An. R. Acad. Nac. Farm., 2006, 72: 27-36

Artículo original

Antiapoptotic proteins Bcl2 and BclX do not protect chronic myeloid leukemia cells from imatinib-mediated growth arrest

Recibido el 31 de enero de 2006

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ABSTRACT

Imatinib (Glivec, Gleevec, STI571), a Bcr-Abl kinase inhibitor, is the most used drug in chronic myeloid leukemia. Imatinib induces apoptosis in a number of CML-derived cell lines, including K562. However, in order to achieve hematological remissions it is required chronic treatment with the drug, a fact inconsistent with a cytotoxic mechanism of imatinib in vivo. In this work we have analysed the effects of imatinib on the proliferation and apoptosis of K562-derived cell lines with constitutive expression of the anti-apoptotic genes Bcl2 and BclX. We found that imatinib-mediated apoptosis was completely abrogated in both Bcl2- and BclX-cell lines. However, imatinib inhibited proliferation, although growth rate was higher than in parental K562. We conclude that, besides its apoptotic effect, imatinib acts through an apoptosis-independent mechanism to arrest cell growth.

Key words: Bcl2.—BclX.—Imatinib.—K562.—CML.

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Abbreviations: CML, chronic myeloid leukemia.

RESUMEN

Las proteínas antiapoptóticas Bcl2 y BclX no protegen a las células de leucemia mieloide crónica de la parada proliferativa inducida por imatinib

El imatinib (Glivec, Gleevec, STI571) es un inhibidor de la quinasa Bcr-Abl, y es el fármaco de más uso en leucemia mieloide crónica (LMC). El imatinib induce apoptosis en varias líneas celulares derivadas de LMC, entre ellas K562. Sin embargo, para obtener remisión hematológica es necesario el tratamiento continuado con imatinib, un hecho no consistente con un mecanismo de acción citotóxico *in vivo* del fármaco *in vivo*. En este trabajo hemos analizado un los efectos del imatinib en la proliferación y apoptosis de líneas celulares derivadas de K562 con expresión constitutiva de las proteínas antiapoptóticas Bcl2 y BclX. Hemos encontrado que la apoptosis mediada por imatinib era completamente abolida en las líneas celulares con expresión de Bcl2 y BclX. Sin embargo, el imatinib inhibía la proliferación, aunque este efecto fue menos severo que en las células parentales K562. Concluimos que, además de su efecto apoptótico, el imatinib actúa a través de un mecanismo independiente de la apoptosis para detener la proliferación.

Palabras clave: Bcl2.—BclX.—Imatinib.—K562.—CML.

INTRODUCTION

Bcl2 and BclX (BclXL) are proteins of the BH2 super family. Both are mitochondria-located proteins with an apoptosis inhibitory activity. Most follicular lymphoma carry a chromosomal translocation involving Bcl2 locus that results in Bcl2 deregulation, and there are consistent reports of Bcl2 and BclX overexpression in many types of human cancer, through mechanisms different from chromosome translocation (1). Leukemia, including CML, is frequently associated to Bcl2 and BclX overexpression (2, 3). Impaired apoptosis is considered a crucial step in tumorigenesis. Indeed, a defective cell death program endows neoplastic cells with selective advantages, and with resistance to cytotoxic therapy.

Most of the patients of chronic myeloid leukemia (CML) are diagnosed in chronic phase, characterized by the clonal expansion of myeloid precursors, followed by a blastic crisis phase. The molecular hallmark of CML is the Bcr-Abl kinase, expressed in more than 95% of CML. This is a fusion protein with constitutive tyrosine kinase activity formed on the Philadelphia chromosome, that arises from a

reciprocal translocation between chromosomes 9 and 22 (4). Bcr-Abl is believed to be the major responsible for CML pathogenesis as it is able to transform hematopoietic cells and to induce myeloproliferative disease in mice after bone marrow repopulation with Bcr-Abl-expressing cells. The transformation pathways induced by Bcr-Abl are not fully understood, but it seems clear that apoptosis protection is a major mechanism by which Bcr-Abl allows the expansion of the leukemic clon (5, 6).

Although the outcome of CML in blast crisis is fatal, the disease can be cured by allogenic bone marrow transplantation during chronic phase. Interferon-a (IFNa) has been used in CML treatment, and in those patients where IFNa treatment failed, the treatment was based on hydroxyurea or busulfan (7). More recently, a new drug, imatinib (formerly STI571, trade name «Gleevec», or «Glivec»), was introduced in CML treatment. Imatinib is a potent inhibitor of the tyrosine kinase activity of Bcr-Abl and it is rapidly displacing other treatments for CML (8). Imatinib is active *in vitro* on CML-derived cells, and it has been proposed that imatinib mechanism of action involves the apoptosis that follows Bcr-Abl inhibition (5, 6, 8).

In the present work we study the effect of Bcl2 and BclX overexpression on the response to imatinib of a CML-derived cell line. Our data indicate that both proteins inhibit the imatinib-mediated apoptosis but imatinib still impairs cell growth. Thus, imatinib acts through apoptosis-dependent and apoptosis-independent pathways. The results suggests that the action of imatinib as anti-CML drug in vivo may depend more on its cytostatic effects than on its apoptotic effects.

MATERIAL AND METHODS

Cell culture and drugs

K562 cell line was obtained from the American Type Culture Collection. Kbcl2v cells are K562 infected with a Bcl-2 retrovirus and expressing high levels of Bcl-2 protein. K562-BclX_L (KbclX) are cells transfected with Bcl-X_L (9). All cell lines were grown in RPMI 1640

medium (Invitrogen) containing 8% fetal calf serum and gentamycin (80 mg/mL). For proliferation assays, exponentially growing cells at a concentration of 250,000 cells per mL were treated with the indicated concentrations of hydroxyurea (Sigma), IFNα (kindly provided by Roche Farma, Madrid, Spain), busulfan (Sigma) and imatinib (kindly provided by Novartis, Basel, Switzerland).

Cell proliferation and apoptosis assays

Cells were counted in a hemocytometer. When appropriate, cell viability was scored with trypan blue vital staining. For thymidine incorporation assays, cells were incubated with 1 μCi/mL of ^{3}H-thymidine for 2 h, harvested onto glass wool filters and the radioactivity was counted by liquid scintillation. Each experiment was repeated at least three times. Cells with apoptotic morphology were analyzed by May-Grünwald-Giemsa staining of cytocentrifuge preparations, and scored by light microscopy. Binding of annexin V to cell surface was carried out by flow cytometry with annexin V-FITC following the manufacturer's instructions (Genzyme Diagnostics).

Northern analysis

RNAs were prepared by the guanidine thiocyanate method. RNAs (15 μg of total RNA per lane) were separated by electrophoresis through agarose-formaldehyde gel and transferred to nitrocellulose. Probe labeling with [α-^{32}P]dCTP and filter hybridization were carried out according to standard procedures. Probe for human *BCL2* was a 1.8 kb fragment from human cDNA.

RESULTS AND DISCUSSION

In order to investigate a possible effect of Bcl2 and BclX on the antiproliferative activity of imatinib , we compared the growth rates of K562 (parental cells), Kbcl2v and KbclX cells in response to imatinib at two concentrations of the drug (0.5 μM and 2.5 μM). The results are shown in Fig. 1A, and demonstrate that imatinib

significantly inhibited the growth of Kbcl2v and KbclX cells, athough to a lesser extent than K562.

It was conceivable that imatinib impaired the expression of the ectopic Bcl2 or BclX genes. To test this possibility we analysed the Bcl2 mRNA in Kbcl2v cells treated with imatinib. The results (Fig. 1B) showed that imatinib did not affect Bcl2 expression at concentrations that were effective on the growth of Kbcl2v cells. As previously described, parental K562 do not express Bcl-2 (9).

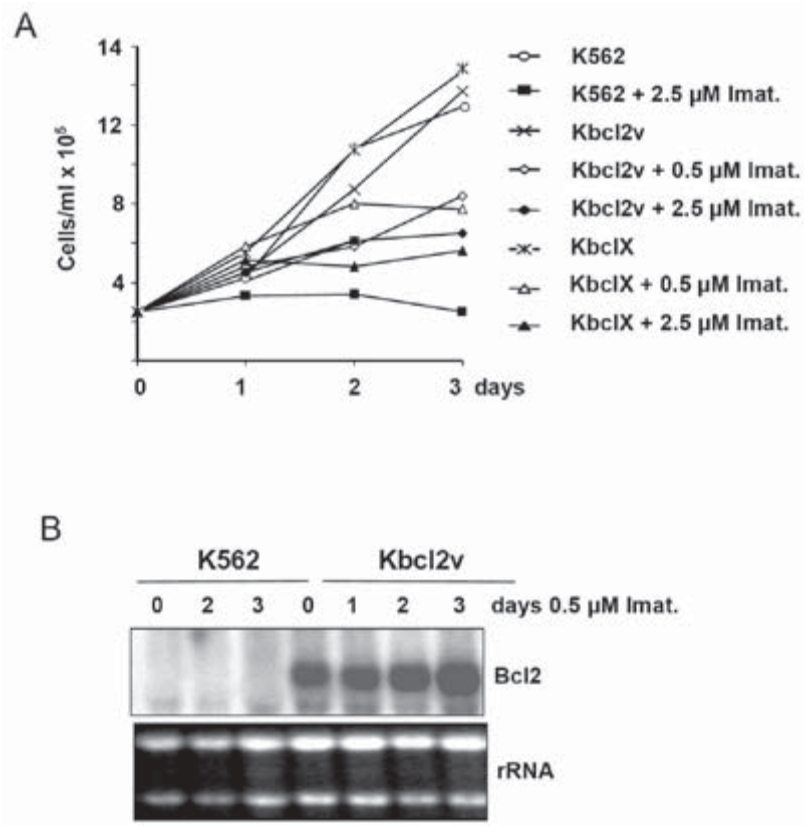

FIGURE 1. *Effect of imatinib on K562 expressing Bcl-2 and Bcl-X_L. (A) Inhibition of proliferation determined by DNA synthesis in K562, Kbcl2v and KbclX treated for the indicated period of times with 0.5 µM and 2.5 µM imatinib. (B) BCL2 expression in K562 and Kbcl2v cells treated for 1, 2 or 3 days with 0.5 µM imatinib, as analyzed by Northern blot analysis. A picture of the filter after transfer showing the rRNAs stained with ethidium bromide is shown to assess the loading and integrity of the RNAs.*

The retardation in growth rate of Kbcl2v and KbclX provoked by imatinib could be explained by reduced cell proliferation or by increased apoptosis. To investigate this point we determined the DNA synthesis rate of DNA by the ^{3}H-thymidine incorporation method in the three cell lines treated with 0.5 µM and 2.5 µM imatinib for 48 and 72 h. The results indicate that DNA synthesis in parental K562 (as well as cells transfected with the empty vector, KXLSN, not shown) was almost completely suppressed after 72 h of treatment with 0.5 and 2.5 µM imatinib. Imatinib also decreased the DNA synthesis in Kbcl2v and KbclX in a time- and dose-dependent manner (Fig. 2A). However, DNA synthesis rate in Kbcl2v and KbclX was not suppressed but dramatically reduced (20% and 30% respectively with respect to controls). These differences in DNA synthesis were roughly consistent with the growth curves of the distinct cell lines (Fig. 1A).

Next we compared the effect of imatinib with the other three drugs used in CML treatment (hydroxyurea, busulfan and IFNα). We chose drug concentrations slightly above the minimal cytostatic concentrations for the three cell lines: 2000 UI of IFNα per mL, 0.5 mM hydroxyurea and 0.5 mM busulfan (data not shown). K562, Kbcl2v and KbclX cells were treated with the drugs at the indicated concentrations for 48 h and the ^{3}H-thymidine incorporation was measured. The results (Fig. 2B) indicate that neither Bcl-2 nor Bcl-X_L conferred resistance to the antiproliferative effect of busulfan, IFNα and hydroxyurea in K562, in accordance with the absence of cells showing apoptotic morphology (not shown).

Next we analysed whether Bcl2v and BclX were capable to antagonize the apoptosis elicited by imatinib in K562. We measured the apoptosis extent by determining the binding of annexin V to cell surfaces. This protein binds to phosphatidyl serine, which is exposed to the outer layer of plasma membrane during apoptosis. K562, Kbcl2v and KbclX cells were treated with annexin V-FITC and binding determined by flow cytometry. The results (Fig. 3A) indicate that imatinib-mediated apoptosis was dramatically reduced in Kbcl2v and KbclX. Similar result was observed by scoring cells with apoptotic morphology after Giemsa staining of the cells. Cytospin preparations of K562 cells treated with imatinib demonstrated a

high fraction of apoptotic cells which were absent in the Kbcl2v and KbclX cells (Fig. 3B), thus confirming the result observed through the annexin V assay.

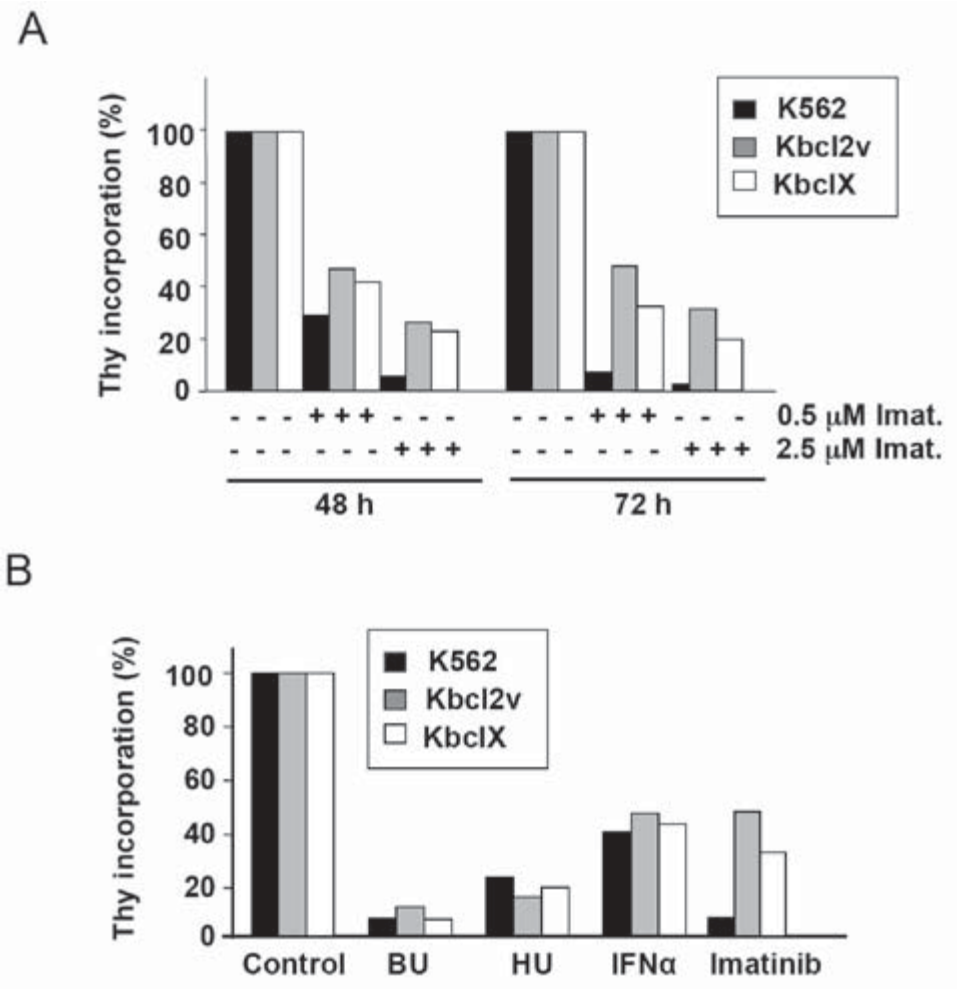

FIGURE 2. *(A) Inhibition of DNA synthesis measured by ^{3}H-thymidine incorporation in K562, Kbcl2v and KbclX treated with 0.5 μM and 2.5 μM imatinib for 48 h and 72 h as indicated at the bottom of the graph. (B) Inhibition of DNA synthesis in K562, Kbcl2v and KbclX treated with 0.5 mM busulfan (BU), 0.5 mM hydroxyurea (HU) and 2000 UI/ mL of IFNa for 72 h. The data obtained with 0.5 μM imatinib is included for comparison.*

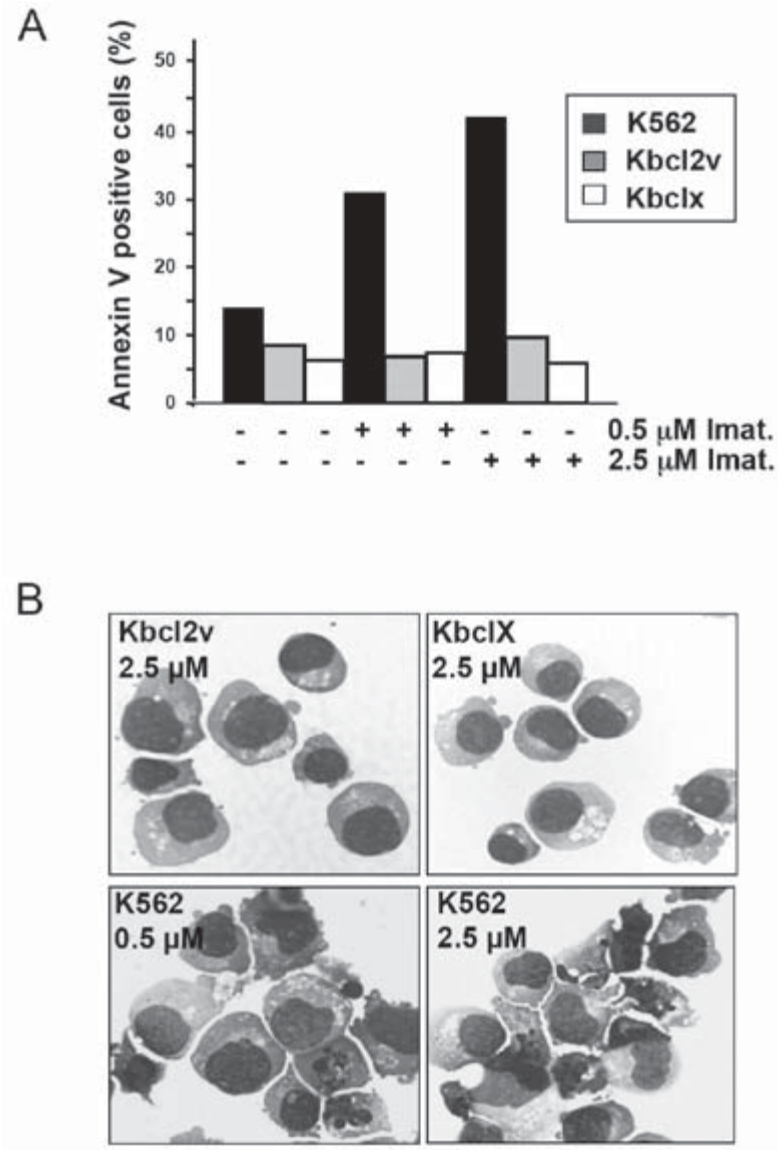

FIGURE 3. *Lack of apoptosis of Kbcl2v and KbclX cells in the presence of imatinib. (A) Cells were treated for 2 days with 0.5 μM or 2.5 μM imatinib as indicated and the extent of apoptosis was assessed by the binding of annexin V to cell surface as determined by flow cytometry. (B) Cytospin preparations stained with May Grumwald-Giemsa of the indicated cells treated with 0.5 μM or 2.5 μM imatinib for 2 days. Note the absence of apoptotic cells in the Kbcl2v and KbclX cell lines.*

These results confirm that Bcl2 and BclX conferred complete resistance to imatinib-mediated apoptosis. Although it has been proposed that apoptosis induction is a major mechanism for the cytotoxic effect of imatinib *in vitro* (5, 6) our results underscores the importance of apoptosis-independent mechanisms for imatinib antiproliferative activity. It is conceivably that non-apoptotic mechanisms are responsible for the cytostatic effect of imatinib, and allows the restoration of cell growth when the drug is removed. In consistency with this result, it has been reported that imatinib selectively suppresses CML primitive progenitors by reversing abnormally increased proliferation but does not significantly increase apoptosis (10).

Thus, our results suggest that the growth inhibitory effect of imatinib is mediated two concomitant mechanisms: one cytotoxic mechanism that triggers an apoptotic program and another cytostatic mechanism unrelated to apoptosis. This second mechanism would be the responsible for the growth retardation induced in cells expressing Bcl2 and BclX. Also, the results suggest that this cytostatic mechanism could be important for the imatinib activity *in vivo*.

ACKNOWLEDGEMENTS

We thank Pilar Frade, María Aramburu and Guillermo Santana for technical assistance, Novartis for imatinib, Roche España for interferon a and Jose Luis Fernández-Luna for KbclX cell line. The work was supported by grant PM98-0109 and SAF2002-04193 from Spanish Ministry of Science and Technology to J.L.

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