ORIGINAL ARTICLE

Synergistic effect of ponatinib and epigallocatechin-3-gallate induces apoptosis in chronic myeloid leukemia cells through altering expressions of cell cycle regulatory genes

Bakiye Goker[1], Cansu Caliskan[1], Hasan Onur Caglar[2], Cagla Kayabasi[1], Tugce Balci[1], Burcu Erbaykent Tepedelen[3], Duygu Aygunes[1], Sunde Yilmaz Susluer[1], Zeynep Mutlu[1], Nur Selvi Gunel1, Mehmet Korkmaz[4], Guray Saydam[5], Cumhur Gunduz[1], Cigir Biray Avci[1]

[1]Department of Medical Biology, School of Medicine, Ege University, Izmir; [2]Ege University, Health Science Institute, Department of Stem Cell, Bornova, Izmir; [3]Department of Molecular Biology and Genetics, Faculty of Science and Letters, Avrasya University, Trabzon; [4]Celal Bayar University, Faculty of Medicine, Department of Medical Biology, Manisa; [5]Department of Hematology, School of Medicine, Ege University, Izmir, Turkey

Summary

Purpose: Ponatinib (P) has been used for the treatment of chronic myeloid leukemia (CML) and it is known that inhibition of BCR-ABL fusion protein by ponatinib induces apoptosis of CML cells. Epigallocatechin-3-gallate (EGCG), which is a polyphenol in green tea, induces apoptosis in different types of cancer cells. The purpose of this study was to determine the cytotoxic and apoptotic effects of ponatinib and EGCG combination in K562 CML cell line. This study also aimed to detect alterations of the expression levels of cell cycle-regulation related genes after ponatinib and EGCG combination in K562 CML cell line.

Methods: The cytotoxic effects of the compounds on K562 cells were determined in a time-and dose-dependent manner by using WST-1 analysis. The combination index (CI) isobologram was used to analyze the data. Apoptotic effects of P-EGCG were defined by flow cytometry and gene expres-sions were detected by RT-qPCR.

Results: IC_{50} values of ponatinib and EGCG were 87.13 nM and 50μM, respectively. CI value of the P-EGCG was 0.658 and the combination showed synergistic effect (ED_{90} value: 28.39 nM ponatinib, 117.12 μg/ml EGCG). Ponatinib, EGCG and P-EGCG induced apoptosis compared to control cells. CyclinD1 and CDC25A were downregulated by P-EGCG by 2.49 and 2.63-fold, respectively. TGF-β2 was upregulated by 4.57-fold..

Conclusion: EGCG possesses cytotoxic and apoptotic properties and may cooperate with the growth inhibiting activity of ponatinib synergistically against CML cells. P-EGCG mediated apoptosis might be associated with up-regulation of TGF-β2 gene and downregulation of cyclinD1 and CDC25A genes.

Key words: chronic myeloid leukemia, epigallocatechin-3-gallate, ponatinib, tyrosine kinase inhibitors

Introduction

CML is a malignant myeloproliferative disorder of adults, originating from hematopoietic stem cells (HSCs), and the median age on presentation is approximately 60 years [1-3]. Because CML is a clonal disorder of HSCs, not only myeloid cells but also other hematopoietic cells in peripheral blood are affected by this condition [1]. Patients with CML are generally diagnosed by the presence of Philadelphia chromosome [4,5]. This chromosome is the result of translocation between chromosome 22 (*BCR* gene), and chromosome 9 (*ABL* gene) [6,7]. BCR-ABL fusion protein which has abnormal tyrosine kinase activity results by this reciprocal translocation [8].

BCR-ABL fusion protein can activate different signal pathways such as MAP kinase, PI3 kinase, and Jak-Stat pathways which are associated with cell proliferation and malignant transformation [9]. BCR-ABL induces uncontrolled G1-S phase transition of the cell cycle and reduces the activ-

ity of cell cycle negative regulators such as p21 and p27 [10-12]. Because of these, inhibition of tyrosine kinase activity of BCR-ABL fusion protein is the main approach to cure CML patients. Although tyrosine kinase inhibitors (TKIs) have been used for the treatment of CML patients, resistance against TKIs can be observed in CML patients. Ponatinib, a third generation TKI, is used for the CML treatment, especially for patients who have resistance or intolerance to other TKIs [13].

EGCG, which is a polyphenol found in green tea, induces apoptosis and autophagy in human mesothelioma cells [14]. However apoptotic effect of EGCG is observed in Ishikawa cells. In Ishikawa cell treated with 17β-estradiol, EGCG increases the expression of caspase 6 and caspase 10, and decreases the anti-apoptotic gene Bcl-XL [15]. Furthermore, the expression levels of cdk1, cdk2, and cyclin D3 are downregulated in Ishikawa cells despite the proliferative effect of 17β-estradiol on Ishikawa cells [15].

This study aimed to determine the cytotoxic and apoptotic effects of ponatinib and EGCG combination in K562 CML cell line. It also aimed to detect alterations of the expression levels of cell cycle-regulation related genes after ponatinib and EGCG combination in K562 CML cell line.

Methods

Cell culture

K562 cells were grown in RPMI 1640 medium, containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were maintained in a standard cell culture incubator at 37 °C in 5% CO_2.

Cell treatment

Stock solutions of ponatinib and EGCG at a concentration of 0.5M were prepared in distilled water and stored at +4 °C until use. Required concentrations (1-100 nM ponatinib; 1-100 μM EGCG, for WST-1 assay) were freshly prepared by diluting the stock solution in culture medium immediately before use. Twenty four hrs after seeding, the cells were treated continuously with appropriate concentrations of ponatinib, EGCG, and combination of ponatinib and EGCG (P-EGCG) for 24, 48 and 72 hrs in culture conditions (37 °C, 5% CO_2). Control cells were incubated under same conditions without any treatment.

WST-1 assay

Determination of IC_{50} doses of ponatinib, EGCG, and P-EGCG in K562 cells was performed by using the WST-1 assay (Roche Diagnostics) as indicated in the

manufacturer's instruction. Cell culture was carried out in 96-well plates and cells were incubated for 24 hrs without any agent. After addition of ponatinib, EGCG, and P-EGCG, cells were incubated for 24, 48 and 72 hrs, and cell proliferation was assessed by using WST-1 kit. WST-1 solution (20 μl/well) was added to cells in 96-well plates followed by incubation for 2 hrs at 37°C. The plate was read spectrophotometrically at 440 nm using a microplate reader (Bio-Rad, Coda, Richmond, CA). The viability of the cells was calculated as the percentage of WST-1 reduction. The absorbance of control cells (subjected to the same procedure) was assumed as 100%. The 50% of lethal doses were calculated by the GraphPad Prism 5.0 program (GraphPad Software, California, USA).

Detection of apoptosis by flow cytometry

To evaluate apoptosis in ponatinib, EGCG, and P-EGCG treated cells, a single step staining assay for labelling DNA breaks with FITCdUTP was performed by using APO-DIRECT kit (BD Pharmingen, New Jersey, USA). Control and cells of treatment groups were incubated for 24, 48 and 72 hrs. After centrifugation the cell pellet was resuspended in 500 μl PBS. Paraformaldehyde (1%) in PBS was then added to the cell suspension and incubated for 15 min on ice. After washing twice with PBS, the cell pellet was again resuspended in 70% ethanol and stored at -20°C. Cell samples were then pelleted, washed, and resuspended in 50μL of staining solution containing reaction buffer, terminal deoxynucleotidyl transferase enzyme, FITC-labeled deoxyuridine triphosphate nucleotides, and deionized water. After 60- min incubation at 37°C, cells were washed again, pelleted, and resuspended in 250 μl of propidium iodine/RNase solution and incubated for 30 min at room temperature. The protocol was provided by the manufacturer and followed accordingly including the use of positive and negative controls. Apoptosis was evaluated using BD Accuri C6 Flow Cytometer (Becton–Dickinson, USA) utilizing a 488 nm argon laser light source for excitation and a 530-nm band-pass filter for FITC fluorescence (FL-1H) and a fluorescence detector equipped with a 585/42 band pass filter for FL2A. A total of 20,000 events were acquired for analysis using Cell Quest Software.

Total RNA isolation

To evaluate gene expression, a total RNA isolation was conducted to extract RNA from the K562 cell line using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's manual. Total RNA was isolated from untreated and treated K562 cells.

Reverse transcription quantitative RT-PCR

Complementary DNA (cDNA) synthesis was performed on the total RNAs obtained from untreated and treated K562 cells. RT2 First Strand Kit (Roche Diag-
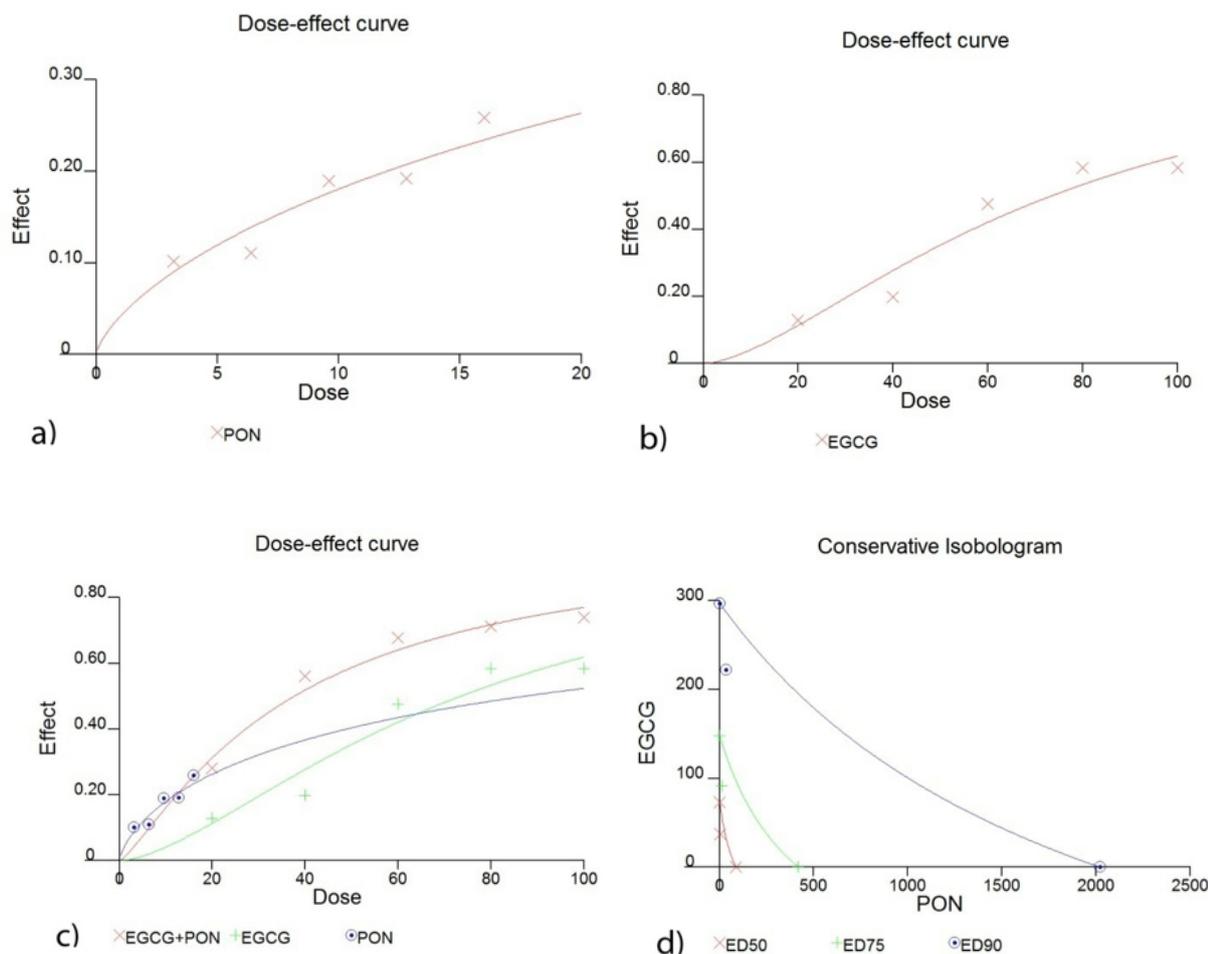
Figure 1. Dose-effect curves for ponatinib **(a)**, EGCG **(b)**, and EGCG+ponatinib **(c)** groups. Combination shows synergism **(d)**. IC_{50} values of ponatinib and EGCG were determined as 87.12 nM and 50 µM, respectively. IC_{50} doses were analyzed according to control cells which were incubated under the same conditions without any treatment.

nostics, Germany) was used for the synthesis of cDNA from cell lines as first step of reverse transcription. Custom RT² PCR Array was used to evaluate the quantitative gene expression analysis including housekeeping genes at LightCycler 480 qRT-PCR platform. The expression analyses of *cyclinD1, CDC25A,* and *TGF-β2* genes were studied on LightCycler 480 qRT-PCR platform. The results of expression were proportioned to *18S ribosomal RNA, GAPDH, Hypoxanthine phosphoribosyltransferase 1,* and *Glucuronidase* genes (housekeeping genes) expressions to calculate the relative expression ratios.

Statistics

Data analysis was evaluated with the ΔΔCT method and quantified with a computer program named "LightCycler 480 Quantification Software" (Roche, Germany). Statistical analyses of gene expressions and activities were performed via the Web-based RT² Profiler PCR Array Data Analysis, version 3.5. P values were calculated by the Student's t-test of the replicate ΔΔCT

values for each gene in both groups (control and treatment groups). A p value <0.05 was considered as statistically significant.

Results

Cytotoxic effects of ponatinib and/or EGCG in K562 cells

Ponatinib and EGCG were treated alone or in combination to the detect the cytotoxic effect on K562 CML cells. IC_{50} values of ponatinib and EGCG were determined as 87.12 nM and 50 µM, respectively. The CI isobologram was used to analyze the data. The analyses were calculated by CalcuSyn software. CI value of the P-EGCG was 0.658 and the combination was accepted as synergistic (ED_{90} value: 28.39 nM ponatinib, 117.12 µg/ml EGCG). Data of cytotoxic effects of ponatinib and/or EGCG is given in Figure 1.
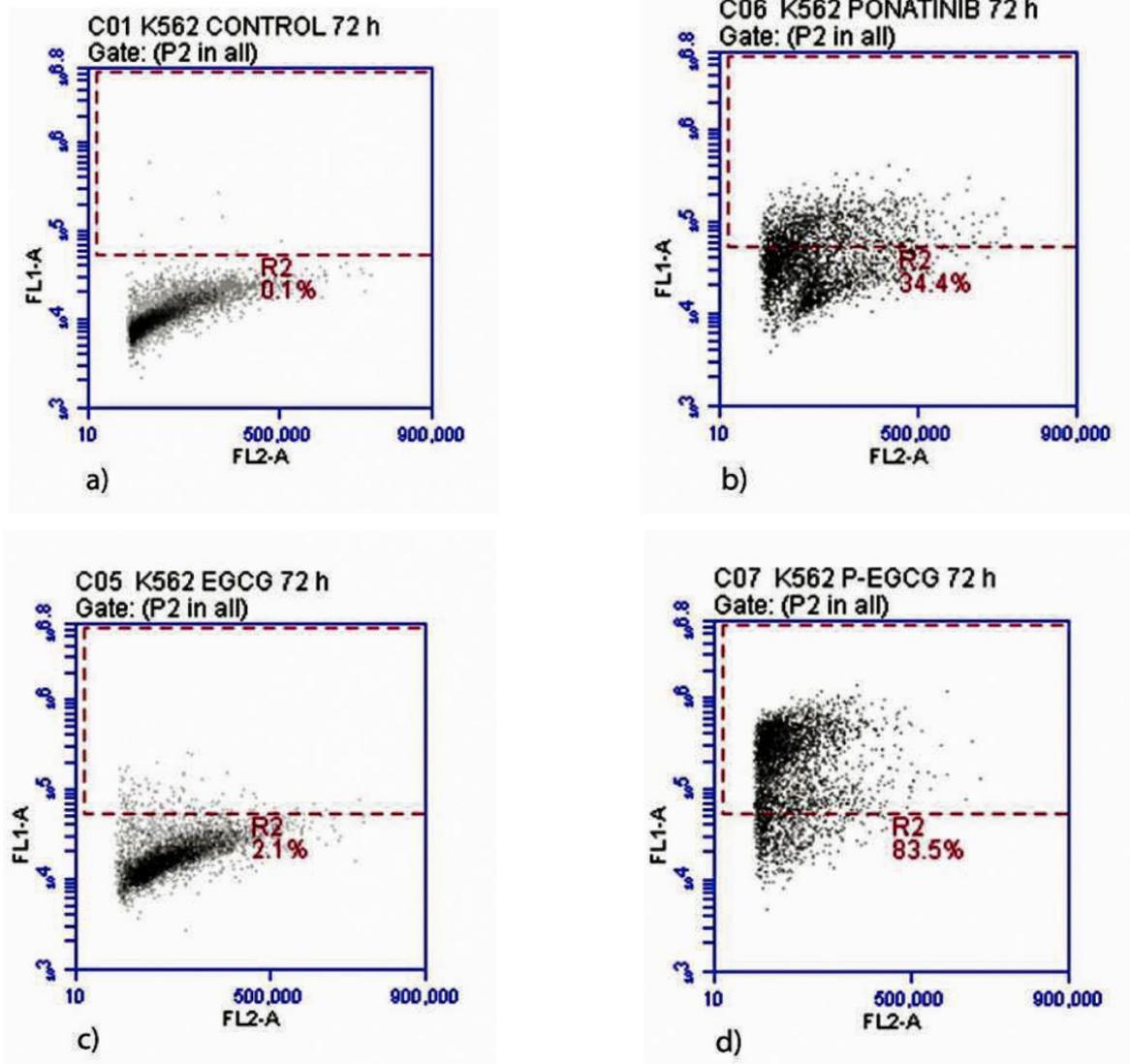
Figure 2. Flow cytometry data of apoptotic cells in 72 hrs for all treatment groups. The percentage of apoptotic cells in the ponatinib treatment group was higher than in EGCG treatment group. The highest percentage of apoptotic cells was observed in ponatinib (P)+EGCG treatment group. **(a)** control, **(b)** EGCG, **(c)** ponatinib, and **(d)** ponatinib+EGCG.

Ponatinib and EGCG induce apoptosis in K562 cells

Apoptosis was detected by flow cytometry using APO-DIRECT kit after ponatinib, EGCG, and P-EGCG treatments. During the time of observation, apoptotic cells were barely observed in the control groups. However, EGCG showed less apoptotic effect than ponatinib treatment group in 24, 48, and 72 hrs. Apoptotic cell percents in the EGCG group were 9.1, 4.4, and 2.1% in 24, 48, and 72 hrs, respectively, whereas apoptotic cell percents in the ponatinib group were 23.2, 48.3, and 34.4% in 24, 48 and 72 hrs, respectively. When the 2 agents were administered alone, ponatinib was more effective in inducing apoptosis in K562 cells in a time-dependent manner. Interestingly, P-EG-CG treatment group induced apoptosis more than EGCG or ponatinib treatment alone. Apoptotic cell percents in P-EGCG were 43.9, 57.6, and 83.5% in a time-dependent manner. Ponatinib and EGCG showed synergistic effect to induce apoptosis in K562 cell line. Flow cytometry data of apoptotic cells in 72 hrs are shown in Figure 2.

Ponatinib and EGCG regulate cell cycle associated genes in K562 cells

qRT-PCR was performed in 72 hrs after P-EG-CG treatment because the highest percents of apoptotic cells were observed in this group. Expression of *cyclinD1*, *CDC25A*, and *TGF-β2* genes were detected by qRT-PCR. Relative quantification method was used for data analysis. In P-EGCG group, *cyclinD1* and *CDC25A* genes were down-

Table 1. Gene expressions of target genes were analyzed for EGCG, ponatinib and ponatinib+EGCG groups in 72 hrs. *18S ribosomal RNA* and *GAPDH* genes were used as control genes. The relative quantification method was used for analysis of data

Genes	$2^{-\Delta\Delta Ct}$ values in treatment groups after 72 hrs		
	Relative expression in the EGCG group	*Relative expression in the ponatinib group*	*Relative expression in the ponatinib and EGCG group*
CDC25A	-0.68	-1.68	-2.63
Cyclin D1	-0.56	-3.28	-2.49
TGF-β2	+0.50	+0.52	+4.57
18S ribosomal RNA (housekeeping)	-0.5	-0.08	0.5
GAPDH (housekeeping)	0.5	-0.2	-0.4
Hypoxanthine phosphoribosyl transferase 1 (housekeeping)	-0.5	-0.46	-0.64
Glucuronidase (housekeeping)	0.5	0.8	0.5

(-) denotes downregulation of target gene expression and (+) denotes upregulation

regulated by 2.49-fold and 2.63-fold, respectively. However, expression of *TGF-β2* gene was upregulated by 4.57-fold in the P-EGCG group. Relative expressions for all treatment groups are presented in Table 1.

Discussion

BCR-ABL chimeric protein has tyrosine kinase activity and is associated with several signaling pathways including PI3K, Jak-Stat, and MAP kinase pathways [9]. BCR-ABL protein can trigger uncontrolled G1-S transition and reduces the activity of cyclin-dependent kinase inhibitors (CDKIs) in the cell cycle process [10-12]. Suppressing of BCR-ABL activity in K562 CML cells results in G1 phase arrest depending on the decreasing expression level of cyclin D1 and increasing expression levels of p21 and p27 [16]. EGCG, which is a polyphenol in green tea, induces apoptosis in human mesothelioma cells and Ishikawa cells [14,15]. Interestingly, EGCG increases the expression of apoptotic genes including caspase-6, caspase-10 and Bax to induce apoptosis, and reduces the expression levels of cdk1, cdk2, and cyclin D3 in Ishikawa cells [15]. Ponatinib and EGCG might exhibit combined effect to induce apoptosis and might alter the expression profiles of the cell cycle related genes in CML cells. In this study, cytotoxic and apoptotic effects of ponatinib and EGCG were determined in K562 CML cells. Not only ponatinib but also EGCG induced apoptosis and exhibited cytotoxic effect on K562 cells. However, the combination of ponatinib and EGCG (P-EGCG) was more cytotoxic than in the ponatinib or EGCG alone treatment groups. P-EGCG treatment had synergistic effect for inhibition of proliferation in K562 cells. This synergistic effect was also observed in terms of apoptosis. Ponatinib treatment induced apoptosis much more higher than EGCG treatment in K562 cells. When ponatinib and EGCG were combined, the presence of apoptotic cells reached the highest level. The reason of increased apoptosis after P-EGCG treatment might be associated with the expression profiles of cell cycle-related genes. It is known that inhibition of ABL kinases induces p21 expression and decreases *CDC25A* expression in imatinib resistance and BCR-ABL positive subclone of 32D cells [17]. It has been shown that inhibition of NPM/ALK with WHI-154 reduces the level of *CDC25A* in anaplastic large cell lymphoma (ALCL) cells; furthermore, inhibition of *CDC25A* alters the proliferative features of ALCL cells [18]. The PI3K/Akt pathway regulates *CDC25A* expression in ALCL and K562 CML cells because mRNA expression of *CDC25A* gene is decreased, depending on PIK pathway inhibition with LY294002 [18]. The level of cyclin D1 transcripts in the accelerated phase is higher than in the chronic phase of CML patients and mRNA expression level of cyclin D1 is associated with the accelerated phase transformation time in CML patients [19].

In this study, it was investigated whether the expression profiles of cell cycle-related genes might be indicative for apoptosis after P-EGCG treatment in K562 cells. Expression profiles of cell cycle-related genes were detected 72 hrs after P-EGCG treatment because the highest percents of apoptotic cells were observed in this treatment

group. Expressions of *cyclinD1* and *CDC25A* genes were downregulated by P-EGCG in 72 hrs compared to control cells. However, expression of *TGF-β2* gene was upregulated by P-EGCG in 72 hrs. Downregulation of *CDC25A* and upregulation of p15 and p21 are associated with TGF-β related growth inhibition [20-23]. TGF-β can prevent G1 to S phase transition in the cell cycle process and induces apoptosis via downregulation of X-linked inhibitor of apoptosis protein (XIAP) which has a role in the PI3K/Akt pathway [24-27]. In hematopoietic cells, TGF-β induces apoptosis through *SHIP* gene which is regulated by smad pathway and inhibits Akt and protein kinase B phosphoryl-ation [28]. *TGF-β2* suppresses cell proliferation in bovine corneal epithelium cells and inhibits AKT activation [29].

Conclusion

Ponatinib and EGCG exhibited synergistic effect on the proliferation and apoptosis in CML cells. P-EGCG mediated apoptosis might be associated with upregulation of *TGF-β2* gene and downregulation of *cyclinD1* and *CDC25A* genes; furthermore, expression patterns of the defined genes might affect the PI3K/Akt signaling pathway.

References

1. Sawyers CL. Chronic myeloid leukemia. N Engl J Med 1999;340:1330-1340.

2. Sant M, Allemani C, Tereanu C et al. Incidence of hematologic malignancies in Europe by morphologic subtype: results of the HAEMACARE project. Blood 2010;116:3724-3734.

3. Smith A, Howell D, Patmore R, Jack A, Roman E. Incidence of haematological malignancy by sub-type: a report from the Haematological Malignancy Research Network. Br J Cancer 2011;105:1684-1692.

4. Bernstein R. Cytogenetics of chronic myelogenous leukemia. Semin Hematol 1988;25:20-34.

5. Morris CM. Chronic myeloid leukemia: cytogenetic methods and applications for diagnosis and treatment. Methods Mol Biol 2011;730:33-61.

6. Bartram CR, de Klein A, Hagemeijer A et al. Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature 1983;306:277-280.

7. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell 1984;36:93-99.

8. Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science 1990;247:1079-1082.

9. Deininger MW, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. Blood 2000;96:3343-3356.

10. Cortez D, Reuther G, Pendergast AM. The Bcr-Abl tyrosine kinase activates mitogenic signaling pathways and stimulates G1-to-S phase transition in hematopoietic cells. Oncogene 1997;15:2333-2342.

11. Gesbert F, Sellers WR, Signoretti S, Loda M, Griffin JD. BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor p27Kip1 through the phosphatidylinositol 3-Kinase/AKT pathway. J Biol Chem 2000;275:39223-39230.

12. Nimmanapalli R, Fuino L, Bali P et al. Histone deacetylase inhibitor LAQ824 both lowers expression and promotes proteasomal degradation of Bcr-Abl and induces apoptosis of imatinib mesylate-sensitive or -refractory chronic myelogenous leukemia-blast crisis cells. Cancer Res 2003;63:5126-5135.

13. Shamroe CL, Comeau JM. Ponatinib: A new tyrosine kinase inhibitor for the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. Ann Pharmacother 2013;47:1540-1546.

14. Satoh M, Takemura Y, Hamada H, Sekido Y, Kubota S. EGCG induces human mesothelioma cell death by inducing reactive oxygen species and autophagy. Cancer Cell Int 2013;13:19.

15. Park SB, Bae JW, Kim JM, Lee SG, Han M. Antiproliferative and apoptotic effect of epigallocatechin-3-gallate on Ishikawa cells is accompanied by sex steroid receptor downregulation. Int J Mol Med 2012;30:1211-1218.

16. Rangatia J, Bonnet D. Transient or long-term silencing of BCR-ABL alone induces cell cycle and proliferation arrest, apoptosis and differentiation. Leukemia 2006;20:68-76.

17. Mancini M, Brusa G, Zuffa E et al. Persistent Cdk2 inactivation drives growth arrest of BCR-ABL-expressing cells in response to dual inhibitor of SRC and ABL kinases SKI606. Leuk Res 2007;31:979-987.

18. Fernandez-Vidal A, Mazars A, Gautier EF, Prevost G, Payrastre B, Manenti S. Upregulation of the CDC25A phosphatase down-stream of the NPM/ALK oncogene participates to anaplastic large cell lymphoma enhanced proliferation. Cell Cycle 2009;8:1373-1379.

19. Liu JH, Yen CC, Lin YC et al. Overexpression of cyclin D1 in accelerated-phase chronic myeloid leukemia. Leukemia Lymphoma 2004;45:2419-2425.

20. Massague J. TGFbeta signalling in context. Nat Rev Mol Cell Biol 2012;13:616-630.

21. Kang SH, Bang YJ, Jong HS, Seo JY, Kim NK, Kim SJ. Rapid induction of p21WAF1 but delayed down-regulation of Cdc25A in the TGF-beta-induced cell cycle arrest of gastric carcinoma cells. Br J Cancer 1999;80:1144-1149.

22. Miyazaki M, Ohashi R, Tsuji T, Mihara K, Gohda E, Namba M. Transforming growth factor-beta 1 stimulates or inhibits cell growth via down- or up-regulation of p21/Waf1. Biochem Biophys Res Commun 1998;246:873-880.

23. Wang X, Sun W, Bai J et al. Growth inhibition induced by transforming growth factor-beta1 in human oral squamous cell carcinoma. Mol Biol Rep 2009;36:861-869.

24. Caron PL, Frechette-Frigon G, Shooner C, Leblanc V, Asselin E. Transforming growth factor beta isoforms regulation of Akt activity and XIAP levels in rat endometrium during estrous cycle, in a model of pseudopregnancy and in cultured decidual cells. Reprod Biol Endocrinol 2009;7:80.

25. Gottfried Y, Rotem A, Lotan R, Steller H, Larisch S. The mitochondrial ARTS protein promotes apoptosis through targeting XIAP. EMBO J 2004;23:1627-1635.

26. Bachman KE, Park BH. Duel nature of TGF-beta signaling: tumor suppressor vs. tumor promoter. Curr Opin Oncol 2005;17:49-54.

27. Shima Y, Nakao K, Nakashima T et al. Activation of caspase-8 in transforming growth factor-beta-induced apoptosis of human hepatoma cells. Hepatology 1999;30:1215-1222.

28. Valderrama-Carvajal H, Cocolakis E, Lacerte A et al. Activin/TGF-beta induce apoptosis through Smad-dependent expression of the lipid phosphatase SHIP. Nat Cell Biol 2002;4:963-969.

29. Lu J, Lu Z, Reinach P et al. TGF-beta2 inhibits AKT activation and FGF-2-induced corneal endothelial cell proliferation. Exp Cell Res 2006;312:3631-3640.