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Original article

STAT pathway in the regulation of zoledronic acid-induced apoptosis in chronic myeloid leukemia cells

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ABSTRACT

In this study, we aimed to evaluate the cytotoxic and apoptotic effects of zoledronic acid on K562 chronic myeloid leukemia (CML) cells and to examine the roles of STAT genes on zoledronic acid-induced apoptosis. The results showed that zoledronic acid decreased proliferation, and induced apoptosis in K562 cells in a dose- and time-dependent manner. mRNA and protein levels of *STAT3*, *-5A* and *-5B* genes were significantly reduced in zoledronic acid-treated K562 cells. These data indicated that STAT inhibition by zoledronic acid may be therapeutic in CML patients following the confirmation with clinical studies.

1. Introduction

Chronic myeloid leukemia (CML) is a malignant disorder of the haematopoietic stem cell arisen from the reciprocal translocation between the breakpoint cluster region (*BCR*) gene on chromosome 22, and the Abelson (*ABL*) murine leukemia virus gene on chromosome 9, t(9;22)(q34;q11), resulting in the formation of Philadelphia (Ph) chromosome. Ph chromosome encodes the BCR–ABL fusion protein, which has constitutive tyrosine kinase activity, leading to leukemogenesis [1]. Imatinib mesylate, a selective inhibitor of the ABL tyrosine kinase, has demonstrated a remarkable efficacy in the treatment of CML by inducing cytogenetic remissions in over 75% of chronic phase patients, as the first line therapy [2]. However, drug-resistance and early relapses frequently occur in a considerable proportion of patients, as the main limitation for a prolonged survival [3]. Resistance to imatinib is caused primarily by point mutations in the kinase domain of BCR–ABL, which block drug binding [4]. To overcome this problem, more selective second generation ABL tyrosine kinases have been developed, but unfortunately, there is still a group of patients that fail to achieve sustained responses [5–7]. Therefore, new therapeutic approaches are being recently investigated in order to overcome the resistance problem and targeting the alternative downstream regulators of BCR–ABL seems promising.

It is known that BCR–ABL activates multiple signalling pathways, including rat sarcoma (Ras), myelocytomatosis (Myc), Janus kinase/signal transducers and activator of transcription (JAK/STAT) and Phosphoinositide 3-kinase/Akt (PI3K/Akt) that lead to uncontrolled proliferation and inhibition of apoptosis. Since the critical role of JAK/STAT pathway has been demonstrated in myeloid differentiation, novel therapeutic strategies have remarkably focused on targeting this pathway. STAT proteins are a family of latent cytoplasmic transcription factors that are involved in several cellular processes, such as proliferation, survival, apoptosis, and differentiation [8]. Constitutive or aberrant activation of STATs was hypothesized to cause cellular transformation, and in particular, leukemogenesis [9,10]. Seven mammalian STAT proteins have been discovered: STAT1 to -4, STAT5A, STAT5B, and STAT6 [11]. STAT1, -3 and -5 contribute to the progression or suppression of malignant transformation. STAT1, the first STAT to be discovered, is required in innate immunity, and has a tumour-suppressive role in oncogenesis as it mediates growth inhibitory signals and promotes apoptosis. It also contributes to the host rejection of tumours [12,13]. Inhibition of STAT3 pathway has been shown to induce apoptosis in cancerous cells by down-regulating the expressions levels of antiapoptotic B-cell lymphoma-extra large (Bcl-XL) and myeloid cell leukemia-1 (*Mcl-1*) genes [14,15]. Increased or unrestrained JAK2, platelet-derived growth factor receptor (PDGF-R), or ABL signaling by the expression of fusion proteins was shown to cause constitutive activation of STAT5, which is effective in the development of malignancy [13].

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Zoledronic acid, a nitrogen-containing bisphosphonate, is a potent inhibitor of osteoclast-mediated bone resorption, and it is primarily established in the management of metastatic bone disease. Accumulating preclinical data has suggested that zoledronic acid has also remarkable anticancer activities in a variety of solid and haematological malignancies [16–19]. Beside its direct anticancer effects like reducing proliferation and inducing apoptosis, it has also indirect effects like activating anticancer immune responses, inhibiting angiogenesis, and inhibiting interactions with mesenchymal stem cells [20–22]. It was shown that zoledronic acid inhibits the activation of Ras proteins through the suppression of both farnesyl transferase and geranylgeranyl transferase enzymes within the mevalonate pathway. These enzymes are essential for the prenylation of Ras proteins, and blocking prenylation results in reduced cellular growth and proliferation with induced apoptosis of cancer cells [23]. Ras is a major downstream signal transducer of BCR–ABL and on this basis, it has been demonstrated that zoledronic acid has a considerable antileukemic activity in CML cell lines and mouse models [24]. Furthermore, recent evidence suggests that there is a variety of anticancer mechanisms, which have been attributed to the action of zoledronic acid, and it is important to clarify the exact underlying mechanisms in order to determine the future anticancer strategies.

In this study, we aimed to evaluate the cytotoxic and apoptotic effects of zoledronic acid on human K562 CML cells and to clarify the roles of STATs on zoledronic acid-induced apoptosis.

2. Materials and methods

2.1. Cell line, culture conditions and chemicals

K562 CML cells were kindly provided by Dr. Serdar Bedii Omay from Ege University. K562 cells were grown in RPMI 1640 medium (Kibutz Beit Haemeh, Israel), containing 10% foetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA) and 1% penicillin–streptomycin were maintained in a standard cell culture incubator at 37 °C in 5% CO_2. Zoledronic acid was obtained from Novartis Pharma (Basel, Switzerland). Trypan blue dye was obtained from Sigma Chemical (St. Louis, MO, USA). 2,3-*Bis*[2-methoxy-4-nitro-5-sulphophenyl]-2*H*-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.

2.2. Measurement of cytotoxicity

Cytotoxicity assay and determination of the concentration of zoledronic acid that inhibits cell proliferation by 50% (IC_{50}) were performed by XTT cell proliferation assay (Biological Industries, Israel) as indicated in the manufacturer's instructions. Briefly, 1×10^4 K562 cells were plated into 96-well plates containing 100 μL of the growth medium in the absence or presence of increasing concentrations of zoledronic acid and incubated at 37 °C in 5% CO_2. After 24, 48, 72 and 96 h incubation, cells were treated with 50 μL XTT solution for 4 h and then, the plates were read under 492 nm wavelength by ELISA reader. Finally, the IC_{50} dose of zoledronic acid was calculated from the cell proliferation plots [25].

2.3. Evaluation of apoptosis

Zoledronic acid-induced apoptosis of K562 cells was evaluated by fluorescence microscopy using AnnexinV-EGFP Apoptosis Detection Kit (Biovision) as described in the manufacturer's instructions. In short, K562 cells were treated with 60 μM of zoledronic acid and apoptosis was induced. At 0, 24, 48, 72 and 96 h time points, 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 cover glass and morphologically examined by fluorescence microscopy.

2.4. Total RNA Isolation and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from K562 cells treated with 60 μM of zoledronic acid at 24, 48, 72 and 96 h post-incubation using High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) as described by the manufacturer. One microgram of total RNA was reverse transcribed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) 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, Mannheim, Germany). *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-F: 5′-ACCAACAATCCCAAGAATGT-3′, STAT3-R: 5′-CGATGCTCAGTCCTCGC-3′; STAT5A-F: 5′-GAAGCTGAACGTGCACATGAATC-3′, STAT5A-R: 5′-GTAGGGACAGAGTCTTCACCTGG-3′; STAT5B-F: 5′-AGTTTGATTCTCAGGAAAGAATGT-3′, STAT5B-R: 5′-TCCATCAACAGCTTTAGCAGT-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 Light Cycler 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 of the reference gene.

2.5. Western blot analysis

Protein levels of STAT3, STAT5A, STAT5B and β-actin, an internal positive control, genes were detected by Western blot analysis. For protein extraction; the cells were lysed by using ProteoJET Mammalian Cell Lysis Reagent (Fermentas, Maryland, USA), and subsequently incubated for 10 min on ice. Nuclei were removed by centrifugation at 20,000x*g* for 15 min, and the cell lysates were assayed for protein contents using the Bradford protein assay. Protein samples were separated by running on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Richmond, CA, USA), and then transferred onto a PVDF membrane. The primary antibodies used for immunblotting were anti-STAT3 (1:1000, rabbit polyclonal IgG, Upstate-Cell Signalling Solutions, Lake Placid, NY, USA), anti-STAT5A (1:2000, rabbit antiserum, Upstate-Cell Signalling Solutions), anti-STAT5B (1:2000, rabbit antiserum, Upstate-Cell Signalling Solutions) and anti-β-actin (1:1000, Cell Signalling Technology, Danvers, MA, USA). The protein levels of target genes in each fraction were detected by using the enhanced colorimetric detection kit (Amplified Alkaline Phosphatase Goat Anti-Rabbit Immune-Blot Assay Kit, Bio-Rad) according to the manufacturer's instructions.

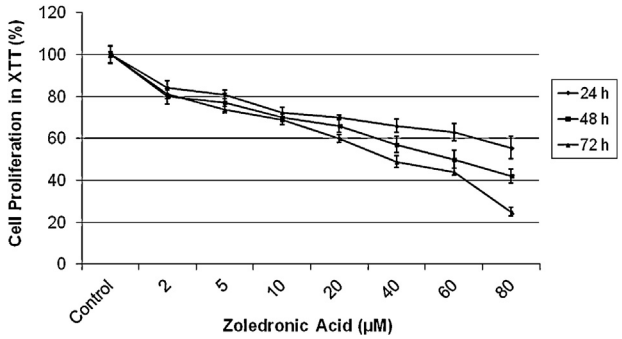

Fig. 1. Cytotoxic effects of zoledronic acid on K562 chronic myeloid leukemia cells. The concentration of zoledronic acid that inhibited cell growth by 50% (IC_{50}) was calculated from cell proliferation plots. The XTT assays were performed using duplicate samples in at least two independent experiments. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance (ANOVA), and $P < 0.05$ was considered to be significant. Control: untreated control cells.

The Western blot results were evaluated with a gel imaging system (Chemi-Smart 2000).

2.6. Statistical analyses

All data were uploaded to excel format and statistical analyses were performed by using SPSS 16.0 version in computer via appropriate method based on data type as parametrical or non-parametrical.

3. Results

3.1. Zoledronic acid reduced proliferation of K562 cells in a time- and dose-dependent manner

The degree of cytotoxicity induced by zoledronic acid on human K562 cells was assessed by XTT assay, and the results showed that zoledronic acid decreased cell proliferation significantly in a time- and dose-dependent manner (Fig. 1). The IC_{50} value of zoledronic acid was calculated from the cytotoxicity plots, and was determined to be 60 and 42 μM for 48 and 72 h, respectively.

3.2. Zoledronic acid-induced apoptosis in K562 cells

Significant increases were observed in apoptotic cell population in a time-dependent manner in zoledronic acid treated K562 cells. There were 23, 31, 34 and 34% apoptotic cell population in 24, 48, 72 and 96 h zoledronic acid exposed K562 cells (Fig. 2A). These values were statistically significant when compared to 10% apoptotic cell population in untreated control group ($P = 0.03$). Apoptotic cells were morphologically examined under fluorescence microscopy, and cellular changes were identified. Apoptotic cells were stained green, whereas necrotic cells were observed in red as a result of PI staining (Fig. 2B) and live cells were observed in blue (Fig. 2C).

3.3. Zoledronic acid decreased mRNA levels of STAT3, STAT5A, and STAT5B genes in K562 cells in a time-dependent manner

Expression levels of *STAT3*, *-5A* and *-5B* genes were examined in K562 cells treated with 60 μM of zoledronic acid by qRT-PCR.

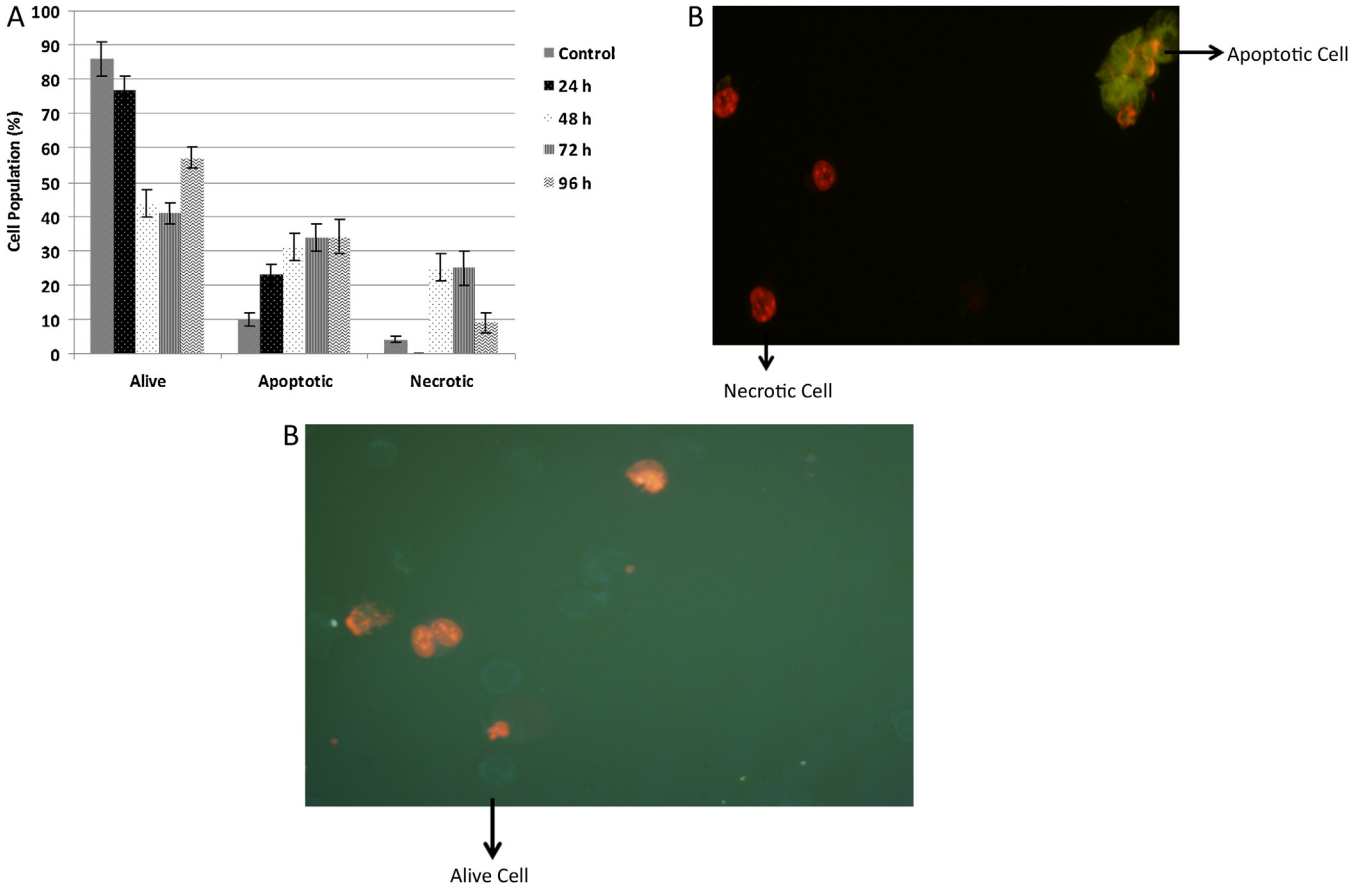

Fig. 2. Percentage changes in apoptotic, necrotic and alive K562 cells treated with 60 μM zoledronic acid (A) and microscopic examination of dead (B) and alive (C) cells. Statistical significance was determined using Student's *t* test, and $P < 0.05$ was considered to be significant.

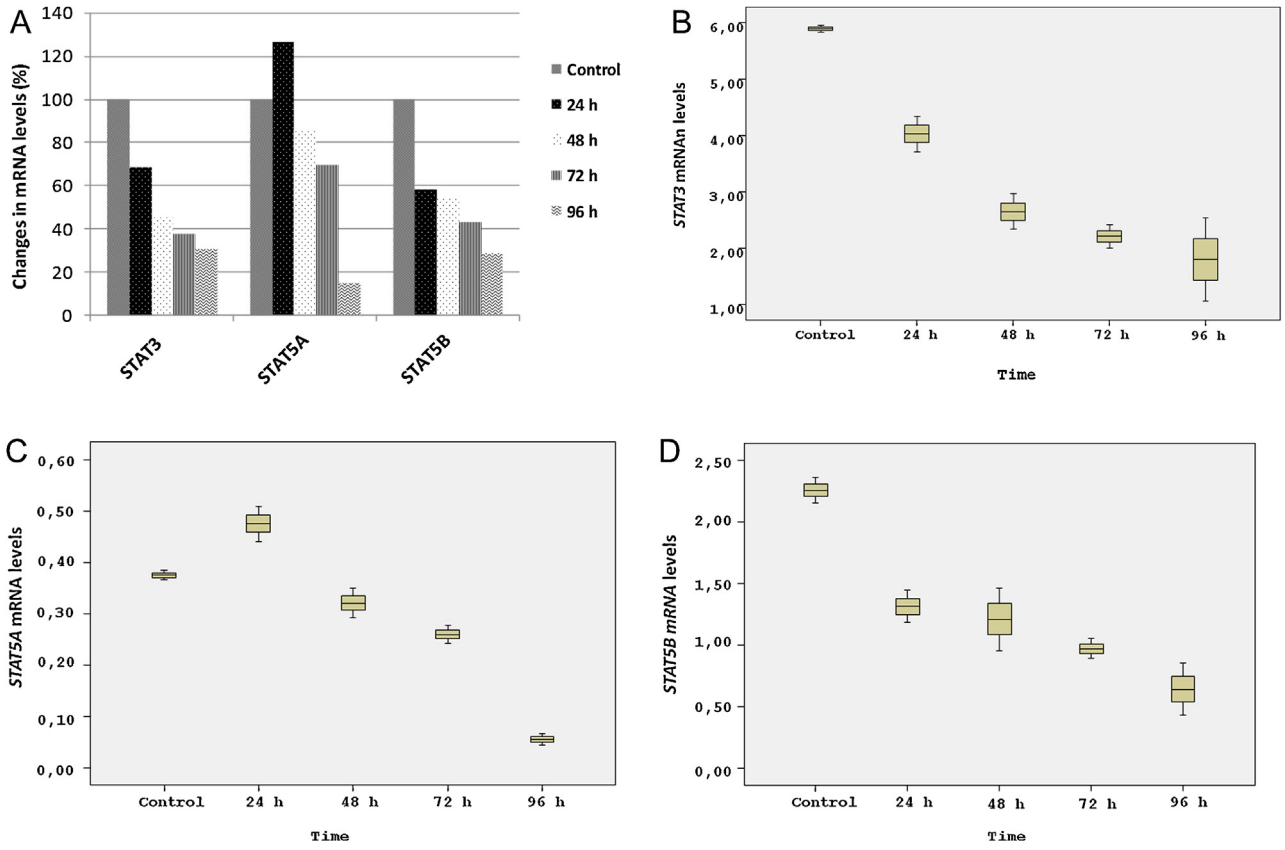

Fig. 3. Percentage changes in mRNA levels of *STAT3* (B), *STAT5A* (C) and *STAT5B* (D) genes in K562 cells exposed to 60 μM zoledronic acid at 24 h intervals over a 4-day period (A). mRNA levels of signal transducers and activator of transcription genes were normalized to G6PDH and compared to the untreated control group. Statistical significance was determined using ANOVA and $P < 0.05$ was considered to be significant.

As shown on Fig. 3A and 3B, zoledronic acid significantly decreased the expression levels of STAT3 in K562 cells in a time-dependent manner. There were 32, 55, 63 and 70% decreases in mRNA levels of *STAT3* gene at 24, 48, 72 and 96 h, respectively, when compared to untreated controls and normalized to G6PDH ($P = 0.014$) (Fig. 3A, B). The expression of *STAT5A* unexpectedly increased in zoledronic acid-treated K562 cells within the first 24 h, whereas there were 15, 31 and 85% decreases in mRNA levels of *STAT5A* gene after 48, 72 and 96 h incubation with zoledronic acid when compared to untreated controls and normalized to G6PDH. The change in the expression levels of *STAT5A* gene over the total duration of experiment was of borderline statistical significance as a result of the discordant values at 24 h time point ($P = 0.06$), but the suppression rates at 72 and 96 h time points were statistically significant as compared to untreated controls ($P = 0.001$ and $P < 0.001$) (Fig. 3A, C). There were 42, 47, 57 and 72% decreases in mRNA levels of STAT5B in K562 cells treated with zoledronic acid for 24, 48, 72 and 96 h, respectively, as compared to untreated controls and normalized to G6PDH and the difference was statistically significant ($P = 0.02$) (Fig. 3A, D).

3.4. Zoledronic acid decreased protein levels of STAT3, STAT5A, and STAT5B genes in K562 cells in a dose-dependent manner

The protein levels of STAT3, STAT5A and STAT5B in zoledronic acid-treated K562 cells were detected by Western blot analysis using specific anti-STAT antibodies. Protein levels of β-actin gene was detected as internal positive control. After alkaline phosphatase colorimetric reaction, protein levels were evaluated according to the thickness and darkness of the bands. The observations revealed that the protein levels of STAT3, STAT5A, and STAT5B were decreased significantly in a time-dependent manner, especially at 72 and 96 h time points (Fig. 4A, B, C). These findings were compatible with the results of the qRT-PCR analysis, thus the reduction of STAT expressions in transcriptional level were confirmed by the reduction in translational level.

4. Discussion and conclusion

Targeting the alternative downstream targets of BCR-ABL is a rational therapeutic strategy in order to overcome the tyrosine kinase inhibitor resistance in CML. Furthermore, to determine the future approaches, it is certainly needed to clarify the possible relations between the signal transduction pathways of cancer and the potential anticancer agents. Accumulating evidence has revealed that nitrogen-containing bisphosphonates, especially zoledronic acid, have potent anticancer activities in a variety of cancer cells [16,17,26–29]. The principal mechanism of apoptosis has been postulated to be through inhibition of the mevalonate pathway, which leads to inactivation of Ras-related proteins [16,26]. Ras is an important downstream target of BCR-ABL, thus it has been proposed that zoledronic acid may be a potential antileukaemic agent. In this study, we showed that zoledronic acid also interacts with the intracellular STAT pathway and induces apoptosis in the leukaemic clone.

Available preclinical data have demonstrated that zoledronic acid has antiproliferative and proapoptotic efficacy on its own, and also acts synergistically with other cytotoxic agents [30]. The first study that assessed the antileukaemic effects of zoledronic acid indicated that it has potent cytotoxicity on Ph(+) leukaemia cell lines, and also significantly augments the effects of imatinib

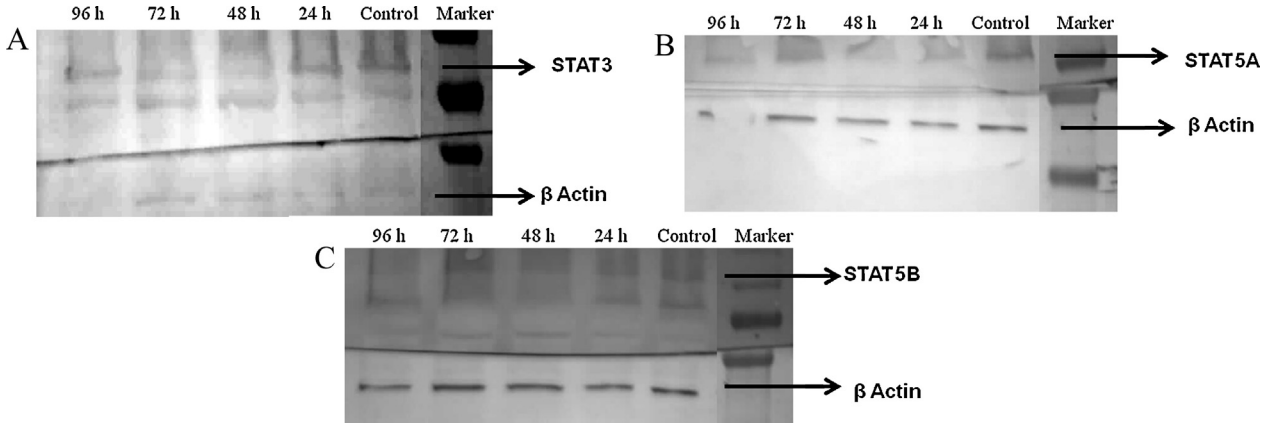

Fig. 4. Protein levels of *STAT3* (A), *STAT5A* (B) and *STAT5B* (C) genes in response to zoledronic acid. Western blot analyses were conducted for three independent experiments.

mesylate both *in vitro* and *in vivo*. It was also shown that zoledronic acid acts additively following the concurrent administration with hydroxyurea, cytarabine and daunorubicin in various leukaemia cell lines. However, the study performed by the same group suggested that zoledronic acid augments the *in vivo* effects of imatinib only against primary Ph(+) leukaemia cells that have not yet acquired resistance to imatinib [24,31,32]. Nevertheless, another striking study revealed that zoledronic acid is equally effective in inhibiting the proliferation and clonogenicity of both imatinib-sensitive and -resistant CML cells, regardless of their mechanism of resistance. The same study showed that the combination of imatinib and zoledronic acid also acts synergistically in imatinib-resistant CML cells [33]. On the basis of these preclinical studies, a phase I/II study was designed to assess the safety and efficacy of the combination of zoledronic acid with imatinib in 10 CML patients with a suboptimal response to imatinib alone. Although there was no haematological toxicity, no responses were demonstrated on the combination after 6 months [34]. These results were explained by low plasma concentrations of zoledronic acid, which were probably 10- to 100-fold less than the levels required to inhibit the cell growth or induce apoptosis in vitro, as previously reported [33]. With the support of several studies that showed tolerable toxicity using the dose of 16 mg administered every 28 days, it was suggested that increasing the dose of zoledronic acid might be effective [34–36]. Further large-scale clinical trials with escalated doses shall be designed to assess the factual antileukemic efficacy of zoledronic acid in CML patients.

In the first stage of our study, we examined the potential cytotoxic and apoptotic effects of zoledronic acid on K562 CML cells, and investigated the possible mechanisms involved in cell death. The results of XTT cell proliferation assays revealed that zoledronic acid decreased proliferation of K562 cells in a dose- and time-dependent manner. Fluorescently labelled annexin-V is widely used for the detection of apoptotic cells by measuring the plasma membrane phosphatidylserine externalization, and is also advantageous as it offers the possibility of detecting early phases of apoptosis before the loss of cell membrane integrity [37]. Apoptosis analyses showed that there was a remarkable increase in the number of apoptotic cells in zoledronic acid-treated K562 cells in a time-dependent manner. This difference was statistically significant when compared to untreated controls ($P = 0.03$) and indicated that zoledronic acid induces apoptosis in K562 cells.

STAT pathways play a pivotal role in oncogenesis and leukemogenesis, and thus targeting the STAT signalling pathway appears to be an effective anticancer treatment strategy. It has been described that constitutive activation of STAT3 and STAT5 plays a pro-oncogenic role both in acute and chronic myeloid neoplasms [38,39]. STAT5 signalling has been shown to play an important role in the antiapoptotic activity mediated by BCR-ABL by up-regulating the expression of the antiapoptotic gene Bcl-XL, and it is known that imatinib mesylate also induces apoptosis by this mechanism [40]. In the second stage of our study, we aimed to identify the pathway(s) that play role in zoledronic acid-induced apoptosis in K562 cells. Therefore, we investigated the mRNA and protein levels of *STAT3*, *-5A* and *-5B* genes in zoledronic acid-treated K562 cells by qRT-PCR and Western blot, respectively. The results demonstrated that there were significant decreases in mRNA and protein levels of STAT3 ($P = 0.014$), STAT5A ($P < 0.001$), and STAT5B ($P = 0.02$) genes. Based on these results, we suggested that STAT3, STAT5A and STAT5B may have a significant role in zoledronic acid-induced leukaemic cell death.

In conclusion, these data indicated that zoledronic acid inhibits cell proliferation and induces apoptosis via the STAT pathway in K562 CML cells, and if it can be conveyed to the clinical area, zoledronic acid may contribute to the treatment of CML as a promising therapeutic agent. Further drug-combination studies are needed to explore whether zoledronic acid could augment the antileukemic effects of tyrosine kinase inhibitors by potent STAT inhibition, and if it could be used as an appropriate approach to overcome drug-resistance or -intolerance in CML. Furthermore, in this study, we have shown for the first time that zoledronic acid triggers apoptosis through inhibiting the expression of STATs in any malignancy. This finding is important to propose that discovery or identification of novel agents targeted to STATs may open a new way for the treatment of different types of haematological and solid malignancies, associated with aberrant STAT expression.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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