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REPORT

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# CDKIs p18$^{INK4c}$ and p57$^{Kip2}$ are involved in quiescence of CML leukemic stem cells after treatment with TKI

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**ABSTRACT**

Chronic Myeloid Leukemia (CML) is sustained by a small population of cells with stem cell characteristics known as Leukemic Stem Cells that are positive to BCR-ABL fusion protein, involved with several abnormalities in cell proliferation, expansion, apoptosis and cell cycle regulation. Current treatment options for CML involve the use of Tirosine Kinase Inhibitor (Imatinib, Nilotinib and Dasatinib), that efficiently reduce proliferation proliferative cells but do not kill non proliferating CML primitive cells that remain and contributes to the persistence of the disease.

In order to understand the role of Cyclin Dependent Kinase Inhibitors in CML LSC permanence after TKI treatment, in this study we analyzed cell cycle status, the levels of several CDKIs and the subcellular localization of such molecules in different CML cell lines, as well as primary CD34$^{+}$CD38$^{-}$lin$^{-}$ LSC and HSC.

Our results demonstrate that cellular location of p18$^{INK4c}$ and p57$^{Kip2}$ seems to be implicated in the antiproliferative activity of Imatinib and Dasatinib in CML cells and also suggest that the permanence of quiescent stem cells after TKI treatment could be associated with a decrease in p18$^{INK4c}$ and p57$^{Kip2}$ nuclear location. The differences in p18$^{INK4c}$and p57$^{Kip2}$activities in CML and normal stem cells suggest a different cell cycle regulation and provide a platform that could be considered in the development of new therapeutic options to eliminate LSC.

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## Introduction

Chronic Myeloid Leukemia (CML) is a haematopoietic disease characterized by the presence of the Philadelphia chromosome (Ph), a shortened chromosome 22 originated by the reciprocal translocation between long arms of chromosomes 9 and 22. This abnormality results in the p210 BCR-ABL fusion protein, involved with abnormalities in cell proliferation, expansion, inability to adhere to marrow stroma, and inhibition of apoptosis.[1,2] Knowledge on the role of p210 BCR-ABL in the pathogenesis of CML leads to the development of drugs that inhibit its tyrosine kinase activity. Current treatment options for CML involve the use of Imatinib, Nilotinib and Dasatinib, 3 drugs that act through competitive inhibition of the ATP-binding site in the BCR-ABL kinase domain and that have proved to be effective in 80% of the patients. However, the other 20% remain insensitive due to mechanisms that involve resistance or intolerance to such drugs.[3-5]

CML is sustained by a small population of cells with stem cell characteristics, known as Leukemic Stem Cells (LSC). Just like normal haematopoietic stem cells (HSC), LSC express CD34, and lack CD38, CD71 and lineage specific markers (lin$^{-}$); however, in contrast to their normal counterpart, CML LSC are positive for CD26 and IL1-RAP.[6-9] It is noteworthy that CML LSC are quiescent, thus, they are insensitive to most drugs used in the clinic. Both normal HSC and LSC coexist in the marrow of CML patients, being the HSC responsible for recovery after treatment with Tirosine Kinase Inhibitors (TKI). However, in recovered patients the quiescent LSC remain viable and insensitivity to TKI, so they can spontaneously exit from quiescence, proliferate and contribute to relapse when TKI treatment is discontinued.[5,10,11]

Different reports have shown that BCR-ABL could be involved in different cell processes, such as the transition from $G_1$ to S in the cell cycle, DNA synthesis, activation of Cyclin-Dependent Kinases (CDK), and deregulation of the cyclin-dependent kinase inhibitors (CKDIs) p27$^{Kip1}$ and p21$^{Cip1}$ by decreasing their nuclear location by cytosolic relocalization and sustaining p27$^{Kip1}$ ubiquitination-dependent proteasomal degradation. Interestingly, treatment of CML cell lines and CD34$^{+}$ cells from CML patients with Imatinib results in the nuclear accumulation of p27$^{Kip1}$ and p21$^{Cip1}$ up regulation.[12-16]

In order to understand the role of CDKIs in the response of CML LSC to TKI, and in trying to explain their possible role in

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CML LSC permanence after treatment, in the present study we addressed different aspects related to cell cycle in CML cells. To this end, we used different CML cell lines, as well as primary $CD34^+CD38^-lin^-$ LSC and HSC, and analyzed their cell cycle status, the levels of several CDKIs and the subcellular localization of such molecules.

## Results

### *Tyrosine kinase inhibitors reduce viability and $G_0$ cell cycle arrest in human CML cell lines*

We first evaluated the effects of both Imatinib and Dasatinib -at different doses- on cell viability, proliferation, and cell cycle of $CD34^+lin^-$ cells from normal marrow, as well as in 2 different CML cell lines. Cells were maintained for 48 hours in the absence or presence of different concentrations of TKI; the latter were based on the level reported in plasma after in vivo treatment.[19] Figure 1 shows that regardless of the concentration of TKI, the frequency of viable cells (identified as 7AAD-negative cells) in the NBM $CD34^+lin^-$ cell population remained with a percent of viability between 85–95%. In contrast, in K562 and MEG01 cell lines, treatment with Dasatinib and Imatinib increased the frequencies of dead cells in a dose-dependent manner (Fig. 1A). With Dasatinib, the percentage of K562 alive cells was reduced to 65%, when comparing 150 nM to control conditions, whereas for MEG-01 cells, the reduction was 80%. For Imatinib, on the other hand, the percentage of alive cells was between 65–75% for K562 and 75% for MEG01 cells (Fig. 1B).

After treatment with TKIs, CFSE levels were determined in the remaining live cells. As shown in Figure 2, both Dasatinib and Imatinib were able to reduce the levels of CFSE in the CML cell lines but not in primary NBM $CD34^+lin^-$ cells, suggesting a selective delay in cell proliferation (Fig. 2A). It is important to mention that in the case of normal cells, there is an increase in CFSE level (Fig. 2B) independently to the TKI and concentration used and suggest that the total cell population was increased unless 1 folds. This same effect is detected in CML cell lines without treatment (control) were the proliferation index increase since 1 to 1.6 or 2 in K562 or MEG-01 cells respectively. However when the cells were treated with Dasatinib the proliferation index was only 1.2 in both cell lines or 1 to 1.3 after treatment with Imatinib in MEG-01 or K562 cells, respectively (Fig 2B). These results suggest an inhibition in cell proliferation in CML cells lines as a consequence to the treatment in vitro with TKI.

In trying to determine the specific point on the cell cycle at which Dasatinib and Imatinib stop cell proliferation; we analyzed the distribution of the different cell cycle phases. To do this, CML cell lines or $CD34^+lin^-$ NBM were cultured in with or without TKI for 48 hours and then were stained with the nuclear proliferative antigen Ki67 and 7AAD. Figure 3 shows that in normal cell no changes were observed in the cell cycle phases; in contrast, a significant accumulation of cells in $G_0/G_1$ was evident in response to Dasatinib or Imatinib. Indeed, for K562 cells an increase from 1.2% to 10% was observed for cells in $G_0$ and from 18% to 60% for cells in the $G_1$ phase with both TKIs. For MEG-01 cells, the frequency of cells in $G_0$ went from 1% to 10% with Imatinib, and cells in $G_1$ went from 50% to 70–80% for Imatinib and Dasatinib. Accordingly, a significant reduction was observed for cells in $G_2$ without major changes in the S phase. These results confirm that TKIs induce accumulation of cells in the early phases ($G_0/G_1$) of the cell cycle at the expenses of the $G_2$ phase (Fig. 3B).

### *Cell cycle arrest by TKIs is related to CDKIs levels*

Considering the above results and knowing that progression from $G_1$ to S phase of cell cycle is regulated by 2 families of Cyclin-Dependent Kinase Inhibitors (CDKIs): the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) and the INK4 family ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ y $p19^{INK4d}$), Kip2 and with the intention to describe the relationship of these molecules with the increase in $G_0/G_1$ phases in CML leukemic cells after treatment with Dasatinib and Imatinib, we analyzed the ARN expression and protein level of each one of the CDKIs after 48 hours of treatment. Figure 4 shows that expression of CDKIs ($p18^{INK4c}$, $p19^{INK4d}$, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) did not change in response to Dasatinib or Imatinib in both CML cells lines; in the case of $p15^{INK4b}$ and $p16^{INK4a}$, the status was not evaluated since has been reported homozygous deletion of p16 in the 50% of CML patients and $p15^{INK4b}$, for his part is frequently loss in myeloid diseases.[20–22]

In contrast to ARN expression, $p18^{INK4c}$ and $p57^{Kip2}$ protein levels had a significant reduction when K562 and MEG-01 cell lines were treated with Dasatinib or Imatinib. Protein levels for $p21^{Cip1}$ and $p27^{Kip1}$, on the other hand, did not change (Fig. 5A). Importantly, in $CD34^+lin^-$ NBM cells there were no changes in CDKI protein levels after TKI treatment; this latter result seems to be in keeping with the cell cycle status and the tendency to reduce the percentage of cells in $G_0/G_1$ (Fig. 5B). These results suggest that permanence in $G_0/G_1$ phases in CML cells lines is directly related to modifications in $p18^{INK4c}$ and $p57^{Kip2}$ protein levels.

### *Cellular relocation of CDKIs after treatment with TKIs*

Several reports have demonstrated that CDKI level and function are controlled by their phosphorylation status and sub-cellular localization; thus, considering that $p18^{INK4c}$ and $p57^{Kip2}$ protein level were reduced in response to TKIs, we evaluated the effect of Dasatinib and Nilotinib on the levels of both proteins in cytoplasm and nucleus in K562 cells. Figures 6A and 6B show that levels of $p18^{INK4c}$ and $p57^{Kip2}$ were increased in the nuclear fraction after treatment with CDKIs, whereas their levels were decreased in the cytoplasm fraction. These results seem to be in keeping with a reduced metabolic activity reported in cells that remain in $G_0/G_1$. It is important to notice that in cultures without TKI treatment, the levels of both proteins were higher in relation to cultures that had been exposed to the inhibitors. However, when the cells were exposed to Dasatinib or Nilotinib the concentration of total protein was reduced and there was a significant increase in the nuclear fraction. In the case of $p27^{Kip1Kip1}$, the level of nuclear or cytoplasmic protein never

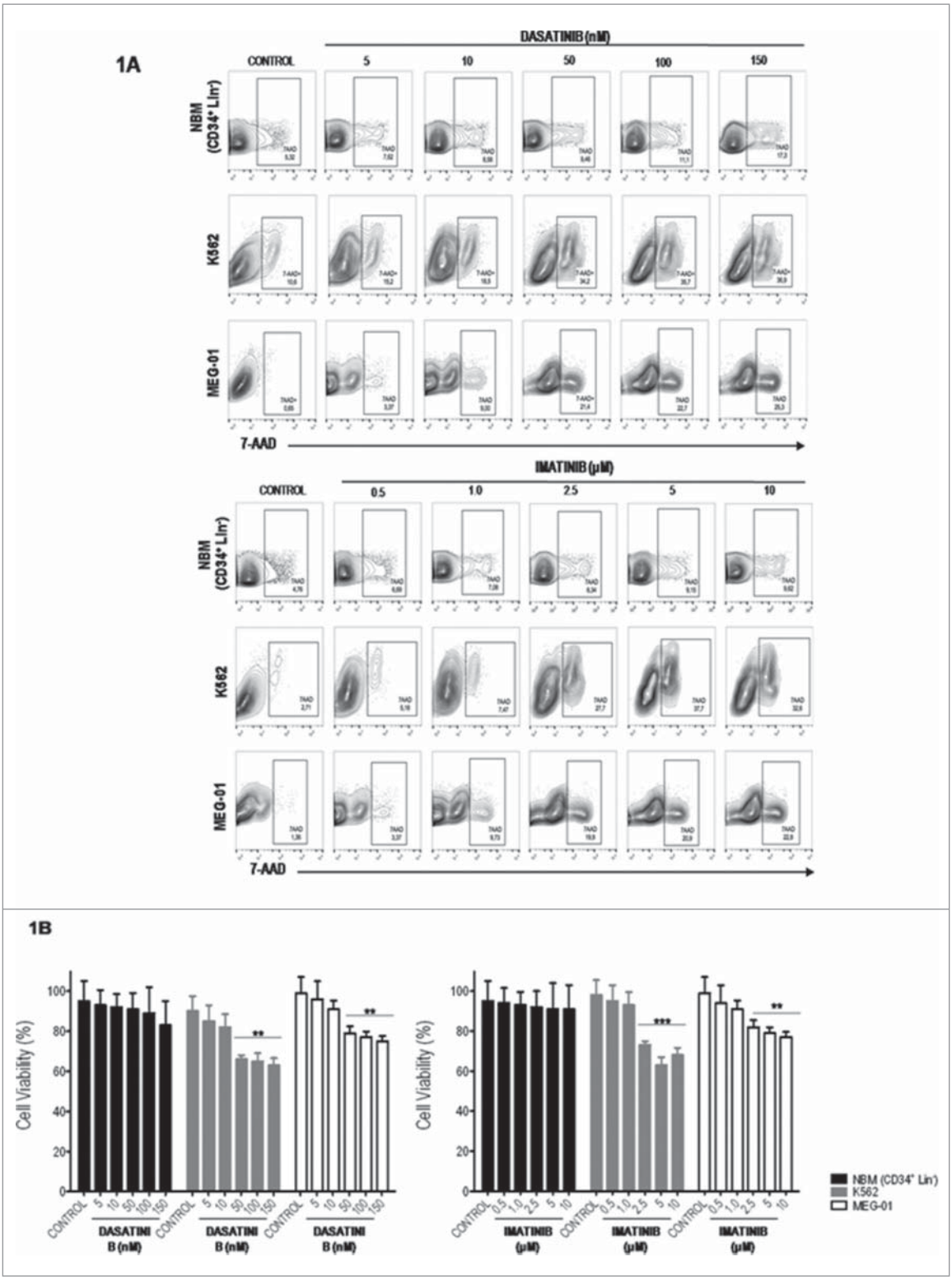


**Figure 1.** Imatinib and Dasatinib reduce viability of CML cells. Normal marrow-derived (NBM) $CD34^{+}Lin^{-}$ cells, K562 and MEG-01 CML cell lines were cultured in the absence (Control) or in the presence of different doses of Dasatinib (5, 10, 50, 100 and 150 nM) or Imatinib (0.5, 1, 2.5, 5 and 10 uM). Results in 1A correspond to a representative analysis of viability as a measure of 7AAD and 1B represent mean ± SD values from 5 different experiments. $^{**}p < 0.001$ as compared to the corresponding control.

changed in response to TKI treatment and this finding correlated with the results showed in Figure 5.

To confirm the subcellular location of both proteins, an immunofluorescence analysis was performed (Figs. 6C and 6D). Results also showed that $p57^{Kip2}$ and $p18^{INK4c}$ are expressed in the cell cytoplasm before treatment with Dasatinib and Imatinib but when cells were exposed to these molecules there was an increase of both TKIs in the nucleus. It is also noteworthy that, after treatment, the size of the cells was reduced and this may be related to the reduced amount of total cell protein and the decreased metabolic activity.

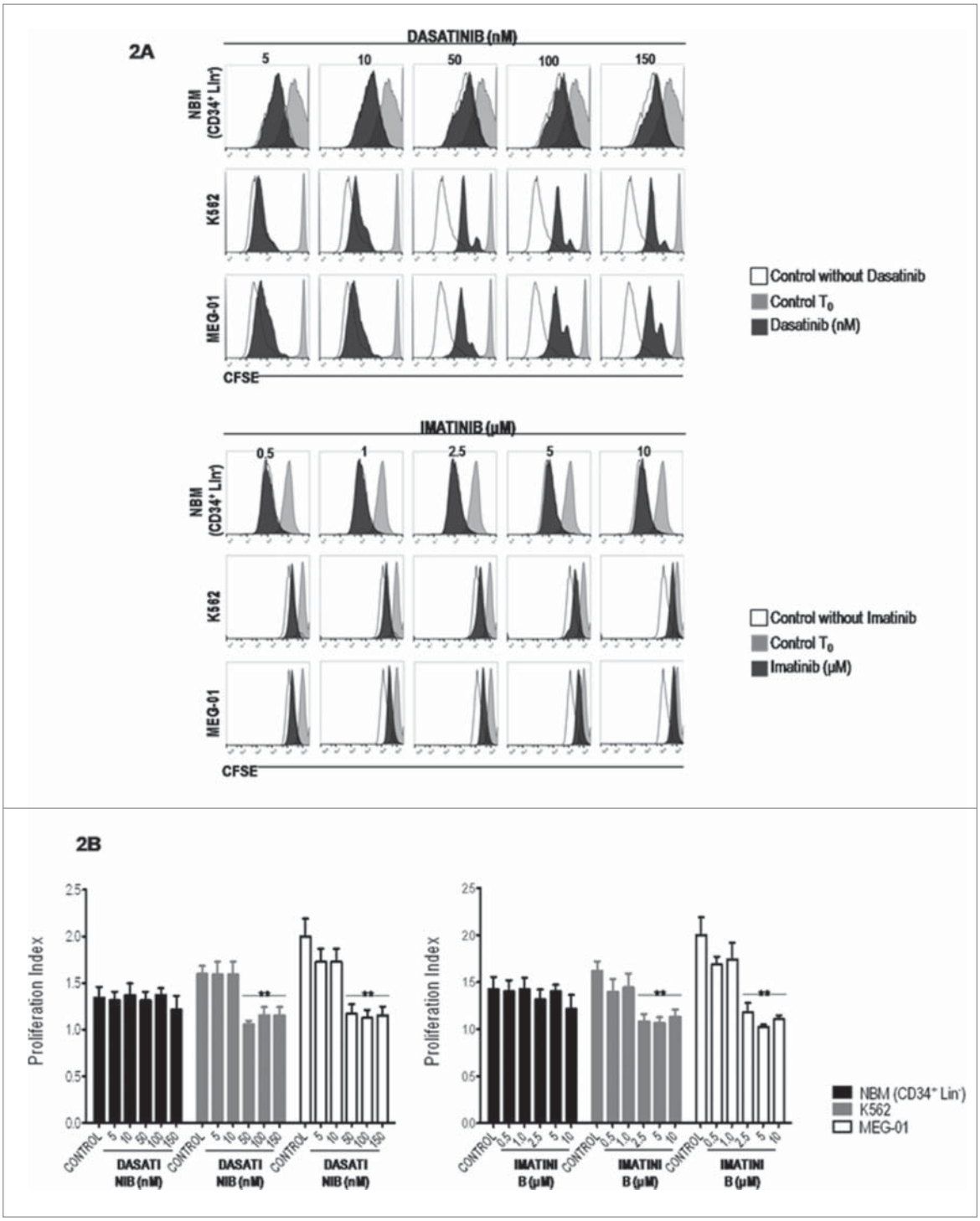


**Figure 2.** Imatinib and Dasatinib delay proliferation index of CML cells. Normal marrow-derived (NBM) $CD34^{+}Lin^{-}$ cells, K562 and MEG-01 CML cell lines were cultured in the absence (Control) or in the presence of different doses of Dasatinib (5, 10, 50, 100 and 150 nM) or Imatinib (0.5, 1, 2.5, 5 and 10 uM) and compared with their correspondent CFSE content at the beginning of culture (Control T0). Results in 2A correspond to a representative analysis of CFSE content after 48 hours of TKI treatment and 2B represent mean ± SD of Proliferation Index (evaluating according flowjo analysis) from 5 different experiments. $^{**}p < 0.001$ as compared to the corresponding time cero control.

### *Permanence of LSC in $G_0$ cell cycle phase is related to $p18^{INK4c}$ and $p57^{Kip2}$*

In order to determine if $p18^{INK4c}$ and $p57^{Kip2}$ had the same expression patterns in CML primitive cells as in CML cell lines, bone marrow aspirates from newly diagnosed CML patients in Chronic Phase were enriched for $CD34^{+}lin^{-}$ cells and subsequently stained with CD34 and CD38 antibodies to analyze the Leukemic Stem Cell-enriched cell fraction ($CD34^{+}CD38^{-}lin^{-}$ cells). As shown in Figure 7, the LSC fraction represented around 7% of $CD34^{+}lin^{-}$ cells and the majority of these primitive cells (62%) were in $G_0/G_1$ after 48 hours in culture with cytokines but without TKI treatment; 27% and 11% of the cells were in S and $G_2$/M, respectively. When $CD34^{+}lin^{-}$ cells were cultured with Dasatinib and Imatinib, there was an increase in the proportion of

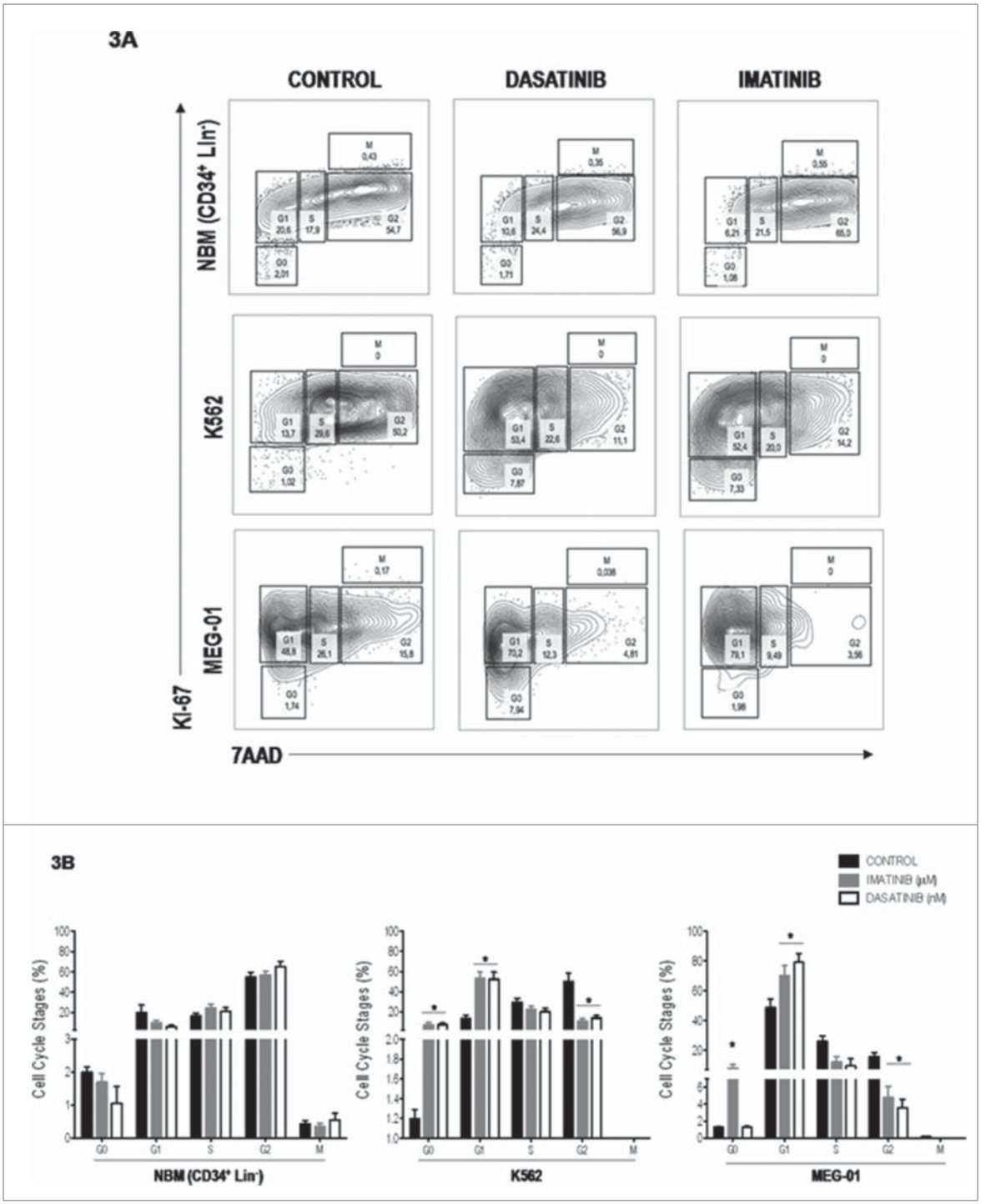


**Figure 3.** Imatinib and Dasatinib increase quiescent cells in CML cells. Normal marrow-derived (NBM) $CD34^{+}Lin^{-}$ cells, K562 and MEG01 CML cell lines were cultured in the absence (Control) or in the presence of Dasatinib (50 nM) or Imatinib (2.5 uM) for 48 hours to analyze cell cycle distribution as a measure of 7AAD and Ki67 contain. Results in 3A correspond to a representative plot of cell cycle distribution and 3B represent mean ± SD from 5 different experiments of cell cycle phase distribution in the different cells analyzed. $^{**}p < 0.001$ as compared to the corresponding time cero control.

$CD34^{+}CD38^{-}lin^{-}$ cells in $G_0/G_1$ (88% in average), whereas cells in S and $G_2$/M were reduced to 11% and 2%, respectively (Fig. 7). Similar to our observations with the leukemic cell lines, when $p18^{INK4c}$ and $p57^{Kip2}$ levels were evaluated in primary $CD34^{+}CD38^{-}lin^{-}$ cells, a significant reduction in both proteins was observed, indicating that $p18^{INK4c}$ and $p57^{Kip2}$ may be involved in the permanence of LSC in quiescence after treatment with TKIs.

## Discussion

Several reports have demonstrated that TKIs can efficiently reduce proliferation of mature and proliferative cells in CML patients; these drugs, however, do not kill non-proliferating CML primitive cells, including progenitor and stem cells. This contributes to persistence of leukemic cells after treatment (residual disease) and its corresponding impact in terms of side

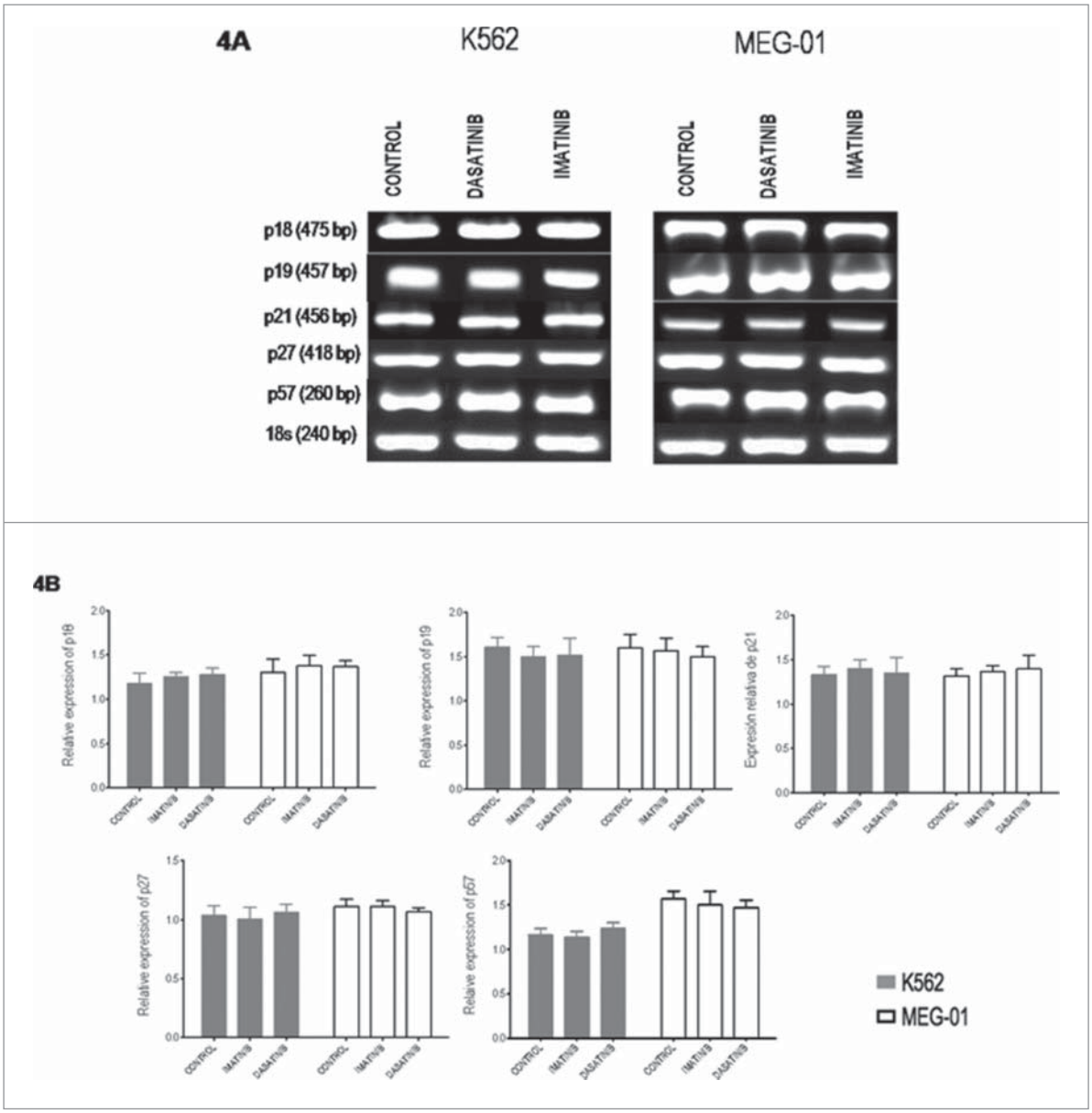


**Figure 4.** Treatment with Imatinib and Dasatinib do not change the expression of Cyclin-Dependent Kinase Inhibitors in CML cells. K562 and MEG-01 CML cell lines were cultured in the absence (Control) or in the presence of Dasatinib (50 nM) or Imatinib (2.5, uM) and the ARN expression of each CDKI was evaluated. Results in 4A correspond to a representative figure for PCR analysis and 4B represent mean ± SD values from densitrometric analysis from 5 different experiments.

effects, pharmacological and economic issues.[3,7,17,18] This persistence of CML quiescent stem cells when BCR-ABL activity remains blocked could suggest that signals provided by the bone marrow microenvironment support CML stem cells survival and allow for the coexistence of both CML and normal stem cells within the marrow cavity. We have hypothesized that coexistence of normal and CML stem cells could be explained, at least in part, by differences in cell cycle regulation; thus, its analysis could offer important information on CML stem cells biology and the effect of TKIs on primitive normal and CML cells.[23,24]

In keeping with previous reports, herein we observed a significant reduction in CML cell viability and delay in proliferation in response to TKIs.[5,25] Importantly, when we analyzed the effects of these molecules on primitive $CD34^{+}lin^{-}$ cells from NBM, no significant changes were observed, confirming that these molecules do not have major in vitro effects in normal hematopoiesis, despite increasing concentrations of TKIs. It is noteworthy, however, that Bartolovic et al detected a significant, time-dependent, inhibitory effect of Imatinib on normal cells, probably due to the high concentration of cytokines that could make normal cells more susceptible to TKIs.[26] Weisel et al, on the other hand, showed a non-specific growth inhibition of normal $CD34^{+}$ cells that could be due to hematotoxicity caused by such a TKI.[27] In a more recent report, Tao et al, demonstrated that activity of IL3 on the 32D mouse cell line requires the interaction between c-Abl and Jak 2, suggesting c-Abl inhibition by Imatinib treatment.[28] In addition, has been recently demonstrated that the persistence of the most primitive cells in CML could be associated with interactions between BCR-ABL and JAK2 and with the

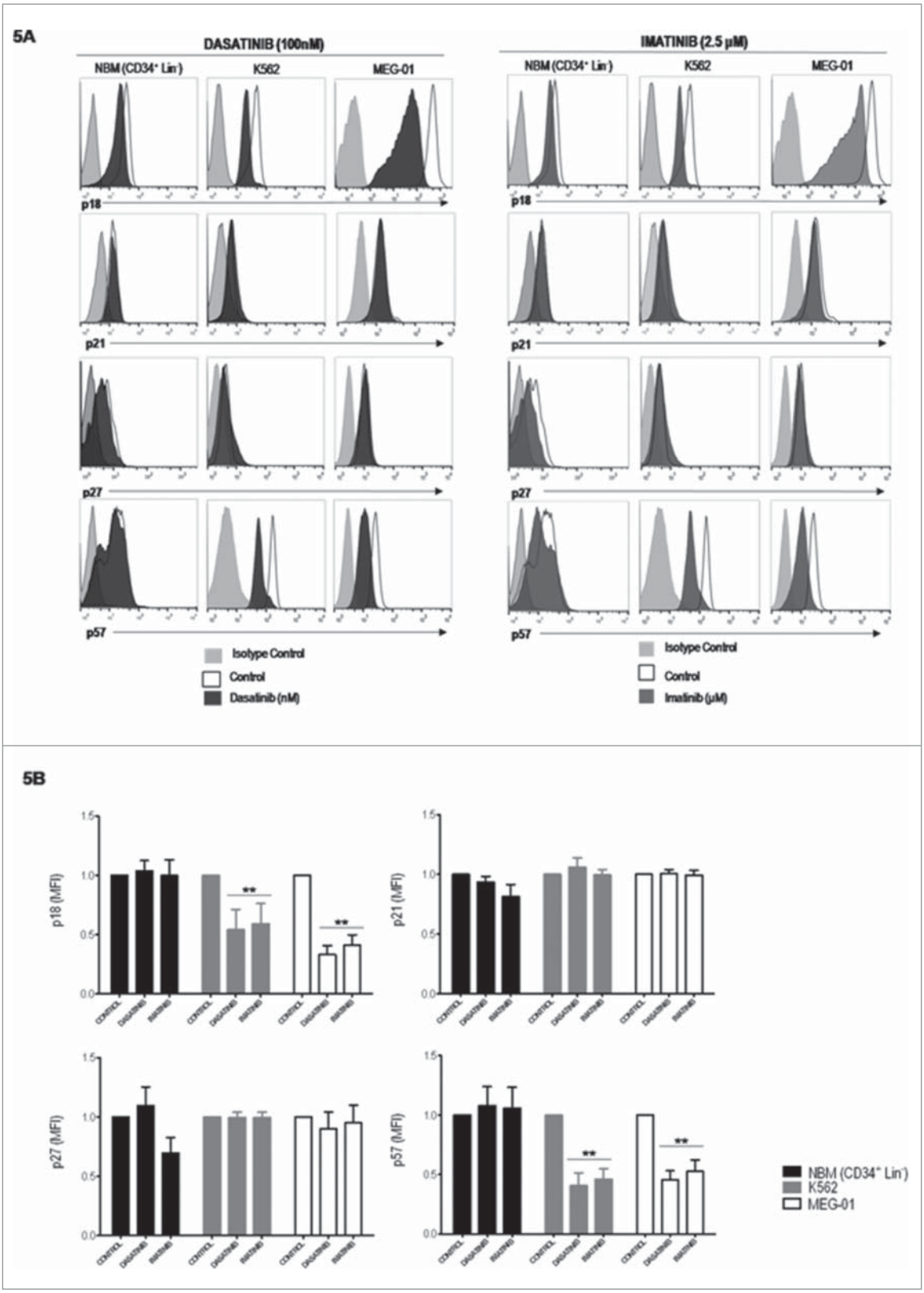


**Figure 5.** Treatment with Imatinib and Dasatinib reduce the protein level of p18 and p57 Cyclin-Dependent Kinase Inhibitors in CML cells. Normal marrow-derived (NBM) $CD34^{+}Lin^{-}$ cells, K562 and MEG-01 CML cell lines were cultured in the absence (Control) or in the presence of Dasatinib (50 nM) or Imatinib (2.5, uM) and the level of CDKI protein was evaluated by flow citometry. Results in 5A correspond to a representative histogram and 5B represent mean ± SD values from MFI for each CDKI from 5 different experiments. $^{**}p < 0.05$ as compared to the corresponding control.

transcriptional activity of STAT 5, which leads to propose these molecules as new targets to reduce and kill primitive CML cells that are resistant to TKIs.[29,30]

Interestingly, when we analyzed the cell cycle status of normal $CD34^{+}lin^{-}$ cells in response to Imatinib and Dasatinb, no significant alterations were detected in their cell cycle phases,

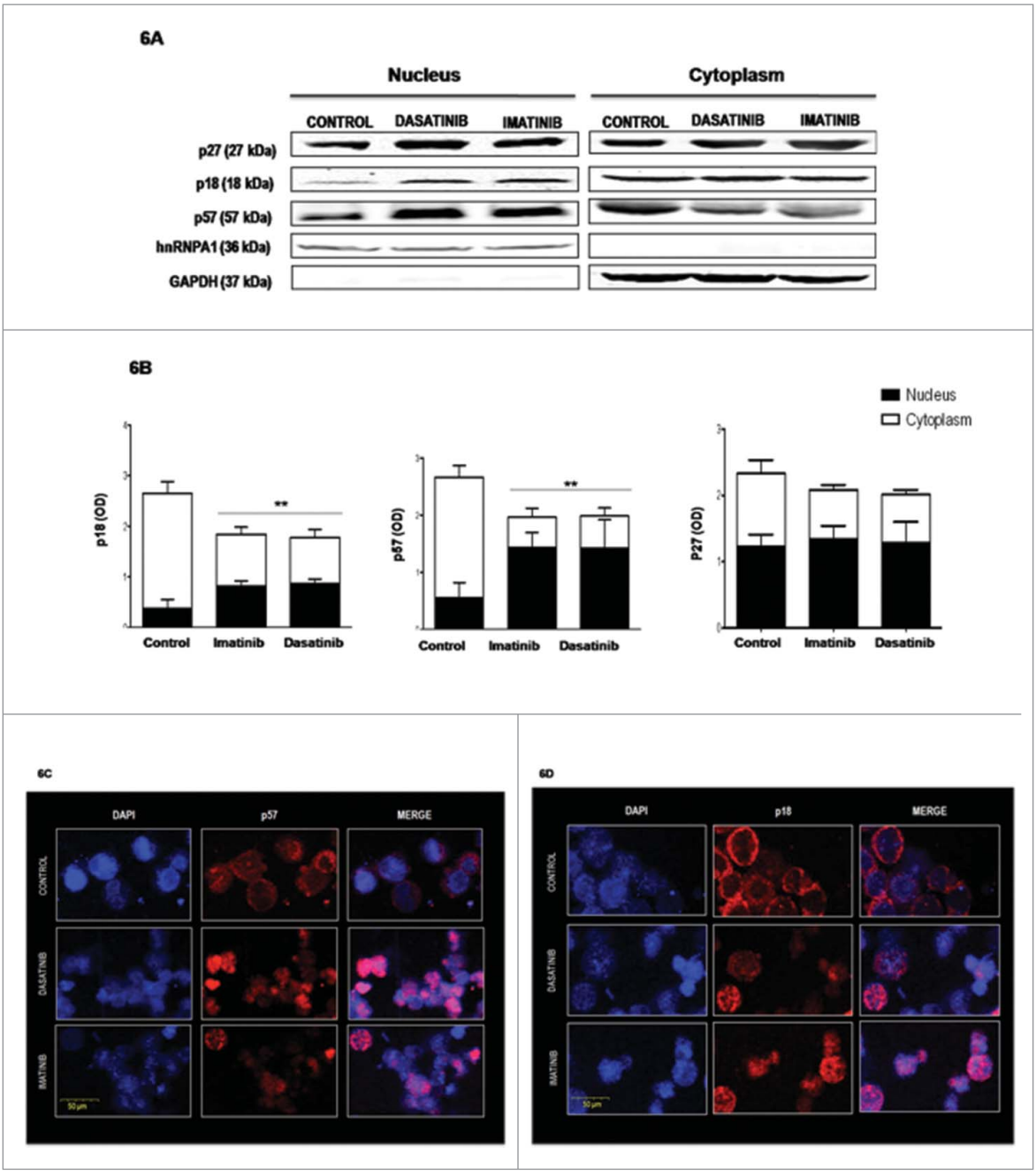


**Figure 6.** Treatment with Imatinib and Dasatinib relocate $p18^{INK4c}$ and $p57^{Kip2}$ Cyclin-Dependent Kinase Inhibitors to nucleo in CML cells. K562 CML cell line was cultured in the absence (Control) or in the presence of Dasatinib (50 nM) or Imatinib (2.5, uM) and nuclear or cytoplasm protein was obtain and analyzed. Results in 6A correspond to a representative Western Blot, 6B) show a densitometric analysis from 5 different experiments for each protein and 6C and 6D represent an inmunofluorescence analysis for $p57^{Kip2}$ and $p18^{INK4c}$ respectively. $^{**}p < 0.05$ as compared to the corresponding control.

and no changes were observed in the expression levels of different CDKIs. These results could explain, at least in part, the presence of active normal hematopoiesis when patients had achieved hematological and/or molecular remission after treatment. In leukemic cell lines, on the other hand, we observed accumulation of cells in $G_0/G_1$. Accumulation of tumor cells in $G_0/G_1$ in response to Dasatinib has been reported in osteosarcoma cells, gastrointestinal stromal tumors, head and neck squamous cell carcinoma, and small cell lung cancer. In all these cases, such an effect was accompanied by alterations in mitotic spindles, reduction in migration and invasion, and induction of $p27^{Kip1}$.[31,32]

It has been demonstrated that BCR-ABL has an effect on some members of the CDKI family. Indeed, transfection of p210 BCR-ABL into human M07 cells and murine Ba/F3 cells induces $p27^{Kip1}$ down regulation.[33] In Ba/F3 cells, such an effect was related with the PI3K/ADT signaling pathway leading to entry into the S phase of cell cycle.[34] These results were in contrast to the report by Jiang et al, who showed higher levels of $p27^{Kip1}$ in CML, but not in NBM $CD34^+$ cells, without changes in mRNA.[15] In the present study, we found that when K562 and MEG-01 cell lines were treated with TKIs, there were no changes in mRNA levels for $p18^{INK4c}$, $p19^{INK4d}$, $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$; in contrast, there was a reduction in the

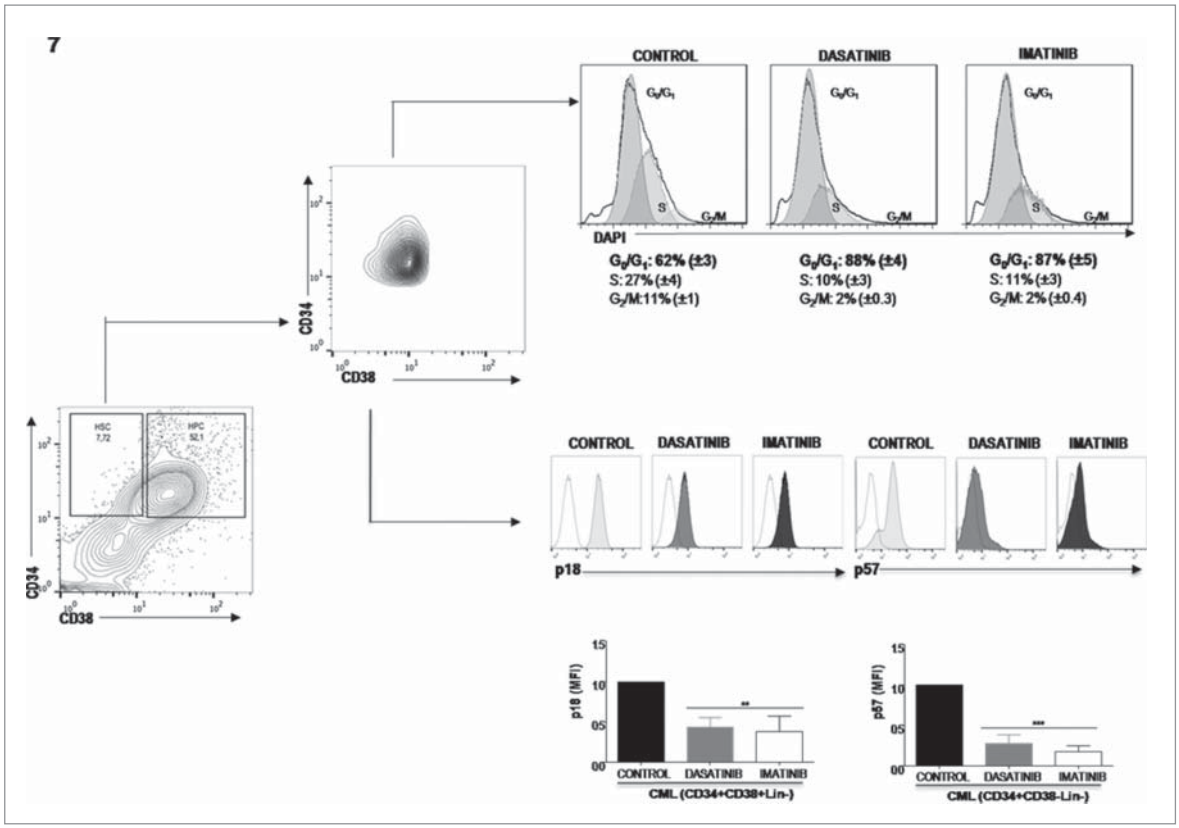


**Figure 7.** Imatinib and Dasatinib increase quiescence in primary CML stem cells and reduce the $p18^{INK4c}$ and $p57^{Kip2}$ Cyclin-Dependent Kinase Inhibitors. LSC derived from CML patients were culture in the absence (Control) or in the presence of Dasatinib (50 nM) or Imatinib (2.5, uM) for 48 hours and their cell cycle status and CDKI content was then analyzed. Results correspond to a representative plot for LSC enrichment and each analysis. Corresponding mean ± SD (from 4 different experiments) for cell cycle status and MFI for each protein are indicated in squares below the each cythometric plot. $^{***}p < 0.05$ as compared to the corresponding control.

protein levels for $p18^{INK4c}$ and $p57^{Kip2}$, suggesting posttranscriptional regulation for these 2 molcules. Thus, our results seem to be in keeping with those of Jiang and colleagues. Contrary to these findings, up-regulation of $p18^{INK4c}$ expression and arrest in $G_1$ were described in the HT93A AML cell line, as a consequence of the treatment with Imatinib.[35]

In terms of $p57^{Kip2}$, decreased protein levels have been documented in several types of cancer cells and during blast crisis progression in CML.[36,37] A recent report, however, describes up regulation of this protein in CML cells in response to Imatinib, but not in $CD34^+$ cells from normal subjects.[16] Some possible explanations for the discrepancies between such a report and our study include the facts that in their study, the experiments were performed after 24 hours of culture (we cultured the cells for 48 hours), and that the primary $CD34^+$ CML cells they used were obtained from peripheral blood, whereas in the present study we obtained $CD34^+lin^-$ cells from CML bone marrow.

As shown by different groups, treatment of CML cells with TKIs induces accumulation of CDKIs in the cytoplasm. Jiang et al reported that BCR-ABL induces an elevation of $p27^{Kip1}$ which is relocated to the cytoplasm and that seems to be involved in the decrease of integrin-mediated adhesion.[15] Such an increase in cytoplasmic $p27^{Kip1}$ in $CD34^+$ cells has been associated with progression from CML chronic phase into blast crisis. The high level of this protein appears to modulate RhoA activity and SAPK/JNK signaling in favor of cell survival in the presence of Imatinib.[38] A similar cytoplasmic location has been reported for $p21^{Cip1}$, which is physically associated with AKT in a PI3K-independent manner; however, when cells are treated with STI571, $p21^{Cip1}$ diminishes and the cells are sensitized to apoptosis.[13] Interestingly, and in contrast to the above mentioned studies, an inducible $p21^{Cip1}$ expression in K562 cell line confers partial resistance to Imatinib-induced apoptosis and it is not related with a cytoplasmic location of $p21^{Cip1}$.[39]

In summary, the present study demonstrates that cellular location of $p18^{INK4c}$ and $p57^{Kip2}$ seems to be implicated in the antiproliferative activity of Imatinib and Dasatinib in K562 and MEG-01 CML cell lines. Also, our results suggest that the permanence of quiescent stem cells after TKI treatment could be associated with a decrease in $p18^{INK4c}$ and $p57^{Kip2}$ levels and their nuclear location. In addition, the differences in $p18^{INK4c}$ and $p57^{Kip2}$ activities in CML and normal stem cells indicate important differences in cell cycle regulation and provide a platform that could be considered in the development of new therapeutic options to eliminate LSC.

## Patients, materials and methods

### *Cell samples and cell lines*

Bone marrow aspirates were obtained from 7 newly diagnosed, untreated CML patients in Chronic Phase (CP) at the Hematology Department, Medical Specialties Hospital, La Raza Medical Center, IMSS, Mexico City. Normal bone marrow (NBM) was obtained from 10 hip replacement surgery patients at General

Regional No. Two Hospital Villa Coapa, IMSS, Mexico City. The Scientific and Ethics Committee of the National Medical Center, IMSS (R-2007-3602-14) had approved these procedures and all of them are in accordance with the Helsinki Declaration. In all cases written informed consent was obtained for each one of the donors. K562 (ATCC, http://www.atcc.org/products/all/CCL-243.aspx) and MEG-01 (ATCC, http://www.atcc.org/products/all/CRL-2021.aspx) 2 BCR-ABL$^+$ CML cell lines were grown in RPMI 1649 culture medium at 10% Fetal Bovine Serum in 5% $CO_2$ atmosphere at 37°C.

### CD34$^+$ enrichment

Mononuclear cells (MNC) were obtained from each bone marrow sample and these were isolated using Ficoll Paque Plus (GE Health Care Life Sciences, http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-mx/products/AlternativeProductStructure_16963/17144002) gradient. CD34$^+$ cells were enriched according to StemSep TM system (Stem Cell Technologies Inc., http://www.stemcell.com/en/Products/Popular-Product-Lines/StemSep/StemSep-Human-Haematopoietic-Progenitor-Cell-Enrichment-Kit.aspx). Briefly, MNC were incubated with an antibody cocktail with the following surface antigens: CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b and glycophorin A, followed by incubation with magnetic colloid. The CD34$^+$lin$^-$ cells were collected in Stem Span medium (Stem Cell Technologies, http://www.stemcell.com/en/Products/All-Products/StemSpan-SFEM.aspx) and subsequently stained with CD34 and CD38 antibodies to analyzed CD34$^+$CD38$^-$lin$^-$ (enriched for LSC). Concomitantly cell cycle status and expression of CDKI was assessed by multicolor flow cytometry using a FACSAria (BD Biosciences, USA).

### Cell viability and proliferation

CD34$^+$lin$^-$ cells from CML and NBM, as well as CML cell lines, were labeled with 10 $\mu$M of carboxy fluorescein diacetate succinimidyl ester (CFSE) (Sigma Aldrich, http://www.sigmaaldrich.com/catalog/product/sigma/21888) at 37°C for 15 minutes and after washed with PBS at 10% FBS to remove CFSE excess. Cells were plated at 2 × 10$^5$ cells/well in 24 well plates and incubated with different concentrations of Imatinib (Gleevec®, Novartis Pharmaceuticals) or Dasatinib (Sprycel®, Bristol-Myers Squibb) for 48 hours. In the case of CD34$^+$lin$^-$ from CML and NBM cells, cultures were maintained in StemSpan media (Stem Cell Technologies Inc., http://www.stemcell.com/en/Products/All-Products/StemSpan-SFEM.aspx), supplemented with 10 ng/ml of TPO (PeproTech Inc., https://www.peprotech.com/en-GB/Pages/Product/Recombinant_Human_TPO/300-18), Flt-3L (PeproTech Inc., https://www.peprotech.com/en-GB/Pages/Product/Recombinant_Human_Flt3-Ligand/300-19) SCF (PeproTech Inc., https://www.peprotech.com/en-GB/Pages/Product/Recombinant_Human_SCF/300-07) IL6 (PerproTech Inc., https://www.peprotech.com/en-GB/Pages/Product/Recombinant_Human_IL-6/200-06), IL3 (PeproTech Inc., https://www.peprotech.com/en-GB/Pages/Product/Recombinant_Human_IL-3/200-03), G-CSF (PeproTech Inc., https://www.peprotech.com/en-GB/Pages/Product/Recombinant_Human_G-CSF/300-23) and GM-CSF (PeproTech Inc., https://www.peprotech.com/en-GB/Pages/Product/Recombinant_Human_GM-CSF/300-03).[11] After culture, cells were stained with 7 Aminoactinomycin D (7-AAD) (BD Biosciences, http://www.bdbiosciences.com/us/applications/research/apoptosis/buffers-and-ancillary-products/cell-viability-solution/p/555816) and analyzed immediately using a FACSCalibur Flow Cytometer (BD Bioscience, USA).

### Cell cycle status and CDKI protein content

CML cell lines and CD34$^+$lin$^-$ cells from NBM (2 × 10$^5$) were plated in 24 well plates and incubated in the presence or absence to 2.5 $\mu$M Imatinib or 100 nM Dasatinib for 48 hours. After this time, cells were collected, washed with PBS, and fixed with formaldehyde at 4% (Sigma-Aldrich, http://www.sigmaaldrich.com/catalog/product/aldrich/f15587) for 15 minutes on ice. After that, cells were permeabilized for 20 minutes with triton 0.1% (Sigma-Aldrich, http://www.sigmaaldrich.com/catalog/product/sial/x100). Cells were washed with PBS at 3% FBS (flow buffer) and then stained with anti Ki67 AF488 antibody (1:100 dilution) (BD biosciences, http://www.bdbiosciences.com/us/applications/research/apoptosis/conjugated-antibodies/alexa-fluor-488-mouse-anti-human-ki-67-b56/p/561165) for 2 hours, washed with flow buffer and a subsequent incubation with 7-AAD for 30 minutes. After this procedure, cells were analyzed using a FACS Calibur Flow Cytometer. To analyze the CDKIs protein content, cells previously cultured in presence or absence of Imatinib or Dasatinib were incubated with anti p21-FITC (1:50) (Santa Cruz, http://www.scbt.com/es/datasheet-6246.html); anti p27 PE (1:50) (Santa Cruz, http://www.scbt.com/es/datasheet-1641.html); anti p18 AF488 (1:100) (Abcam, http://www.abcam.com/ube2i-ubc9-antibody-ep2938y-chip-grade-alexa-fluor-488-ab198588.html) or anti p57 AF647 (1:100) (Abcam, http://www.abcam.com/p57-kip2-antibody-ep2515y-alexa-fluor-488-ab199069.html) antibodies for 2 hours. Cells were washed with flow buffer and analyzed in a FACS Calibur Flow Cytometer. All data was analyzed using the Flowjo Software, version 10.6.

### CDKI expression analysis

Reverse transcription (RT-PCR) and polymerase chain reaction (PCR) were performed to analyze the expression of CDKIs in CLM lines after 48 hours of treatment with TKIs. Briefly, total RNA from different cell cultures was isolated using TriPure (Roche, http://www.sigmaaldrich.com/catalog/product/roche/tripurero) according to the manufacturer's instructions. Concentration and purity of RNA was assessed by spectrophotometry and by 1% agarose gels. cDNA was produced from 1 ug of RNA using Moloney murine leukemia virus reverse transcriptase (M-MLVRT) and random hexameres.[17] PCR specific primers used to detect each CDKI are included in Table 1 and the following conditions were used to detect all molecules: 96°C for 5 min, then 35 cycles of denaturation at 94°C for 30 seconds; annealing at 60°C for 1 min and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min.[18] Ten $\mu$l of each reaction were loaded in 1% agarose gels and densitometry analysis was performed using ImagJ software.

| CDKI | Primers sequence |
|---|---|
| p18 | sense: CATCATGCTGCCTGGTTAGG<br>anti-sense: GCTGGCCGTGTGCTTCACCA |
| p21 | sense: AGCTCAATGGACTGGAAGGG<br>anti-sense: GAGCTGGAAGGTGTTTGGGG |
| p27 | sense: CGGGGTATGAAGAGCTTGCTTTGAT<br>anti-sense: AACATTCAAAACTCCCAAGCACCTC |
| P57 | sense: GATCAAGAAGCTGTCCGGGC<br>anti-sense: TTGCTGCTACATGAACGGTCC |
| 18 s RNA subunit | sense: AAT CCA CGC CAG TAC AAG ATC CCA<br>anti-sense: TTT CTT CTT GGA CAC ACC CAC GGT |

### *CDKI cellular location*

Cytoplasm and nuclear protein extraction were performed to identify cell location of p18 and p57. Briefly, CML cell lines treated with TKI for 48 hours were collected and washed with cold PBS and incubated with nuclear buffer (NaCl 150 nM, MgCl2 10 mM, cOmplete™ EDTA-free (Roche, http://www.sigmaaldrich.com/catalog/product/roche/coedtafro) and Tris 10mM) for 10 min on ice, lysed with NP40 (1%) (Abcam, http://www.abcam.com/np-40-ab142227.html) and centrifuged for 5 min at 2500 rpm. The cytoplasmatic fraction was recovered and centrifuged for 15 min at 13000 rpm. The nuclear fraction was obtained by lyses with M-PER™ (Thermo Fisher Scientific, https://www.thermofisher.com/order/catalog/product/78501) according with manufacturer's instructions. Cell fractions proteins (40 $\mu$g) were electrophoresed through SDS (12%) polyacrilamyde gels, transferred to nitrocellulose membranes and immunoblotted using specific anti-p18 (Cell Signaling Technologies, http://www.cellsignal.com/products/primary-antibodies/p18-ink4c-dcs118-mouse-mab/2896), anti-p57 (Cell Signaling Technologies, http://www.cellsignal.com/products/primary-antibodies/p57-kip2-antibody/2557), annti-hnRNAP1 (Cell Signaling Technologies, http://www.cellsignal.com/products/primary-antibodies/hnrnp-a1-d21h11-rabbit-mab/8443?_=1445364207356) and anti-GAPDH (GeneTex, http://www.genetex.com/GAPDH-antibody-GT239-GTX627408.html) antibodies. Immunostained proteins were detected using LI-COR (Odyssey, USA) and analized using Image Studio software. Immunoflurescence analysis was performed with $5 \times 10^4$ cells (previously treated or untreated with TKI) spread on slide and fixed with acetone (Sigma Aldrich, http://www.sigmaaldrich.com/catalog/product/vetec/v000187) for 5 min at 20°C. The slides were blocked and permeabilized with perm solution (PBS 10%FBS and 0.1%Tween 20) for 1 hour at room temperature and then incubated with anti p18 or anti p57 antibodies diluted in perm solution (1:50) overnight at 4°C and washed with PBS. The secondary antibody was incubated for 45–60 minutes and then washed with PBS. The slides were mounted with Vectashield® mounting media with DAPI (Vector labs, http://vectorlabs.com/vectashield-mounting-medium-with-dapi.html) and analyzed in an Olympus fv1000 confocal microscope (Olympus, Japan).

### *Statistical Analysis*

Comparisons between groups were made with one-way analyses of variance (ANOVA) and data are expressed as mean ± SEM. Analysis was performed using GraphPad Prism software version 5.0.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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## Authors' contributions

D M-L, experimental develops of study. S A-V, RNA expression data. MA S-E, Viability data. A A-M, cell cycle analysis data. V O-N, analysis of data and revising for intellectual content. H T-M, provided samples from NBM. M A-S, provided samples from CML. HM, analysis of data, drafting the article and revising for intellectual content. A C-G, conception and design of the study, analysis an interpretation of data, drafting of the article and revising for intellectual content.

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