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 expressions were detected by RT-qPCR.

Results: IC50 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 (ED90 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 upregulation 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% CO₂.

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% CO₂). Control cells were incubated under same conditions without any treatment.

WST-1 assay

Determination of IC₅₀ 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 50 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-
Ponatinib and epigallocatechin in leukemia

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. IC₅₀ 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. The CI value of the P-EGCG was 0.658 and the combination was accepted as synergistic (ED₉₀ 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 1. Dose-effect curves for ponatinib (a), EGCG (b), and EGCG+ponatinib (c) groups. Combination shows synergism (d). IC₅₀ values of ponatinib and EGCG were determined as 87.12 nM and 50 μM, respectively. IC₅₀ doses were analyzed according to control cells which were incubated under the same conditions without any treatment.
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-EGCG 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-EGCG 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-

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.
regulated by 2.49-fold and 2.63-fold, respectively. However, expression of \( \text{TGF-\beta2} \) 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 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 resistant 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 phosphorylation [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

7. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G. Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, and upregulation CDC25A genes; [28].


