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p21^{Cip1} confers resistance to imatinib in human chronic myeloid leukemia cells

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ABSTRACT

Imatinib is a Bcr-Abl inhibitor used as first-line therapy of chronic myeloid leukemia (CML). p21^{Cip1}, initially described as a cell cycle inhibitor, also protects from apoptosis in some models. We describe that imatinib down-regulates p21^{Cip1} expression in CML cells. Using cells with inducible p21 expression and transient transfections we found that p21 confers partial resistance to imatinib-induced apoptosis. This protection is not related to the G2-arrest provoked by p21, a decrease in the imatinib activity against Bcr-Abl or a cytoplasmic localization of p21. The results suggest an involvement of p21^{Cip1} in the response to imatinib in CML

Key words: p21, imatinib, chronic myeloid leukemia, apoptosis

1. Introduction

p21^{Cip1} (p21 herein after) is a member of the Cip/Kip family of inhibitors of cell cycle progression. The first discovered and so far best studied biochemical activity of p21 is to inhibit Cdks, consistent with its activity as cell cycle inhibitor [1-4]. Also, p21 is a relevant mediator of p53-cell cycle arrest [5-7]. However, more recent studies have shown that p21 has additional functions as differentiation inducer [8], transcriptional co-regulator [9, 10] and as an inhibitor of apoptosis induced by DNA-damaging agents [11-13]. The mechanism for p21-mediated apoptosis protection is unclear and is not operative in all cell types [14]. However, since cancer cells can escape death induced by chemotherapeutic drugs, the antiapoptotic p21 activity might be critically important in human cancer (reviewed in [15]). Actually, p21-null mice develop tumours spontaneously [16] and show increased tumour susceptibility using chemical carcinogenesis [17-20].

Imatinib is the main drug used in chronic myeloid leukemia (CML) treatment. The hallmark of this leukemia is the Bcr-Abl kinase, encoded by a fusion gene produced by the t(9;22) translocation [21, 22]. Several previous studies, including ours, have demonstrated that imatinib induced cell death in CML-derived cells, including K562 [23-25].

It has been reported that imatinib down-regulates p21 in mouse cells transfected with Bcr-Abl [26, 27]. However, it is unknown whether imatinib regulates p21 expression in human CML cells and the effects of p21 in these cells. In this work we describe that Bcr-Abl inhibition results in low p21 levels and that ectopic p21 expression antagonizes imatinib-mediated apoptosis, suggesting a role of p21 in the apoptosis response to Bcr-Abl inhibition.

2. Materials and methods

2.1 Cell culture and transfections

BV173 [28], KBM5 [29], MEG01 [30] and K562 [31] cell lines derive from human chronic myeloid leukemia in blast crisis. Kp21-4 subline, a K562 derivative with inducible expression of p21 was generated as previously described [32]. The cells were grown in RPMI 1640 medium supplemented with 8 % fetal calf serum and antibiotics. Unless otherwise stated, cells at a density of 2.5 x 10^5cells/ml were treated with 75 µM $ZnSO_4$ to induce p21 expression. Imatinib mesylate (provided by Novartis, Basel, Switzerland) was added one hour after $ZnSO_4$. K562 were transiently transfected by nucleofection (Amaxa) following the manufacturer indications with 4,75 µg of pCEFL-p21 [32] or empty vector and 0.25 µg of a Green Fluorescent Protein vector (pmaxGFP, Amaxa) to assess transfection efficiency.

2.2 Proliferation, cell cycle and apoptosis assays

Cell proliferation was assayed with a Nucleocounter (Chemometec). For cell cycle analysis, cells were fixed in 90% ethanol at 4ºC and resuspended in PBS-sodium citrate buffer containing 10 µg/ml bovine serum albumin, 200 µg/ml RNAse and 50 µg/ml propidium iodide (Sigma). Stained cells were analyzed by flow cytometry as previously described [14]. Apoptosis was assessed by annexin V binding and TUNEL assays. Annexin V binding was detected by flow cytometry using the BD-Pharmingen kit. TUNEL assays were performed using In situ Cell Death Kit (Roche Applied Science). Apoptosis in each cell cycle phase was determined by incubating cells with Hoescht 33342 (Sigma) (10 µg/ml for 90 min) to stain nuclei, and then annexin V binding was analyzed as above.

2.3 RNA extraction and expression analysis

Total RNA from cell lines and from bone marrow cells was isolated using the RNeasy kit (Qiagen). For reverse transcription and polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 1 μg of total RNA using SuperScript™ II RNase reverse transcriptase (Invitrogen) and random primers. Quantitative RT-PCR was performed as described [33] with a QuantiTect™ SYBR green PCR kit (Qiagen). The following primers were used: for p21 5'-AAGACCATGTGGACCTGTCA-3' and 5'-GGCGTTTGGAGTGGTAGAAA-3'; for ribosomal protein RPS14, 5'-GGCAGACCGAGATGAATCCTC-3' and 5'-CAGGTCCAGGGGTCTTGGTCC-3'. For each gene, duplicate PCR reactions were performed on the same 96-well plate on an ICycler iQ™ apparatus (Bio-Rad). The expression levels of p21 were normalized by the internal control ribosomal protein RPS14.

2.4 Immunofluorescence staining, immunobloting and cellular fractionation

Cytospin preparations were fixed with paraformaldehyde and the presence of p21 was detected by immunofluorescence using a rabbit polyclonal antibody (C-19 from Santa Cruz Biotech) and Texas Red conjugated secondary antibody (Dako). Actin cytoskeleton was detected using phalloidin-FITC (Sigma). Samples were mounted with Vectashield (Vector) containing 4'-6-diamidino-2-phenylindole (DAPI) to stain nuclei and examined under a fluorescence microscope (Zeiss-Axioskop2). Immunoblots were performed as previously described [14]. Blots were revealed with the ECL system (GE Healthcare). Antibodies used were: anti-actin (I-19), anti-poly(ADP-ribose)polymerase (PARP) (H250), anti-p27 (C-19), anti-UBF (F-9), anti-RhoGDI (A-20) and anti-phospho-tyrosine (PY20) all from Santa Cruz Biotech; anti-c-Abl from BD Pharmingen; anti-p21 (P-184) from Sigma, and anti-α-tubulin antibody kindly provided by Nicholas Cowan (New York University, New York). For cellular fractionation, cells were treated in 0.1% paraformaldehyde in PBS during 5 min and washed with 50 mM glycine in PBS. Nuclear and cytoplasmic extracts were then obtained essentially as described [34] using 50 mM Tris, 1% Nonidet P-40, 0.2% SDS, 1 mM EDTA, 10% glycerol as nuclear lysis buffer.

2.5. CML Patients

Bone marrow mononuclear cells from 17 healthy individuals and from 58 CML patients and were studied. CML cases included 36 samples collected at the time of diagnosis, 7 in blast crisis and 15 in chronic phase treated with imatinib. In this case samples were taken up to 24 months of treatment. The patients are from two hospitals:

Hospital Universitario Marqués de Valdecilla (Santander, Spain) and Hospital Universitario Dr. Negrín (Las Palmas, Spain). The study was approved by the ethics committees according to procedures approved by the two hospitals providing the samples. Statistical analysis was carried out with the SPSS software.

3. Results

We studied the expression of p21 in four CML cell lines (K562, MEG01, KBM5 and BV173) in response to imatinib treatment. p21 levels were low although detectable in growing cells, and decreased upon imatinib treatment (Fig. 1A). In sharp contrast $p27^{Kip1}$ expression, which was also very low, was unchanged or moderately up-regulated by imatinib. p21 down-regulation by imatinib in K562 was also detected at the mRNA level, as assayed by qRT-PCR (Fig. 1B). We next asked whether p21 down-regulation was involved in the cell death mediated by imatinib. To test this hypothesis we used a K562 subline with inducible p21 expression, termed Kp21-4 [32]. In these cells the expression of p21 is induced by $ZnSO_4$ and p21 levels were dramatically increased 3 h after $ZnSO_4$ addition and depended on the inducer concentration (Fig. 1C). Next, we demonstrated that imatinib did not modify the induction of p21 by Zn^{2+} in Kp21-4 cells (Fig. 1D). In these conditions, both p21 and imatinib provoked growth arrest (data not shown). It is reported in many models, including K562, that p21 induces accumulation of cells in the G2 cell cycle phase [32, 35]. We found p21 induced G2 accumulation in the presence of imatinib after 48 h of treatment, in contrast to the G1 arrest mediated by imatinib alone (Fig. 1E), thus confirming that the induced p21 was functional in the presence of imatinib. Altogether, the results indicate that imatinib did not impair p21 activity on cell cycle in the K562 model. At longer times (3-5 days) p21 induces polyploidy in K562 cells [32] but imatinib induced extensive apoptosis after long treatment periods [25], which precludes the analysis after long treatments.

We next asked whether p21 modified the apoptosis induced by imatinib. The results showed that p21 significantly reduced the imatinib-mediated apoptosis. This was assessed by several techniques as PARP proteolysis (Fig. 2A), TUNEL assay (Fig. 2B) and the fraction of cells with sub-G0/G1 DNA content (Fig. 2C). The protection that the induction of p21 exerts against the imatinib-mediated apoptosis was also demonstrated by determining the binding of annexin V to cell surface (Fig. 2D). As p21 induced G2 phase accumulation, we asked whether cells in G2 were less sensitive to imatinib-mediated apoptosis. However, in double-staining experiments (Hoechst

33342 and annexin V) we found that the fraction of apoptotic cells was similar in the G1 and G2 cell cycle phases (Fig. 2E).

To confirm that p21 was indeed the responsible for the apoptosis protection we first performed a dose-response experiment. Different p21 levels were induced with two $ZnSO_4$ concentrations (40 and 75 µM). As expected, the higher p21 levels correlated with a faster cell growth arrest (Fig. 3A) and with less apoptosis, as assessed by PARP proteolysis (Fig. 3B). The above results were obtained with the p21-inducible cell line Kp21-4. As this cell line was generated by selection of stable transfectants, [32] we wanted to rule out the possibility that the p21 effect was due to the specific behavior of the Kp21-4 cell line. Therefore, we transiently transfected parental K562 with an expression vector for p21. p21 overexpression upon transfection was confirmed by immunoblot (Fig. 3C). The results showed that p21-expressed cells were more resistant to imatinib-mediated apoptosis (Fig. 3D). Taken together, these results demonstrate that p21 antagonizes imatinib-induced apoptosis in K562 cells.

It has been reported that cytoplasmic but not nuclear p21, protects leukemia cells from apoptosis [36]. In addition, it is reported that in murine myeloid cells overexpressing Bcr-Abl, p21 is predominantly cytoplasmic and imatinib treatment results in decreased cytoplasmic p21 expression [37]. Thus, we studied the cellular localization of p21 in our model of Kp21-4 cells. Immunofluorescence studies showed that the induced p21 was found in the nuclei and remained nuclear after imatinib treatment (Fig. 4A). We also carried out nucleo-cytoplasmic fractionation studies of Kp21-4 extracts. Immunoblot analysis showed that p21 was predominantly found in the nuclear compartment and that imatinib did not modify this localization (Fig. 4B). p21 was also predominantly nuclear in untreated K562 (not shown). Finally, as imatinib-induced apoptosis depends on Bcr-Abl inhibition we assayed the effect of p21 on Bcr-Abl activity by determining the levels of phospho-tyrosine-Bcr-Abl. The results demonstrated that imatinib efficiently inhibited Bcr-Abl activity, and that the induction of p21 protein levels did not significantly modify this inhibition (Fig. 4C).

Although it is known that p21 is expressed in hematopoietic precursors (reviewed in [38]), the expression of p21 has not been systematically analysed in CML patients. We asked whether p21 levels correlated with CML progression. We determined the expression of p21 by quantitative RT-PCR in bone marrow samples of 36 CML patients at diagnosis, 15 in chronic phase under imatinib treatment and 7 cases of blast crisis (the final and fatal stage of CML) [39] as well as in 17 healthy bone marrow samples as controls. The results indicate that the expression of p21 mRNA was similar in all groups (Fig. 4D). The highest expression was observed cells from

patients in blastic phase. However, the difference was not statistically significant ($P > 0.05$).

4. Discussion

Here we show first that imatinib, the drug almost universally used in CML treatment, results in p21 down-regulation in human CML cells. Imatinib represses p21 expression at the transcriptional level. The mechanism is unknown, but it is unrelated to p53 as three of the four cell lines tested (K562, KU812 and MEG01) do not carry active p53 alleles [40]. p21 gene has a complex promoter, which is regulated by many transcription factors [41]. Imatinib effect could be mediated by STAT5, which transactivates p21 [42] and is activated by Bcr-Abl [43, 44].

It is striking that inhibition of Bcr-Abl activity results in the down-regulation of a protein known to stop cell cycle. However, p21 exerts apoptosis protection in many models [11-13]. We show here that induction of p21 protects K562 cells from imatinib-induced apoptosis. In our model, p21 did not rescue the growth-arrest induced by imatinib, conversely to the result reported with mouse cells [27]. The reasons for this discrepancy may lie, first, in that we are using human CML cells expressing endogenous Bcr-Abl instead of murine cells transfected with Bcr-Abl gene, and second, that we use an inducible p21 gene and transient p21 transfections, rather than stable selected clones with high constitutive p21 expression. Given the depressing effects of p21 on proliferation it is likely that stable constitutive transfectants have acquired other epigenetic changes.

The induced p21 remains in K562 cell nuclei in the presence of imatinib, ruling out in our system the described anti-apoptotic effect of cytoplasmic p21. In K562, as other cell models, p21 provokes the accumulation of cells in the G2 phase of the cycle, but imatinib did not modify this pattern. Moreover, we ruled out the hypothesis the drug is less active in G2 cells so as to explain p21-mediated protection. Thus, the mechanism for p21-mediated apoptosis protection in K562 remains unclear, as it is also the case in the other cell models where this effect has been observed [12, 13]. However, in K562 the mechanism operates downstream of Bcr-Abl inhibition as there is no significant change in the activity of Bcr-Abl mediated by p21. We also observed an increased level of p21 in cells from blastic crisis CML. This result seems consistent with the refractoriness of these cells to treatment with imatinib, although the difference in expression did not get statistical significance. Regardless of the mechanism involved,

our report describes for the first time the p21-mediated protection to apoptosis by the most used drug in CML, suggesting that p21 levels could modulate the response to the drug in CML treatments.

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Legends to Figures

Fig. 1. Effects of imatinib on p21 expression in CML cells. (A) Immunoblot for p21 and p27, and actin as loading control, in CML-derived cell lines treated with 1 μM imatinib for 24 h. (B) qRT-PCR analysis showing the down-regulation of p21 mRNA in K562 cells treated with imatinib. Data are expressed as means ± SEM. (C) Immunoblot analysis showing Inducible p21 expression in Kp21-4 cell line. Cells were treated with 75 μM $ZnSO_4$ and 0.5 μM imatinib for 3 to 48 h (left panel) or 40 and 80 μM $ZnSO_4$ for 6 h (right panel). (D) K562 and Kp21-4 cells were treated for 24 h with 0.5 μM imatinib and 75 μM $ZnSO_4$. The blot for p21 in K562 was overexposed with respect to Kp21-4. (E) Cell cycle analysis of K562 and Kp21-4 cells treated for 48 h with 1 μM imatinib and 75 μM $ZnSO_4$. The arrow indicates the position of G2 phase and the percentage of cells in G2 is indicated in each case.

Fig. 2. Ectopic expression of p21 confers partial resistance to imatinib-induced apoptosis. (A) Apoptosis was assessed by proteolytic cleavage of PARP determined by immunoblot in K562 and Kp21-4 cells treated with 75 μM $ZnSO_4$ and 1 μM imatinib for 48 h. (B) Kp21-4 cells were treated as in (A) for 24 and 48 h and TUNEL-positive cells were analyzed by fluorescence microscopy. Data are expressed as means ± SEM. (C) Kp21-4 cells were treated for 48 h as in (A). DNA content was measured by iodide propidium staining and flow cytometry and the fraction of cells with DNA content lower than 2C was scored. (D) Kp21-4 cells were treated for 48 h as in (A) and the fraction of cells annexin V-positive cells was determined by flow cytometry. Data are expressed as means ± SEM. (E) Cells were treated as in (A) and the apoptosis in each cell cycle phase was assessed by annexin V binding measured by flow cytometry. Black bars correspond to the fraction of annexin V positive cells, and the percentages of annexin V-positive cells within each cell cycle phase are indicated in each case.

Fig. 3. p21 antagonizes imatinib-induced apoptosis. (A) Cell proliferation analysis of Kp21-4 cells treated for 48h with 1 μM imatinib and 40 or 75 μM $ZnSO_4$. (B) Apoptosis of Kp21-4 cells assessed by PARP proteolysis in cells treated for 24 h with 1 μM imatinib and 40 or 75 μM $ZnSO_4$. (C) Expression of p21 in K562 cells nucleofected with a p21 expression vector. Cell lysates were prepared 24 h after nucleofection and analyzed by immunoblot. (D) Apoptosis assessed by cytometry in K562 transfected with p21. Imatinib (1 μM) was added 24 h after transfection and cells were further incubated for 24 h. Data are expressed as means ± SEM.

Fig. 4. Nuclear localization of p21 (A) p21 subcellular localization in Kp21-4 cells treated for 24 h with 75 μM $ZnSO_4$ and 1 μM imatinib. Cytospin preparations were subjected to immunofluorescence with anti-p21 and stained with phalloidin-FITC and DAPI to visualize cytoskeleton and nuclei, respectively. (B) Cellular fractionation of Kp21-4. Extracts from nuclear and cytoplasmic fraction were analyzed by immunoblot with anti-p21, anti-UBF (nuclear fraction control) and anti-RhoGDI (cytoplasmatic fraction control). (C) Inhibition of Bcr-Abl activity as determined by phospho-tyrosine-Bcr-Abl. Extracts of K562 and Kp21-4 cells were treated as in (A) and subjected to immunoblot using the antibodies against Bcr-Abl, c-Abl, p21, actin and phospho-tyrosine. The blot for p21 in K562 was overexposed with respect to Kp21-4. (D) p21 mRNA expression assayed by qRT-PCR in samples from CML patients at different stages of the disease. p21 mRNA levels were normalized with RPS14 expression. Each box refers to the range defined by the 25th and the 75th percentiles and the line indicates the median value. HC, healthy controls; CP-Dg, chronic phase at diagnosis; CP-Im, chronic phase treated with imatinib; BC, blastic crisis. In all comparisons the differences were not significant ($P > 0.05$).

Figure 1

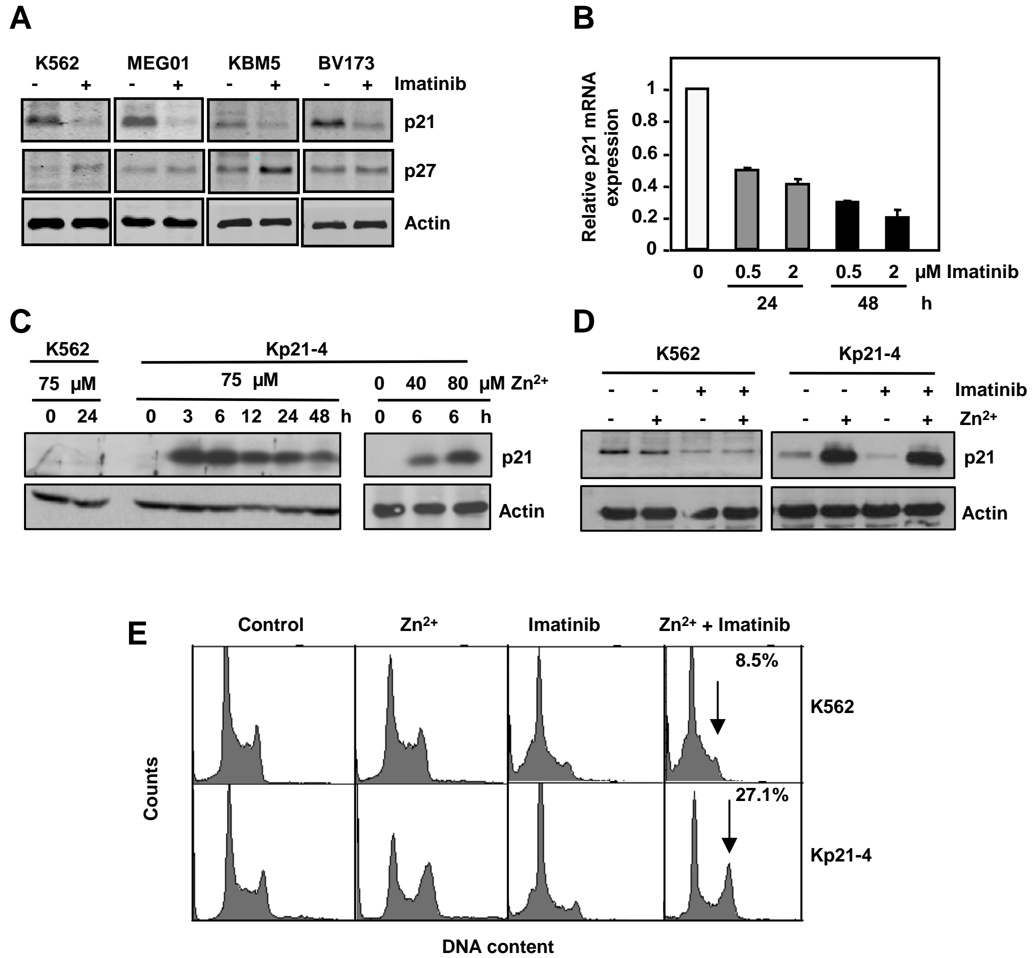

Figure 2

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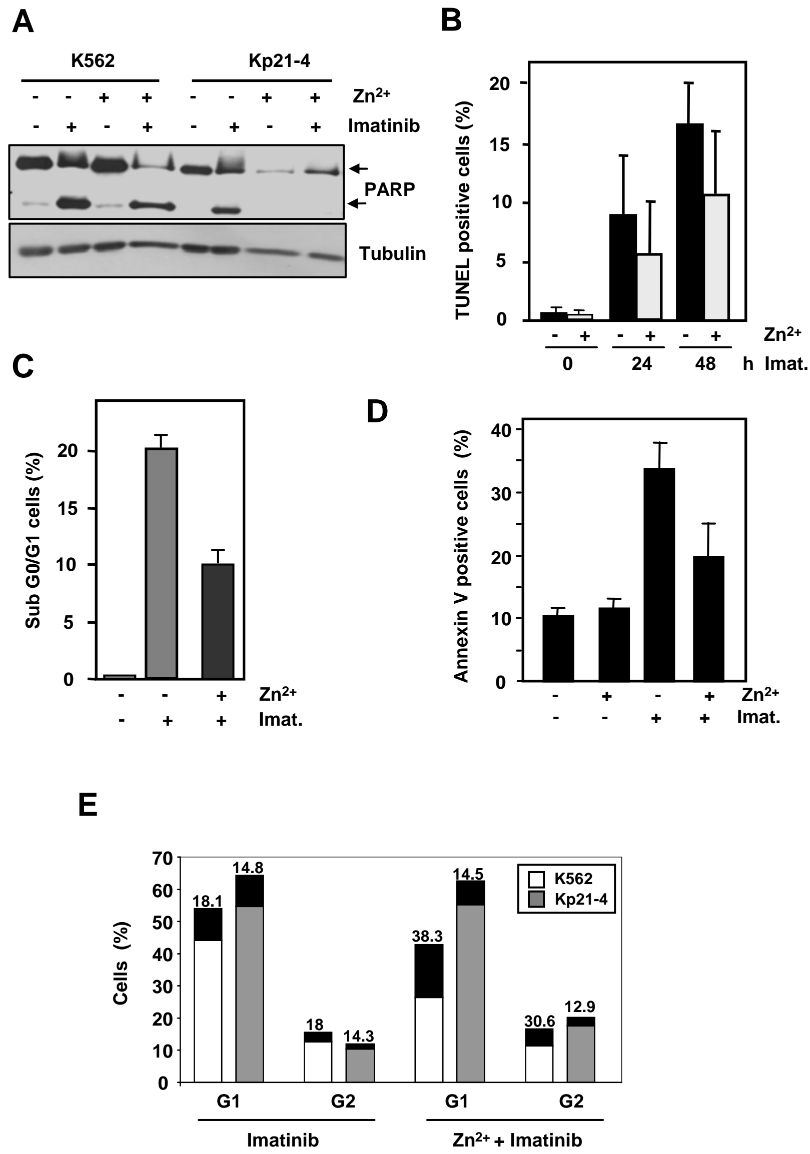

Figure 3

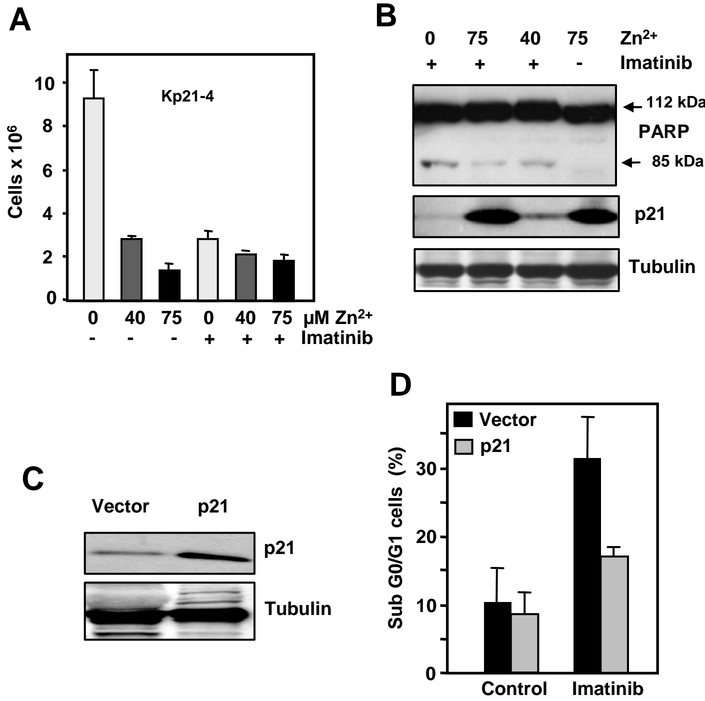

Figure 4