expression was downregulated during the treatment of cell lines with Imatinib. The downregulation was more prominent in Genistein treatment and even more with combination treatment compared to Imatinib single treatment ($p<0.01$)(Figure4B). Concerning CCRF cell line, combination treatment causeda dramatic elevation in p53, p21, and cdc2 ($p<0.001$), and downregulation in cdc25c ($p=0.01$)(Figure.4C).

DISCUSSION

This study proposes that Genistein, a flavonoid drug with a protein kinase inhibitory function, could be combined with Imatinib(21, 22). It has been demonstrated that Genistein could have an inhibitory effect on cell growthand proliferation(23). The estrogenic effect of Genistein usually occurs in lower dosages, which can inhibit apoptosis and increase cell proliferation. In comparison, higher dosages of Genistein usually have an inhibitory effect on cell proliferation, antioxidant activity, and apoptosis induction in cancer cells (24).

Previous studies have demonstrated thatkinase activity inhibition of BCR-ABL1by Imatinibcan causeG1arrestin cell cycle analysis(25). Treatment with Imatinib will cause the activation of tumor suppressors such as P16NK4 and Rb1.This is followed by the binding of these proteins to cyclin-dependent kinases such as CDK4, 6, leading to overexpression of CDK inhibitor families such as Cip/Kip like p21waf1, p27kip1, and p53 and binding to CDK2/Cyclin E,S complex, which eventually leads to G1 cell cycle arrest(26, 27). All data from our studies show the same pattern in both Philadelphia positive K562 and KCL22. It has been demonstrated that Genistein has an

essential role in the G2/M phase of cell cycle arrest in many cancer cells such as prostate, breast, and testis cancer (28). Also, it has been demonstrated that the most critical factor for the transition of growing cellsfrom G2 to M is the activation of cyclin B1/cdc2 (29).

When the cells move to the G2 stage, the cyclin B1 that binds to cdc2 increases(30, 31). Also,the basic phosphorylation of cdc2 has a critical role in arresting or movingcells inthe G2 stage. If Tyr 15 in the ATP binding domain of cdc2 is phosphorylated by Wee1 kinase, it causes inactivation of cdc2 followed by G2 cell cycle arrest.However, if Tyr 15 is dephosphorylated by cdc25c,it leads to the formation of active complex cdc2/cyclin B1 and transfers to mitosis or M stage(32, 33). Several studies have shown that Genistein causes a decrement in cdc25cactivity and stabilization of Tyr 15 cdc2 phosphorylation and G2 stage cell cycle arrest(34).

We have hypothesizedthat using the combination ofImatiniband Genistein as the effecter of the G1 and G2 cell cycle will have a supportive effect on cell suppression and apoptosis caused by Imatinib. It has been shown that the combination has a repressor effect on cdc2 expression caused by Imatinib and even down-regulation of cdc25c in Philadelphia positive cell lines,which indicates more focus on the G2 phase.

DuringImatinib treatment, the expression ofCdk inhibitors, such as P21 waf/cip1, increases in G1. Thisis possibly related to P53 for G1 cell arrest. In the case of Genistein, it has been shown that P21 could be associated with Tyr 15 Cdc2, in which overexpression of P21 decreases the expression of cdc2. This can prevent active Cyclin B1/cdc2 complex formation, or ifit is formed, lack of kinase activity causesG2 stage cell cycle arrest(35). Also, it has been shown that Genistein could inhibit the kinase activity of cdc2 in some cancer cell lines like MDA-MB-468(36).

In line with the previous studies, this studydemonstrates that P53 and P21 increase during treatment with Genistein, which was more evidentin Philadelphia positive K562, CML cell line than ALL cell line KCL22. Also, the expression of p21 and p53 were higher. In addition, the expression of Cdc2 and Cdc 25c decreased more obviously in K562 than in KCL22. Altogether, it demonstrates that Genistein is an inhibitor of the G2 step.

Cell treatment with Imatinib has been shown toenhance p53 and p21 expression, which, unlike Genistein,it is more evident in the case of p53 than p21(37). Imatinib has little effect on Cdc2 and Cdc25c expression. Thus, itmay not have any role in cell cycle arrest in the G2 stage through Cdc2. Our study demonstrated that concomitant use of Imatinib and Genistein increases the expression of p53 and p21 in both K562 and KCL22 cell lines. In contrast, the expression of Cdc25c declined.Since the enhancement of both G1 and G2 arrestdue to the increase in the expression of p53 and p21was observed more in combination treatment, it could be suggested that the combination of Genistein and Imatinib is effective on tumor suppressors and CDK inhibitor expression in both G1 and G2 stages compared to Imatinib treatment alone. On the other hand, downregulation in Cdc2, which has a vital role in G2 cell cycle arrest, is more evident during concomitant treatment thanin Imatinib alone. More decrement in Cdc25c during concomitant treatment causes an increase in G2 cell cycle arrest.

Moreover, by investigatingthe path of Caspase 3 breakage,an effective caspase both extrinsically and intrinsically,we could demonstrate the effective role of Genistein and Imatinib concomitant treatment. It is well shown that Imatinib causes apoptosis in CML cancer cell line K562 through activation of the intrinsic path of apoptosis and activation of pro-apoptotic molecules such as Caspase 9 (33).Also, other studies have shown that treatment with Genistein leads to an increase in apoptosis (38). Genistein can increase the level of Bax protein, a pro-apoptotic protein, and decrease Bcl2 protein, an anti-apoptotic protein (39). In this study, it has been demonstrated that the breaking amount of Caspase 3 during concomitant treatment of Imatinib and Genistein is more than treatment with Imatinib or Genistein alone, suggesting that more cells are arrested in the cycle and going through apoptosis. This was more obvious in CML than ALL cell lines. Induction of apoptosis and inhibition of the cell cycle are critical mechanisms of a drug used for cancer treatment.

CONCLUSION

We have found that concomitant treatment with Imatinibmesylate, an established drug, and Genistein, an herbal drug with no toxic effect, could inhibit Philadelphia positive leukemic cell proliferation more effectivelythana single treatment, even in increasing G1 and G2 stage of cell cycle arrest. Also, based on our results, the combination treatment should be performed in the preclinical stage by using different leukemia cell lines, preferably tyrosine kinase-dependent immortality (such as Philadelphia), for new hope in cases with drug resistance or relapses.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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