Enalapril-induced Apoptosis of Acute Promyelocytic Leukaemia Cells Involves STAT5A

OZLEM PURCLUTEPE1, GUNIZ ISKENDER2, HATICE DEMET KIPER1, BURCIN TEZCANLI3, NUR SELVI1, CIGIR BIRAY AVCI1, BUKET KOSOVA3, AYSUN ADAN GOKBULUT2, FAHRI SAHIN2, YUSUF BARAN2 and GURAY SAYDAM4

Departments of 1Internal Medicine, 2Medical Biology and 3Hematology, School of Medicine, Ege University, Bornova, Izmir, Turkey; 4Department of Molecular Biology and Genetics, School of Science, Izmir Institute of Technology, Urla, Izmir, Turkey

Abstract. Background: In this study, we aimed at evaluating the cytotoxic and apoptotic effects of enalapril on human HL60 acute promyelocytic leukaemia cells and at clarifying the roles of signal transducers and activator of transcription proteins (STATs) on enalapril-induced cell death. Materials and Methods: Cell viability and cytotoxicity tests were conducted by Trypan blue dye exclusion and 2,3-Bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assays, respectively. Apoptotic analyses were performed by the AnnexinV-enhanced green fluorescent protein (EGFP) staining method and by fluorescence microscopy. Expression levels of STAT3, -5A and -5B genes were analysed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Results: The results showed that enalapril reduced viability and proliferation, and induced apoptosis in HL60 cells in a dose- and time-dependent manner as compared to untreated controls. The expression levels of STAT5A gene were significantly reduced in enalapril-treated HL60 cells as compared to untreated controls. Conclusion: Taken together, all data showed for the first time that enalapril has significant anticancer potential for the treatment of acute promyelocytic leukaemia.

Acute promyelocytic leukaemia (APL), a distinct variant of acute myelogenous leukaemia (AML), is characterized by clonal expansion of promyelocytes in the bone marrow and in the bloodstream resulting from the arrest of the differentiation of myeloid cells at the promyelocyte phase (1). In the majority of APL cases, a reciprocal chromosomal translocation occurs involving the retinoic acid receptor-alpha (RARα) gene on chromosome 17 and the promyelocytic leukaemia (PML) gene on chromosome 15 (2). The resultant PML-RARα fusion protein plays a critical role in the pathogenesis of APL. Induction therapy for patients with APL used to be similar to that for AML and included a standard chemotherapy regimen of anthracycline and cytarabine arabinoside (3, 4). Complete remission was sustained following standard chemotherapy; however, most patients experienced relapse and long-term disease-free survival was only 30-40% (5). The introduction of all-trans retinoic acid (ATRA) (6, 7) has changed the treatment course of APL, and its combination with anthracycline-based chemotherapy has become the standard treatment regimen for newly diagnosed APL patients. Following a combination therapy, the complete response rates were found to be more than 90% and cure rates were approximately 80% (8-10). Arsenic trioxide (ATO) in treatment of APL relapse has also given excellent results (11).

Haematopoietic cell proliferation and differentiation is regulated by cytokines that are also known as interferons, interleukins and colony-stimulating factors. Upon the binding of the cytokine with the cell surface receptor that is lacking intrinsic tyrosine kinase ability, the signalling cascade is induced and associated Janus kinases (JAKs) are activated by cross-phosphorylation. Activated JAKs first phosphorylate the receptor itself and then the signal transducers and activator of transcription proteins (STATs) (12). STATs are latent cytoplasmic transcription factors that upon activation function as signal transducers and transcription factors. Seven mammalian STAT proteins have been discovered: STAT1 to -4, and STAT5A, STAT5B, and STAT6 (13). They are known to be involved in several cellular processes, such as cell growth, cell differentiation, apoptosis and immune responses. Therefore, dysregulation of STATs, either due to constitutive activation or functional
clarify the mechanisms of enalapril-induced cell death.

on human HL60 acute promyelocytic leukaemia cells and to
cytotoxic and apoptotic effects of the ACE inhibitor enalapril

treatment of acute leukaemia yet, to date, there is no
works by regulating the RAAS.

inhibitor used for the treatment of high blood pressure that
gained significance (32). Enalapril is a carboxyl-group ACE
examining the anti-tumoural effects of ACE inhibitors have

disease and diabetes mellitus. Recently, studies

Hodgkin’s disease (28) has already been shown. In addition,
and skin (20). The RAS is also a vital element in the process

of development of neoplastic haematopoiesis (25). The
expression of

be expressed in several adult tissues (21). The presence of a
local bone marrow RAS, affecting physiological and

haematopoietic stem cells (HSC), progenitors and bone marrow
microenvironment (22-24). It is the paracrine action of
locally expressed RAS, instead of its circulating counterpart,
that appears vital for tumourigenesis. Expression of
components of the RAS such as renin, angiotensin (Ang),
angiotensin I-II receptor (AT1R, AT2R) and antigen-
converting enzyme (ACE) are identified in various types
of carcinoma, including brain, lung, breast, prostate, colon,
and skin (20). The RAS is also a vital element in the process
of development of neoplastic haematopoiesis (25). The
presence of ACE surface antigen (CD143) within leukemic
bone marrow (26), on the K562 erythroleukaemia cell line
(27) and ACE-expressing macrophages in lymph nodes in
Hodgkin’s disease (28) has already been shown. In addition,
CD143 has been observed to be over expressed in leukemic
blast cells, and it has been found that ACE is directly
correlated to the bone marrow blast count (29). Renin
activity has also been described in leukemic blasts (30, 31).

ACE inhibitors have been widely used in clinical studies
for the treatment of hypertension, heart failure, coronary
artery disease and diabetes mellitus. Recently, studies
examining the anti-tumoural effects of ACE inhibitors have
gained significance (32). Enalapril is a carboxyl-group ACE
inhibitor used for the treatment of high blood pressure that
works by regulating the RAAS.

Many chemotherapeutic agents have been used in the
treatment of acute leukaemia yet, to date, there is no
definitive treatment. In this study, we aimed to evaluate the
cytotoxic and apoptotic effects of the ACE inhibitor enalapril
on human HL60 acute promyelocytic leukaemia cells and to
clarify the mechanisms of enalapril-induced cell death.

Materials and Methods

Cell line, culture conditions and chemicals. Human HL60 acute
promyelocytic leukaemia cells were kindly provided by Dr Serdar
Bedii Omay from Ege University. HL60 cells were maintained in
RPMI-1640 medium containing 10% foetal bovine serum (FBS) and
1% penicillin–streptomycin at 37°C in 5% CO2. Enalapril was obtained from Sigma Chemical (St. Louis, MO, USA). Enalapril (50 mg)
dissolved in 1 ml RPMI-1640 medium and stock solutions
were prepared. Trypan blue dye was obtained from Sigma Chemical.
2,3-Bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-
carboxanilide inner salt (XTT) Cell Proliferation Assay was obtained from
Biological Industries (Israel). AnnexinV-EGFP Apoptosis
Detection Kit was obtained from Biovision (CA, USA). All the other
chemicals and tissue culture supplies were obtained from Sigma
Chemical unless otherwise specified.

Measurement of cell viability and cytotoxicity. In order to measure the
cytotoxic effect of enalapril on HL60 cells, 2×10^5 cells were
seeded into 24-well plates containing 1 ml growth medium in the
absence or presence of increasing concentrations of enalapril (1 nM,
10 nM, 100 nM, 5 μM and 10 μM), and incubated at 37°C in 5%
CO2. Cell viability was assessed at 0, 24, 48, 72 and 96 h post-
incubation by Trypan blue dye exclusion assay as indicated in the
manufacturer’s instructions.

The concentration of enalapril that inhibited cell growth by 50%
(IC50) was determined by XTT assay. Briefly, 2×10^4 HL60 cells
were plated into 96-well plates containing 100 μl of the growth
medium in the absence or presence of increasing concentrations of
enalapril (1 nM, 10 nM, 100 nM, 5 μM and 10 μM) and incubated at
37°C in 5% CO2 (PI). After 24, 48, 72 and 96 h incubation, cells were
treated with 50 μl XTT solution for 4 hours and the absorbance
was recorded at 492 nm using an ELISA reader and the IC50 dose of
enalapril was calculated from the cell proliferation plots (33).

Evaluation of apoptosis. Apoptosis induced by enalapril in HL60 cells
was detected by fluorescence microscopy using AnnexinV-EGFP
Apoptosis Detection Kit (Biovision) as described in the manufacturer’s
instructions. Briefly, HL60 cells were treated with 7 μM of enalapril
and apoptosis was induced. At 0, 24, 48, 72 and 96 h time points, 1-
5×10^5 cells were collected and 500 μl 1X binding buffer was added to
the cell suspension. Subsequently, 5 μl annexinV-EGFP and 5 μl
propidium iodide (PI) were added, and the cells were incubated in the
dark for 5 min at room temperature. Consequently, the cells were
transferred onto a coverglass and morphologically examined by
fluorescence microscopy.

Total RNA isolation and quantitative reverse transcriptase-polymerase
chain reaction (qRT-PCR). Total RNA was isolated from HL60 cells

treated with 7 μM (IC50 value) of enalapril at 24, 48, 72 and 96 h post-
incubation using Trizol reagent (including guanidium thiocyanate,
phenol and sodium citrate) as described by the manufacturer. One
microgramme of total RNA was reverse transcribed using FirstStrand
cDNA Synthesis Kit (Life Sciences GE Healthcare, Piscataway, NJ,
USA) in accordance with the instructions provided by the
manufacturer. After 1 h incubation at 37°C, the reaction was stopped
at 65°C for 10 min. The resulting total cDNAs were then used to
determine the mRNA levels of STAT3, STAT5A, and STAT5B. Q-PCR
was performed with gene-specific primers and probes using the Fast
Start DNA Master Hybridisation Probes (Roche Applied Science,
Penzberg, Upper Bavaria, Germany) and Glucose-6-phosphate dehydrogenase (G6PDH) House Keeping Gene Kit (Roche Applied Science). The G6PDH gene was used as an internal positive control in all PCR reactions, and its amplification product provided both a control for PCR performance and a reference for quantification of PCR products. The primer sequences were (TibMolBiol, Berlin, Germany): STAT3 forward: 5’-ACCAACAATCCCAAGAATGT-3’, reverse: 5’-CGATGCTCAGTCCTCGC-3’; STAT5A forward: 5’-GAAGCTGACCTGACGCGCACTGAA CGTGACACATGAATC-3’, reverse: 5’-GTAGGGAGACTGCTCCTCA CCTGG-3’; STAT5B forward: 5’-AGTTTGGATTCTCAGGAAAGAA TGT-3’, reverse: 5’-TCCATCAACAGCTTCTTAG CAGT-3’. Two microlitres of the reverse transcriptase reaction was amplified using these primers for 50 cycles (95˚C, 10 s; 56˚C, 10 s; 72˚C, 5 s). The mRNA levels of STAT3, -5A, and -5B were determined in a Real-Time LightCycler Instrument (Roche Applied Science). The relative expression level for each gene was calculated by dividing the mRNA copy number of the target gene by the G6PDH mRNA copy number. qRT-PCR experiments were performed at least in three independent trials. Statistical significance was analysed by using ANOVA (analysis of variance) and \( p<0.05 \) was considered significant.

### Results

#### Enalapril reduced viability and proliferation of HL60 cells in a time- and dose-dependent manner.

In order to detect the potential cytotoxic effects of enalapril on human HL60 cells, we used both Trypan blue dye exclusion and XTT cell proliferation assays. The results of the Trypan blue dye exclusion assay showed that there was a dose- and time-dependent reduction in cell viability as compared to untreated controls (Figure 1). Viability of untreated HL60 cells remained constant for the initial 72 hours, before declining in number due to over-confluence in the spent medium. Viability of HL60 cells treated with increasing concentrations of enalapril (3-10 μM) was observed to drop steadily over the experiment. The IC\(_{50}\) value of enalapril was calculated from cell survival plots and was determined to be 7 μM (Figure 1).

The degree of cytotoxicity induced by enalapril was assessed by XTT assay. Enalapril reduced cell proliferation significantly in a time- and dose-dependent manner (Figure 2).

#### Enalapril induced early and late apoptosis in HL60 cells.

Apoptotic cells were morphologically examined under fluorescence microscopy, and cellular changes were identified. Apoptotic cells were stained green, whereas necrotic cells were stained with PI and observed in red. Cells shown as both green and red were defined to be in late apoptosis (Figure 3A) and live cells were observed in blue (Figure 3B). Following the evaluation of apoptosis in HL60 cells treated with 7 μM enalapril, an increase was observed in the number of early apoptotic cells at 48 hours and the rate was found to be 20% (Figure 4). However, this value was statistically insignificant when compared to that of untreated controls, \( (p>0.05) \) and the difference between enalapril-treated and untreated control groups disappeared after 72 hours. At later time points, early apoptotic HL60 cells were not observed; only HL60 cells in late apoptosis were present (Figure 4).
Differential expression patterns of STAT3, STAT5A, and STAT5B in enalapril-treated HL60 cells. Expression levels of STAT3, -5A and -5B genes were examined in HL60 leukaemia cells treated with 7 μM of enalapril, by qRT-PCR. The results revealed that the expression of STAT3 decreased in enalapril-treated HL60 cells within the first 24 hours and the difference was of borderline statistical significance when compared to untreated controls (p<0.065). However, the change in the expression of the STAT3 gene for the total duration of the experiment was statistically insignificant as compared to the control group (p>0.05) (Figure 5A). At 24, 48 and 72 hours post-incubation, the expression of the STAT5A gene was significantly reduced in enalapril-treated HL60 cells when compared to untreated controls (p<0.05) (Figure 5B). The changes in the expression levels of the STAT5B gene were found to be statistically insignificant in enalapril-treated HL60 cells when compared to the negative control group (p>0.05) (Figure 5C).

Discussion

Many chemotherapeutic agents have been used for the treatment of acute leukaemia, but no definitive treatment has yet been described. Therefore, study of novel treatment regimens, including patient-based, in vitro and in vivo, are underway. For the first time, with this study, we evaluated the potential cytotoxic effect of enalapril, an ACE inhibitor generally used for treatment of high blood pressure, on human HL60 acute PML cells.

It is well known that haematopoiesis is regulated by several factors including growth factors, cytokines, cell surface receptors and the microenvironmental signals in the bone marrow. Since haematopoietic bone marrow is an area of excessive cell growth, the presence of a local, intrinsic RAS within the bone marrow was suggested (34). As ACE and other angiotensin peptides have a role in cellular processes, such as cell proliferation and migration, but also in angiogenesis (35-37), the involvement of RAS in the development of malignant haematopoiesis has gained significance (22, 25, 38). Recent data showed the existence of ACE in human primitive lympho-haematopoietic cells, and embryonic, foetal and adult haematopoietic tissues (39, 40). ACE inhibitors are widely used as anti-hypertensive medicines but lately they have gained significance as anticancer agents (32). Their antitumoural effects were first described in a study by Lever et al. (41) in which breast and lung cancer incidence decreased in patients receiving ACE inhibitors, including enalapril.

In the first stage of our study, we examined the potential cytotoxic and apoptotic effects of enalapril on HL60 cells, and investigated the possible mechanisms involved in cell death. The results of Trypan blue cell viability and XTT cell
Figure 3. Morphological examination of apoptosis in HL-60 cells treated with 7 μM of enalapril. AnnexinV-EGFP and PI staining method was used and cells were visualized by using fluorescence microscopy. The nuclei of cells that have lost membrane integrity are stained red (PI) and their plasma membranes are stained green (EGFP) (A). Cells that are stained both red and green are determined to be necrotic whereas green cells are apoptotic. Viable cells are shown in blue (B).
proliferation assays revealed that enalapril reduced viability and proliferation of HL60 cells in a dose- and time-dependent manner. Previously, the potential antiproliferative effect of captopril, another ACE inhibitor, was observed on primitive haematopoietic stem and progenitor cells in vitro (42), however, it was shown to have no effect on proliferation of myeloid leukaemia cells (43). Similarly to our results, in a recent study, the growth and colony-forming ability of AML cells was shown to decrease in vitro in a dose-dependent manner following incubation with an ACE inhibitor (44). In a different study, ACE inhibitors captopril and trandolapril, and losartan, which is an angiotensin II receptor antagonist, were shown to inhibit cell proliferation and induce apoptosis in K562 chronic myeloid leukaemia cells which seemed to be related to Ang II-induced small mothers against decapentaplegic (SMAD) activation (45).

Measurement of plasma membrane phosphatidylserine externalization, using fluorescently labelled annexin V, is widely used for the detection of apoptotic cells and is advantageous as it offers the possibility of detecting early phases of apoptosis before the loss of cell membrane integrity (46). In order to see if enalapril induces apoptosis in HL60 acute leukaemia cells, we incubated HL60 cells with a 7 μM dose of enalapril and applied the AnnexinV-EGFP staining method at certain time points. Apoptotic cells were morphologically examined by fluorescence microscopy and the results showed that necrotic cells were in abundance when compared to apoptotic cells. The results showed that there was a slight increase in the number of early apoptotic cells in enalapril-treated HL60 cells within the early stages of experiment, however, this difference was not permanent and remained statistically insignificant when compared to untreated controls until the end of the experiment.

STAT proteins play an important role in cellular processes. Dysregulation of the STAT pathway may lead to formation of malignant cells (15). It is already known that the JAK-STAT pathway is a vital element in the interaction between the components of RAS present in the bone marrow and haematopoiesis (47). In the second stage of our study, we aimed to identify the pathway(s) that play a role in the cytotoxicity of enalapril towards HL60 cells. Therefore, we investigated the expression levels of STAT3, STAT5A and STAT5B genes in enalapril-treated HL60 cells by qRT-PCR. The results showed that the change in expression levels of STAT3 and STAT5B were statistically insignificant (p>0.05) in enalapril-treated HL60 cells when compared to untreated controls, whereas the expression of STAT5A significantly decreased in a time-dependent manner in enalapril-treated HL60 cells when compared to the control group (p<0.05). These results suggest that STAT5A might have a significant role in enalapril-induced leukaemia cell death. Previously, enalapril was shown to inhibit Ang II-induced proliferation of rat cardiac fibroblasts in vitro by blocking the phosphorylation of STAT3 (48). In a recent study, retinal expressions of STAT3/5 were investigated in chemically induced diabetes in rats, in enalapril-treated and untreated control groups. Following the RT-PCR, STAT3 and STAT5 expression was found to be absent in the enalapril-treated group (49).
In conclusion, our study has shown for the first time that enalapril has cytotoxic and apoptotic effects on HL60 acute promyelocytic leukaemia cells and STAT5A may have significant roles in enalapril-induced cell death.

References


Received January 24, 2012
Revised February 26, 2012
Accepted February 29, 2012