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Cox-2 inhibitors induce early c-Myc downregulation and lead to expression of differentiation markers in leukemia cells

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Key words: COX-2 inhibitors, c-Myc, cell proliferation, cell differentiation, myeloid markers

Abbreviations: AML, acute myeloid leukemia; C/EBPα, CCAAT/enhancer binding protein α; COX, cyclooxygenase; Coxib, celecoxib; Cdk, cyclin dependent kinase; CHX, cycloheximide; CML, chronic myelogenous leukemia; ERK, extracellular signal-regulated kinase; Nime, nimesulide; PCNA, proliferating cell nuclear antigen; PGE2, prostaglandin E2; $p38^{MAPK}$, p38 mitogen-activated protein kinase; pRb, retinoblastoma protein; 3'UTR, 3'-untranslated region

It is well described that cyclooxygenase-2 (COX-2) inhibitors counteract cancer cell proliferation by preventing the G_1/S transition. This effect has been associated with the inhibition of COX-2 enzymatic activity but also as an off-target effect essentially in adherent cancer cell models. In this study, we investigated the effect of three COX-2 inhibitors (nimesulide, NS-398 and celecoxib) on cell proliferation of leukemic and lymphoblastic cells expressing COX-2 at high (U937, Jurkat, Hel and Raji) and very low (K562) protein levels. We found that the inhibitors reduce cell proliferation in all COX-2-expressing cells leading to an accumulation in the G_0/G_1 phase of the cell cycle. We provide evidence that this modulation corresponds to an accumulation of cells in G_0 paralleled by the expression of cell differentiation markers in U937 (CD15) and Hel (CD41a and CD61) cells but not in the insensitive K562. These events are associated with a rapid downregulation (within one hour) of c-Myc expression, accompanied by the upregulation of p27 and the downregulation of PCNA and cyclin D1. Our study suggests c-Myc as a crucial early target of COX-2 inhibitors.

Introduction

The cell cycle is a highly regulated process involving various steps orchestrated by many modulator proteins that are triggered according to a precise chronology. Any perturbation of the cell cycle, if unresolved, may be followed by the activation of specific cell defense responses, such as apoptosis.[1] Alternatively, cells may react by activating a senescence program.[2] Both events have a protective effect for the entire body and represent a mechanism to limit the proliferation of potentially damaged cells.[3-6] These events may therefore serve as a barrier against the development of pathological conditions such as cancer.[2]

Carcinogenesis is characterized at its earliest steps by the loss of important cell cycle checkpoints, as a consequence of mutations in genes involved in cell cycle modulation, such as p53 and c-Myc.[7] These aberrations lead to uncontrolled and exacerbated cell proliferation, accompanied by the establishment of an undifferentiated state[7-9] and by the impairment of the ability of mutated cells to activate cell death.[10] These events then promote the progression toward the final malignant steps of cancer.

In a number of cases, dysfunction of the cell cycle in cancer cells is due alterations in G_1/S transition[7,11] and consequently, targeting pathological pathways implicated in G_1 and S phase progression may represent an important strategy for anti-cancer therapies.

c-Myc is one of the most commonly upregulated oncogenes in cancer.[12] This transcription factor plays an important role in cell cycle, as well as in apoptosis, cell differentiation, cell adhesion, metabolism and senescence.[9,13,14] It promotes the progression of cells from G_0/G_1 into S phase by regulating the transcription of different genes implicated in the G_1/S phase transition. It directly promotes cyclin D1 expression.[15] Cyclin D1 activates kinases, such as Cdk2 and Cdk4, that control the phosphorylation of the retinoblastoma protein (pRb).[16] Once phosphorylated, Rb triggers a cascade of events that starts with its release from the E2F transcription factor and culminates in the indirect transcriptional activation of additional cell cycle mediators that operate at the G_1/S transition, such as cyclin E and the proliferating cell nuclear antigen (PCNA).[17]

c-Myc also binds to the promoter and represses the expression of the cell cycle inhibitor p27, a cyclin-dependent kinase inhibitor belonging to the Cip/Kip family, which blocks the entry of cells into S phase via inhibition of the cyclin-dependent kinases Cdk2, Cdk4 and Cdk6 and promotes the arrest of the cells in G_0 phase.[7,18]

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Targeting c-Myc overexpression in cancer, therefore, plays a cytostatic role in cancer cells, precisely working at the G_1/S transition. Moreover, c-Myc plays an important role in differentiation of many cell types, including hematopoietic cells, where c-Myc downregulation is a pre-requisite to induce pro-differentiated states in cancer cells.[9]

Cyclooxygenases (COX) are a family of enzymes that catalyze the biosynthesis of prostaglandin from arachidonic acid. The two main isoforms, COX-1 and COX-2, have been intensively studied. COX-1, which is ubiquitously expressed, is implicated in tissue homeostasis[19] and seems to play a role in Alzheimer disease by favoring neuroinflammation.[20] The inducible form of COX, cyclooxygenase-2 (COX-2), is a major enzyme involved in inflammation and carcinogenesis.[19,21] Constitutive upregulation of COX-2 has been found in early pre-neoplastic stages of colon, prostate or breast tumors.[22] COX-2 overexpression has been linked to the promotion of cell proliferation in pre-malignant as well as malignant stages of cancer, increased angiogenesis, chemotherapeutic failure and pro-metastatic functions.[23] The importance of COX-2 in tumor promotion primarily comes from studies using inhibitors of COX-2 enzymatic activity, which represent the only therapeutic strategies to target COX-2 function. The prostaglandins produced by COX-2 enzyme activity have been frequently considered to be responsible for the stimulation of cell proliferation and the inhibition of apoptosis in different kinds of cancers.[21] Prostaglandins control the expression of several cell cycle genes through different signaling pathways regulated by prostanoid receptors.[24]

Targeting COX-2 has been reported to inhibit the proliferation of tumor cells. This cytostatic effect has been described for several solid tumors, where an arrest of the cells in G_0/G_1 has been frequently reported in reference 23. However, there is no consensus about the mechanisms responsible for these effects. More recently, COX-2 overexpression has also been described for hematological malignancies, where a role in carcinogenesis has not yet been elucidated.[25]

In this study, we investigated the effects of the COX-2 inhibitors nimesulide, NS-398 and celecoxib on cell proliferation in a panel of hematopoietic cancer cell lines. We show that all the three COX-2 inhibitors affected cell growth in different leukemic and lymphoblastic cell lines, including the acute myeloid leukemia cell lines (Hel and U937), Burkitt's lymphoma cell line (Raji) and acute lymphoid leukemic T cells (Jurkat) with the exception of the chronic myeloid cell line K562 which were insensitive. The mechanism of this regulation involved modulation of different cell cycle regulators with a specific timing, starting with a very early c-Myc downregulation and involving upregulation of p27 and downregulation of cyclin D1 and PCNA. Effects of COX-2 inhibitors culminated in the inhibition of G_1/S transition and accumulation of cells in G_0 phase of the cell cycle, which is associated with the expression of differentiation markers in the cell lines sensitive to COX-2 inhibitors.

Results

COX-2 inhibitors slow down cell proliferation of leukemic and lymphoblastic cell lines. Hel, Jurkat, K562, Raji and U937 cell lines were treated for up to 48 h with nimesulide (10, 40, 100 μM), NS-398 (10, 40, 100 μM) or celecoxib (10, 20, 30, 40 μM). The effect of the inhibitors on cell growth was then assessed. All three COX-2 inhibitors were able to inhibit cell growth in a dose-dependent manner in the panel of cell lines investigated with the exception of K562, which were mildly affected only at the highest concentrations (**Fig. 1A and Fig. S1**).

We confirmed that the impact of COX-2 inhibitors was on cell growth/proliferation rather than on cell viability. Up to one week of treatment with the different COX-2 inhibitors showed no accumulation of cells with apoptotic or necrotic nuclear features (**Fig. S2A and B**; U937 cells were chosen here as a representative cell line).[26] These results were confirmed by WB analysis of caspase-3 cleavage (**Fig. S2C**). Next, we monitored cell proliferation with the carboxyfluorescein succimidyl ester (CFSE) cell tracker (see Materials and Methods). **Figure 1B** shows a typical shift of CFSE fluorescence intensity, as determined by FACS analysis in untreated U937 and K562 cells after up to 48 h of cell culturing. The treatment with nimesulide and celecoxib prevented the reduction in CFSE fluorescence in a dose-dependent manner in U937 treated cells; conversely, K562 cells generally maintained the same pattern of CFSE fluorescence as observed for the untreated cells (**Fig. 1B**). Together, these data confirm that COX-2 inhibitors affect cell proliferation in hematopoietic cell lines.

COX-2 inhibitors counteract cell cycle progression by affecting the G_1/S transition. We next determined whether COX-2 inhibitors affected cell cycle progression. For further mechanistic studies, we selected U937 and K562 cells, which are the most sensitive and the least sensitive cell lines to COX-2 inhibitors, respectively. Cell cycle analysis was performed by flow cytometry with propidium iodide (PI) staining. The analysis showed that all three COX-2 inhibitors led to an accumulation of U937 cells in G_0/G_1 phase (**Fig. 2A and B and Fig. S3A, C and D**). In K562 cells, COX-2 inhibitors did not induce any significant accumulation in any particular phase of the cell cycle (**Fig. 2B and Fig. S3B**). We verified that the modulation of the COX-2 inhibitors on cell cycle progression was common to the other selected sensitive cell lines (**Fig. S3C–E**).

The cell cycle is regulated by many factors and each phase is controlled by specific proteins whose levels of expression follow a specific timing.[7] We therefore analyzed the mRNA and protein levels of a panel of factors that specifically act during the G_1/S transition. In **Figure 2C and D**, we show that a group of these factors are differentially regulated in U937 vs. K562 cells, as assessed after 48 h of treatment with different concentrations of nimesulide. In treated U937 cells (**Fig. 2C**), the cyclin-dependent kinase inhibitor p27 was upregulated at both the mRNA and protein levels; this change was accompanied by a downregulation of cyclin D1 and PCNA. No modulation of these factors was detected in the K562 cell line (**Fig. 2D**). The same analysis performed earlier, after only 24 h of treatment with nimesulide, revealed that the same cell cycle modulators already started to be affected (**Fig. S4A and B**). Moreover, NS-398 and celecoxib induced similar patterns of modulation at the level of mRNA (**Fig. S4C and D**). Altogether data suggest a modulation

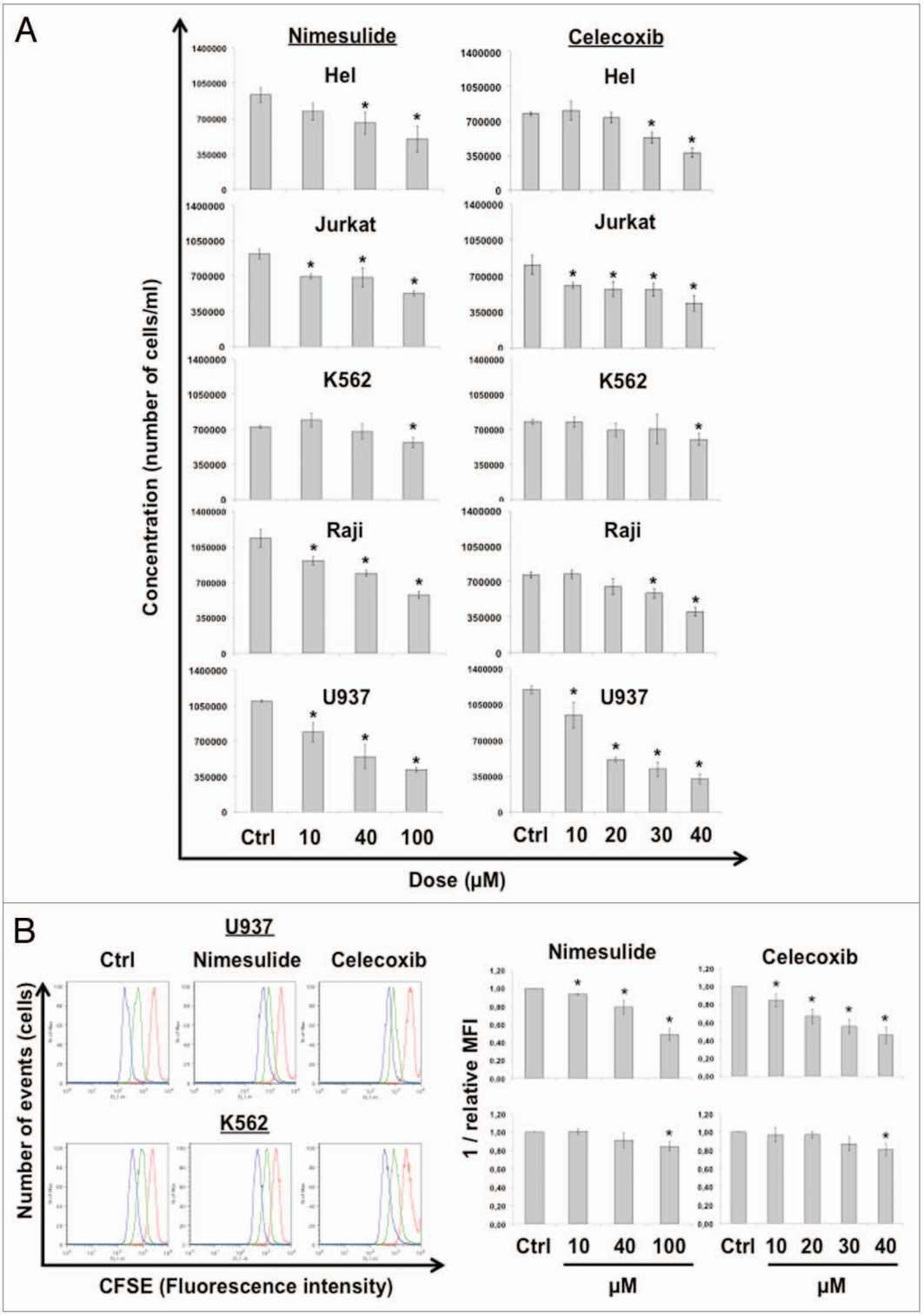


Figure 1. COX-2 inhibitors inhibit cell proliferation in the different cell lines. (A) Effect of nimesulide and celecoxib. Cells were seeded at time 0 at 2 x 10^5 cells/ml and then treated with the indicated concentration of nimesulide or celecoxib for up to 48 h. Cell proliferation was studied using the trypan blue exclusion assay. The experiment is representative of five independent experiments ± SD. (B) CFSE staining analysis to measure cell proliferation in U937 and K562 cells treated with nimesulide (10, 40, 100 μM) or celecoxib (10, 20, 30, 40 μM). Cells were stained with CFSE (0.1 μM), seeded at 2 x 10^5 cells/ml and then treated with the COX-2 inhibitors. CFSE fluorescence was measured after 0, 24 and 48 h by flow cytometry. Quantification of CFSE analysis after 48 h of treatment is represented (bottom parts). The fluorescence values were calculated as the ratio between the fluorescence estimated at 48 h and 0 h for each sample. The values obtained were further normalized with respect the value of the control (relative mean fluorescence intensity, MFI). As the fluorescence intensity is inversely proportional to the rate of proliferation of the cells, the quantification was represented as the inverse of relative MFI (1/relative MFI). The data are representative of four independent experiments (mean ± SD).

of p27, cyclin D1 and PCNA in U937 cells as part of the modulatory mechanism triggered by COX-2 inhibitors on cell cycle.

COX-2 inhibitors lead to an early downregulation of c-Myc protein. It is known that the oncogene c-Myc binds to the promoters of the p27 and cyclin D1 genes to transcriptionally regulate them in opposing ways. c-Myc represses p27 transcription[27] but promotes cyclin D1 expression.[15] We showed that COX-2 inhibitors upregulated p27 and downregulated cyclin D1; the modulation of this expression was also detectable at the mRNA level and was already apparent after 24 h of treatment (**Fig. 2C and Fig. S4C and D**). This similarity prompted us to investigate whether COX-2 inhibitors might affect c-Myc expression in our cell lines.

When U937 cells were incubated for 24 h with different concentrations of nimesulide, a dose-dependent reduction in the protein level of c-Myc was detectable (**Fig. 3A**). Celecoxib gave similar results (**Fig. S5A**). This effect was not limited to U937 cells, since c-Myc protein also appeared downregulated in the other cell lines sensitive to COX-2 inhibitors (**Fig. 3B**). Conversely, no significant changes were seen in K562 cells (**Fig. 3C and Fig. S5B**). A more stringent time-course analysis of c-Myc protein levels revealed that COX-2 inhibitors strongly impacted c-Myc expression already within the first hour of incubation (**Fig. 4A**).

The effect on c-Myc protein might be the result of increased protein instability and/or the modulation of earlier events in c-Myc gene expression, i.e., acting at the transcriptional or translational level. To differentiate between these two scenarios, we first analyzed the ability of COX-2 inhibitors to modulate c-Myc protein stability by using the protein synthesis inhibitor cycloheximide (CHX).

The reduction of c-Myc protein with CHX occurred within the timing of c-Myc downregulation that occurred upon treatment with nimesulide (**Fig. 4B**). When used in combination with CHX, nimesulide did not accelerate the disappearance of c-Myc protein (**Fig. 4C**). This excluded a direct modulatory role of COX-2 inhibitors on c-Myc protein stability and prompted us to determine the impact of COX-2 inhibitors on the upstream level of c-Myc expression.

We next investigated the potential of COX-2 inhibitors to affect c-Myc mRNA. As shown in **Figure 5**, we observed a significant reduction of c-Myc mRNA within 1 h of incubation with nimesulide (**Fig. 5A**). Since c-Myc mRNA possesses a 3'UTR region that can be targeted by specific factors that may affect its stability,[28] we determined whether COX-2 inhibitors may be implicated in this kind of modulation. **Figure 5B** shows no change in mRNA stability upon treatment with nimesulide or celecoxib, even at the highest drug doses. These results strongly suggest an affection of c-Myc transcription. c-Myc promoter activation may be controlled by several kinase-mediated pathways such as extracellular signal-regulated kinase 1/2 (ERK1/2),[29] and $p38^{MAPK}$.[30] We found that nimesulide very rapidly inhibited ERK1/2 phosphorylation with a similar extent to those observed using ERK inhibitor PD98059 (25 μM; 1 h of treatment; **Fig. 5C**) whereas p38 phosphorylation remained unaffected by the treatment (**Fig. S6**).

Altogether, these results indicate that the very early downregulation of c-Myc induced by COX-2 inhibitors is not dependent on a modulation of protein or mRNA stability and strongly suggests that this might be due to a regulation of transcriptional events controlling c-Myc expression where ERK-mediated pathways may be implicated.

Cyclooxygenase expression in leukemic and lymphoblastic cell lines. The modulation of cell proliferation and c-Myc expression by COX-2 inhibitors may be due to their specific ability to inhibit COX-2 enzymes or, alternatively, correspond to an off-target effect. To address the question, first we analyzed the level of expression of the two major COXs enzymes, COX-1 and COX-2 in our panel of hematopoietic cell lines at both the mRNA (by real-time PCR) and protein (by western blot analysis) levels (**Fig. 6**). COX-1 was expressed in all the cell types (**Fig. 6A**). For COX-2 (**Fig. 6B**), a similar trend of mRNA expression was observed. However, K562 cells express COX-2 protein at very low levels. These data show a correlation between COX-2 expression and the sensitivity of the cell lines to COX-2 inhibitors. The same analysis aimed at analyzing the level of expression of p53 or p73 (**Fig. S7A and B**), which are also implicated in cell cycle regulation,[31-33] did not show any correlation with the sensitivity to COX-2 inhibitors effect.

The biological properties of COX-2 enzymes are mainly due to prostaglandin E2 (PGE2),[21] which is well known to be involved in cell proliferation[34,35] and to transactivate ERK.[36] Therefore, we analyzed whether the effect of COX-2 inhibitors on c-Myc expression might be reversed by the exogenous administration of PGE2. To this purpose, U937 cells were pretreated with different concentrations of PGE2 before incubation with nimesulide. However, in presence of PGE2, nimesulide maintained its ability to induce c-Myc protein downregulation (**Fig. 6C**). These data suggest that, despite the good correlation between COX-2 expression and the sensitivity of the cell lines to COX-2 inhibitors, COX-2 inhibitors might affect c-Myc expression independently of PGE2 synthesis.

COX-2 inhibitors induce myeloid markers in sensitive acute myeloid leukemia cell lines. c-Myc expression is associated with the maintenance of cancer cells in an undifferentiated state. Consequently, its downregulation may promote accumulation of the cells in G_0 phase of cell cycle and cell differentiation.[9,37] The pattern of cellular alterations so far described as induced by COX-2 inhibitors may fit with the establishment of a more advanced pro-differentiated state. Therefore, we investigated any potential of COX-2 inhibitors to promote cell differentiation. We have shown that U937 and Hel are among the most sensitive cell lines to COX-2 inhibitors effects whereas K562 are practically unaffected. These cell lines are also eligible cell models to study an eventual induction of cell differentiation by COX-2 inhibitors. Because the promyeloid U937 cells have the potency to differentiate toward monocytes or granulocytes, we assessed a possible induction of myeloid differentiation in this cell line treated with nimesulide or celecoxib by analyzing the expression of the markers CD11b, CD14 which are more expressed in monocytes[38,39] and CD15 which is preferentially expressed in granulocytes[40,41] (**Fig. 7A and B**). From 10 μM of nimesulide and celecoxib, we witnessed a dose-dependent induction of CD15 already after 48 h of treatment whereas no increase of CD11b or CD14 might

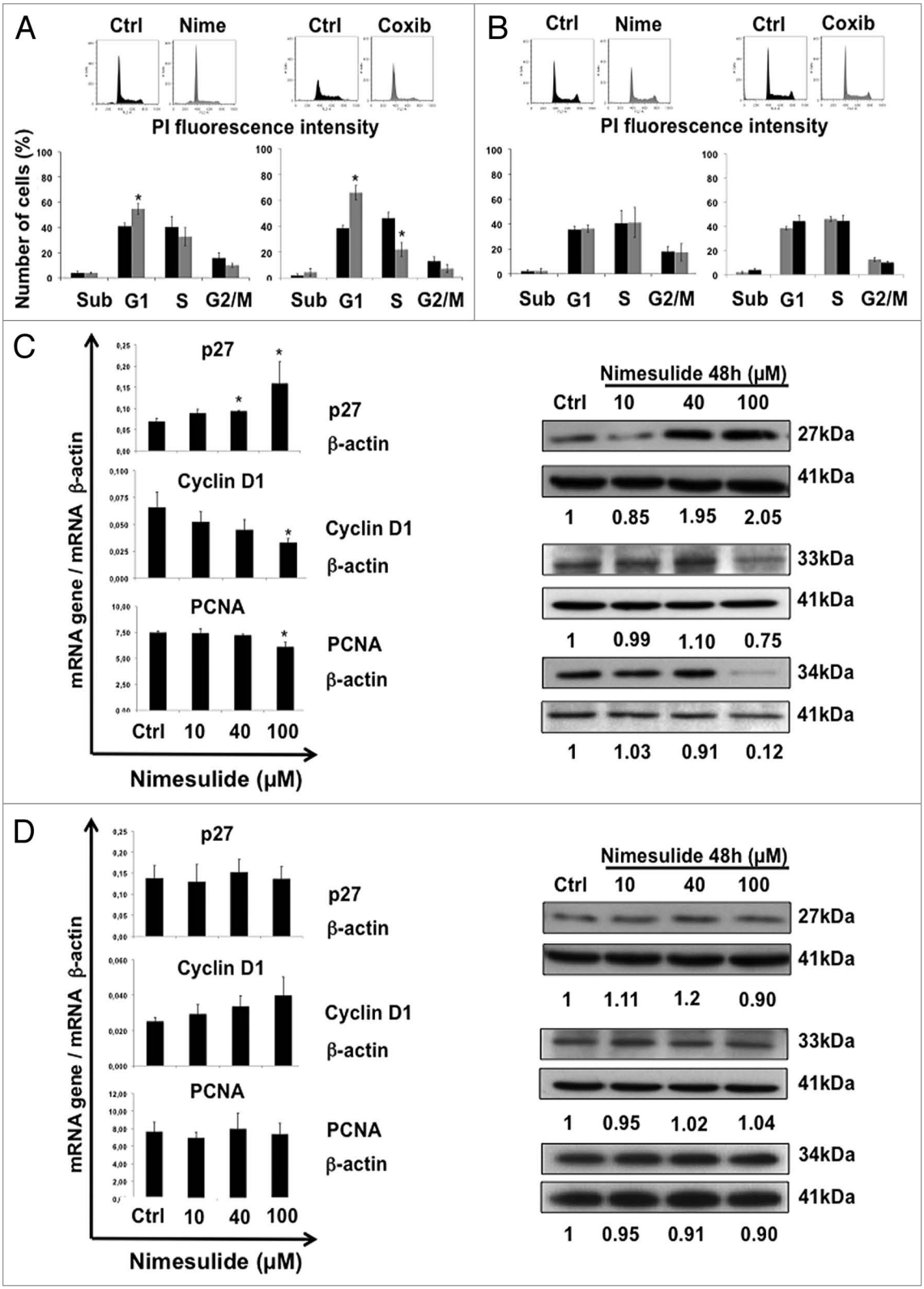


Figure 2. COX-2 inhibitors differentially affect G_1/S transition modulators in U937 and K562. Cell cycle analysis of U937 (A) or K562 (B) cells treated with nimesulide (100 μM) or celecoxib (40 μM) for 48 h. Cells were stained with propidium iodide (1 μg/ml) and cell cycle distribution was analyzed by flow cytometry. The data are representative of three independent experiments (mean ± SD). The level of cell cycle regulators (p27, cyclin D1 and PCNA) determined by real-time PCR analysis (left; mean of three independent experiments ± SD) and western blot (right; one of three independent experiments) in U937 (C) and K562 (D) cells treated for 48 h with nimesulide at the indicated concentrations. The data are representative of three independent experiments showing similar results.

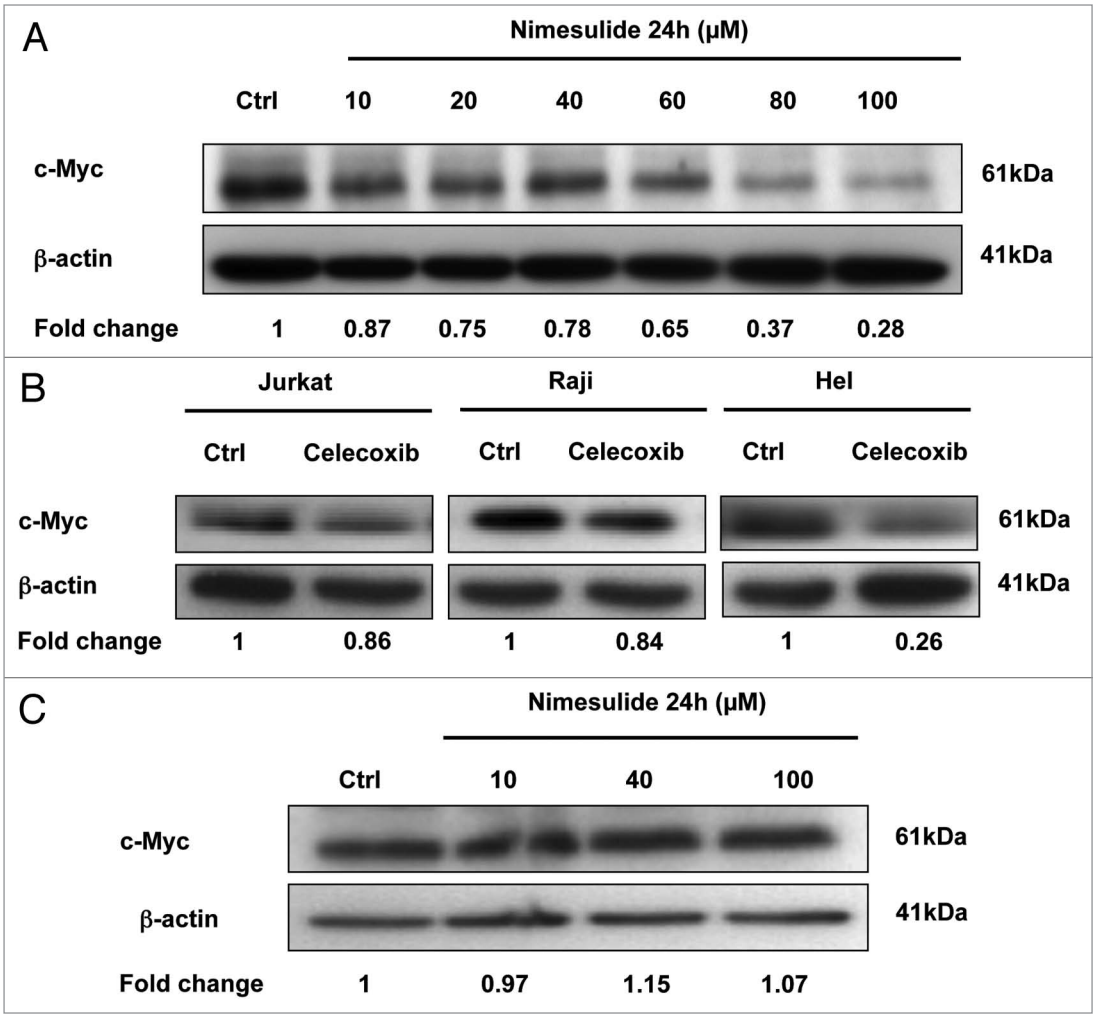


Figure 3. Nimesulide downregulates c-Myc in COX-2-positive cell lines. Effect of nimesulide (24 h of treatment) on c-Myc protein in: (A) U937 cells (0–100 μM); (B) the other COX-2 expressing cells, including Jurkat, Raji, Hel (100 μM); and (C) the COX-2-nonexpressing K562 cells (10, 40 or 100 μM). The data are representative of three independent experiments showing similar results.

be detected even after longer times, i.e., 72 h, of treatment. Only celecoxib at the highest dose mildly inhibits CD11b expression. The expression of CD15 suggests the promotion by COX-2 inhibitors of a granulopoietic-like cell differentiation.

Cells committed to differentiation typically accumulated in G_0 phase of the cell cycle, the only phase during which the cells do not express the nuclear protein K_i-67.[42] **Figure 7C and D** reports the in situ analysis of K_i-67 protein expression by fluorescence microscopy and quantification by flow cytometer of untreated U937 cells compared with the ones treated with various concentrations of nimesulide and celecoxib. A significant dose-dependent increase in the percentage of K_i-67-negative cells might be appreciated, thus proving that a consistent fraction of U937 cells accumulate in G_0 phase of the cell cycle upon treatment. The pattern of alterations observed may be compatible with senescence or quiescence. The induction of senescence is accompanied by a strong increase of the size of the cells and a strong positivity to β-galactosidase staining.[43-45] Therefore, we performed a β-galactosidase assay on U937 cells treated with nimesulide or celecoxib (**Fig. S8**). In our positive control (K562 treated during 4 d with 100 nM of doxorubicin), we confirmed the expected pattern of alterations. In contrast, in U937 cells, COX-2 inhibitors did not lead to any relevant modifications.

We next verified any ability of COX-2 inhibitors to promote differentiation in Hel and K562 cells, which share the common property to differentiate in megakaryocytes or erythrocytes.[46] First, the expression of megakaryocytic markers, CD41a and CD61,[47] were investigated. In Hel, we witnessed a significant increase of both markers as early as 3 d of treatment (**Fig. 8A and B**). The same analysis performed at longer times (6 d of treatment) confirmed the same pattern of alterations, thus suggesting a megakaryocytic commitment (**Fig. 8A and B**). In K562 cells up to 6 d of treatment, no expression of CD41a and CD61 has been detected (**Fig. 8A**). Second, we verify the potential of K562 and Hel to differentiate in erythrocytes upon COX-2 inhibitors treatment by performing a benzidine assay (**Fig. 8C and D**). No positivity for Hel cells was detected up to 6 d of treatment. In K562 cells, the experiment revealed a slight increase of

benzidine-positive cells after 3 d of treatment only with nimesulide, which was not maintained at longer times of treatment (6 d), thus suggesting a mild and transient drug-specific effect (**Fig. 8D**). These data fit with the absence of c-Myc modulation in K562 cells upon COX-2 inhibitors treatment (**Fig. 3C**).

Altogether, these results suggest that COX-2 inhibitors lead to a pro-myeloid state of both acute myeloid cell lines, with a tendency to the granulocytic way for U937 cells and the megakaryocytic one for Hel.

Discussion

COX-2 inhibitors impair c-Myc expression. In the literature, an accumulation of cancer cells in G_0/G_1 has been reported to be a consequence of treatment with COX-2 inhibitors. In this context, meloxicam has been found to downregulate PCNA and cyclin A in a hepatocellular carcinoma cell line.[48] Similarly, in the MC-26 cell line, a highly invasive mouse colorectal cancer constitutively expressing COX-2,[49] NS-398 has been demonstrated to exert similar effects, including a reduction of cyclin D1 and PCNA, thus perturbing the G_1/S transition. Studies of hematopoietic cancers have shown an upregulation of p21 due to NS-398 and an accumulation of the cells in the G_0/G_1 phase of cell cycle.[50] However, no clear upstream events or regulators responsible for these patterns of cell cycle alterations have so far been identified.

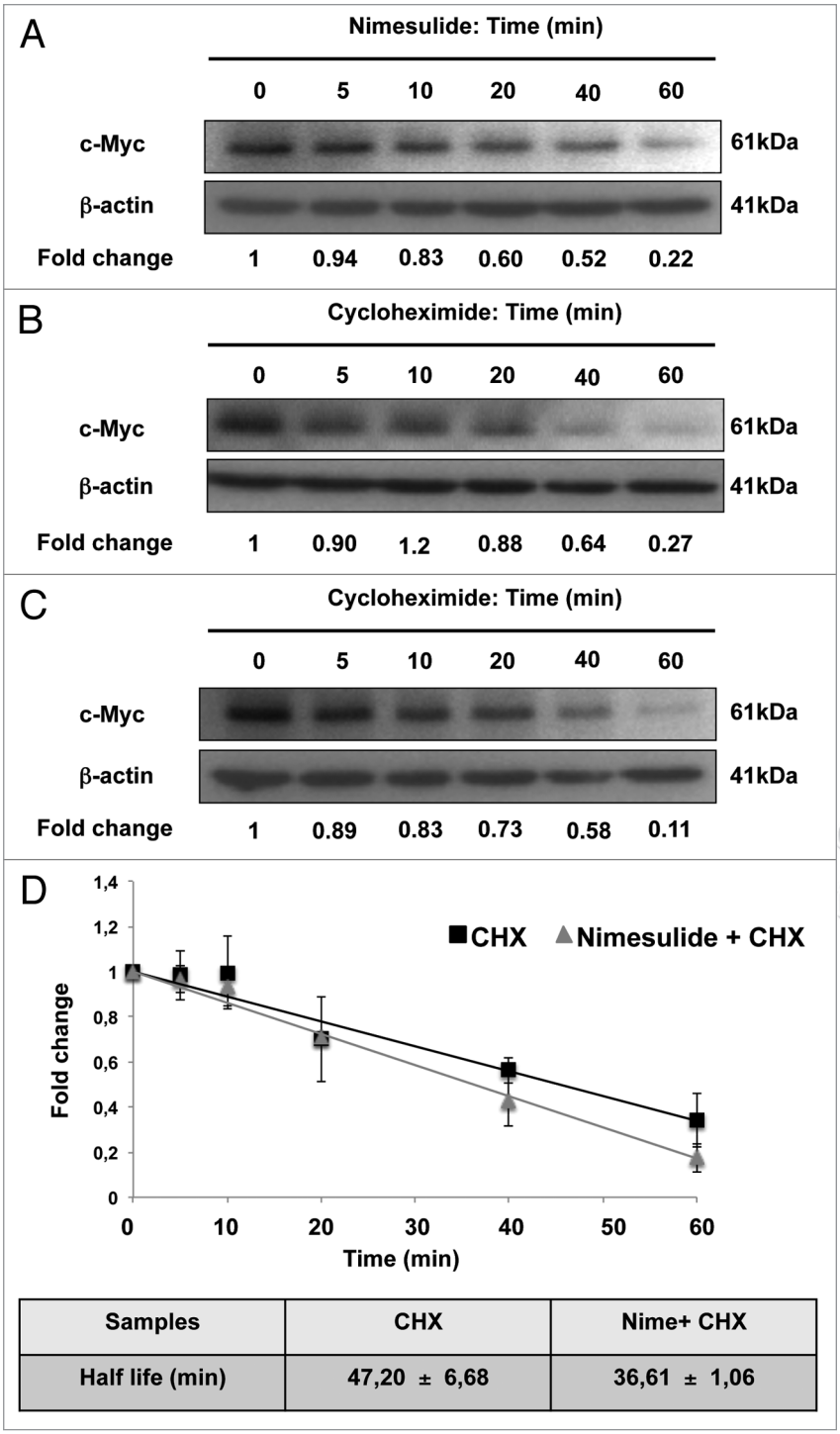
The downregulation of cyclin D1 and the upregulation of p27 at the mRNA level led us to investigate a possible impact of COX-2 inhibitors on upstream transcription factors regulating the expression of these genes. One of these factors is the oncogene c-Myc, which is known to be a positive regulator of cyclin D1 transcription[15] and a repressor of p27.[27] Our study demonstrates that COX-2 inhibitors are able to downregulate c-Myc and to trigger this alteration very efficiently, even within one hour. Our findings exclude protein stability from being the step of c-Myc expression that is impaired by COX-2 inhibitors (**Fig. 5**). Our study therefore suggests that the early downregulation of c-Myc occurs via other mechanisms that operate upstream of protein synthesis. The quantification of c-Myc mRNA showed a significant reduction within one hour of treatment. c-Myc mRNA is known to possess a 3'-untranslated region (UTR) targeted by specific factors that may modulate its stability.[28] However, the stability of the mRNA remained statistically unchanged at each measured time-point, as assessed by our time-course analysis with the transcription inhibitor actinomycin D. This indicates that the transcriptional repression of c-Myc appears to occur quite rapidly after COX-2 administration. This does not exclude that an inhibition of translation may stably take place that accompanies transcriptional repression.

We confirmed in our cell systems that the inhibition of ERK phosphorylation with the specific inhibitor PD98059 downregulates c-Myc expression. Here, we highlight that nimesulide is able to inhibit ERK1/2 phosphorylation to a similar extent. This modulation precedes c-Myc protein downregulation (**Fig. 5C**). This strongly suggests that the COX-2 inhibitors may mediate an attenuation of ERK-mediated pathways, which, in turn, may be responsible for the inhibition of c-Myc transcription. Our finding also supports this hypothesis that c-Myc mRNA pool is early reduced without affecting mRNA stability. However, we cannot exclude that other transcriptional and translational mechanisms as well may be implicated. Published data described that celecoxib can inhibit $p38^{MAPK}$ in hepatocarcinoma.[51] However, in our study, no modulation of p38 phosphorylation has been observed, thus suggesting a cell type-dependent mechanism. Further studies are needed to identify all the mechanisms responsible for the patterns of c-Myc mRNA/protein alterations. We suggest some other potential molecular candidates to be investigated in the future. For example, Wnt/β-catenin is one of the intracellular signaling pathways that may control transcription factor binding to the c-Myc promoter. Interestingly, the Wnt/β-catenin pathway has been reported to be a target of COX-2 inhibitors.[52] mTOR is another kinase to be considered. Indeed, the mTOR pathway is also implicated in G_1/S transition by inhibiting p27 expression and by stimulating cyclin D1 expression.[53] Moreover, a link between c-Myc and mTOR has been already described in different cell models.[53-55]

In our study, a correlation between the constitutive expression of COX-2 and the effects of COX-2 inhibitors has emerged. The modulatory effect on cell cycle and differentiation triggered by COX-2 inhibitors was not observed in K562 cells, which we found very mildly expressing COX-2 protein. Moreover, the analysis of p53 and p73, which play also a role in cell cycle,[32,33,56] did not reveal any correlation with the sensitivity of hematopoietic cancer cell models to COX-2 inhibitors. These findings may suggest a COX-2-dependent effect. Celecoxib showed the strongest effect on cell proliferation, and this was correlated with the lower IC_{50} reported for this compound toward COX-2 (0.04 μM vs. 1.27 μM for nimesulide and 1.77 μM for NS-398).[57] In addition, similar results have been obtained with hematological malignant cell lines such as Raji, BjAB (Epstein-Barr virus negative cell line) and BL41 (Burkitt's lymphoma).[58] In these cell lines, celecoxib was also able to inhibit cell proliferation.[59] An ability of nabumetone and NS-398 to inhibit proliferation of the U937 and ML-1 cell lines and induce an accumulation of cells in the G_0/G_1 phase of cell cycle has been also described in reference 50. In the same study, the use of anti-sense RNA against COX-2 showed that this was at least in part a COX-2-dependent effect.[50]

In our study, the pre-treatment of U937 cells with PGE2, the upstream product of COX-2 enzyme activity, was unable to revert nimesulide-induced c-Myc downregulation (**Fig. 6C**). These results suggest that COX-2 inhibitors might act independently of PGE2 but the involvement of other prostaglandins should be considered. Indeed, other prostaglandins can promote a proliferative state of the cells.[23,60] Besides, the exacerbation of peroxisome proliferator-activated receptor (PPAR)gamma-dependent pathways occurring downstream to arachidonic acid production affects cell proliferation and may promote cell differentiation.[61,62] Therefore, the involvement of pathways downstream to the COX-2 substrate arachidonic acid may be also conceivably implicated.

COX-2 inhibitors induce myeloid differentiation markers expression. Our study reveals an induction of specific differentiation markers in U937 cells (granulocytic CD15) and Hel

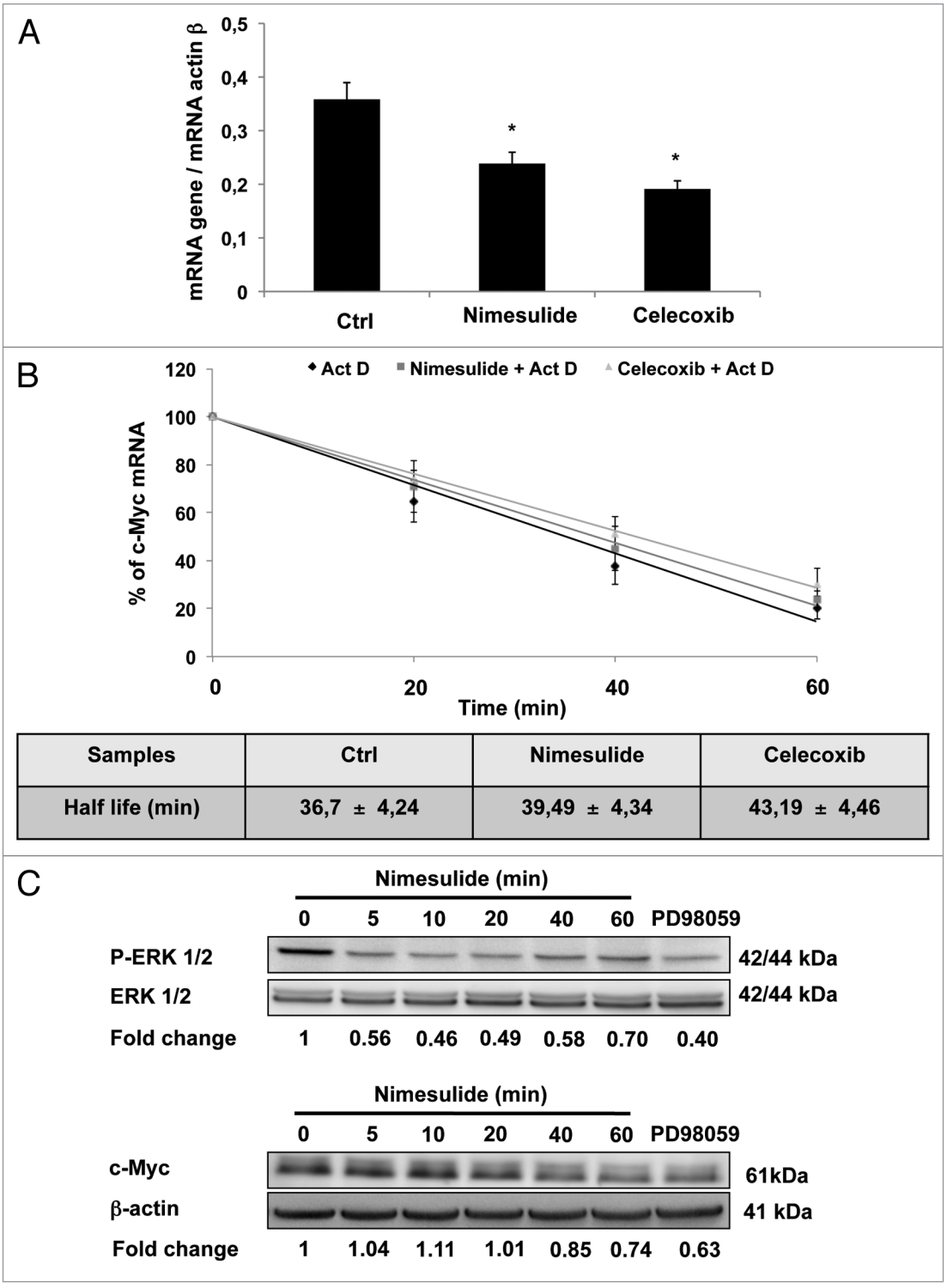


Samples	CHX	Nime+ CHX
Half life (min)	47,20 ± 6,68	36,61 ± 1,06

Figure 4. Effect of COX-2 inhibitors on c-Myc protein stability. Time course of c-Myc protein downregulation following incubation with nimesulide (100 μM) in U937 cells (A). The time-course analysis with the protein synthesis inhibitor cycloheximide (CHX), as determined in U937 cells: untreated (B), pre-treated for 1 h with (C) nimesulide (100 μM). The data are representative of three independent experiments showing similar results. (D) Quantification of the c-Myc downregulation time courses: (D) represents (B and C); (mean of three experiments ± SD). The corresponding half-life values ± SD are reported in the bottom part.

(megakaryocytic CD41a and CD61), both sensitive to the anti-proliferative effects of the COX-2 inhibitors. Remarkably, K562 cells, which are not affected in their proliferation by COX-2 inhibitors, do not express differentiation markers. U937 cells may also differentiate in monocytes, frequently associated with the upregulation of CD11b and CD14,[39] that we did not observed. In our study COX-2 inhibitors inhibit ERK1/2 phosphorylation and in literature, a causal link between ERK phosphorylation and the granulocytic differentiation of U937 cells has been described in reference 63. In this study, the importance of the transcription factor CCAAT/enhancer binding protein α (C/EBPα) has been underlined.[63] The phosphorylation of this protein on serine 21 by ERK is responsible for the inhibition of granulopoiesis. Moreover, it has been suggested that c-Myc represses C/EBPα transcription,[9] thus showing the importance of c-Myc in granulopoiesis. Moreover, c-Myc transcription is negatively regulated by C/EBPα.[64] We suggest this factor as a possible key element in our system to investigate in the future. c-Myc seems to play also a direct role in cell differentiation. Indeed, recently, a link between c-Myc and megakaryocytic commitment has been demonstrated in some studies showing an increase of megakaryocytic differentiation in mice c-Myc$^{-/-}$.[65]

Even if COX-2 inhibitors trigger the expression of differentiation markers, however, the sensitive cells never reach a state of terminal differentiation. The May-Grünwald/Giemsa staining performed in U937 cells cultivated up to one week with COX-2 inhibitors do not reveal any acquisition of the nuclear features expected in case of a more advanced granulopoietic differentiation (not shown). Besides, cell viability continues to be not affected (**Fig. S1**). Thus, COX-2 inhibitors seem to push the cells toward a more advanced differentiated state without allowing them to reach the terminal differentiation. In this instance, the cells may conceivably just cycle much slower. The analysis of the expression of the nuclear antigen K_i-67 suggests an interpretation. After mitosis, the cells may be entrapped for some periods in G_0 phase followed by their re-entering in G_1 and cell cycle progression. Interestingly, p27, which is a factor promoting the quiescence of mammalian cells[66] is upregulated by COX-2 inhibitors. Our findings seem to exclude an effect of COX-2 inhibitors on cellular senescence; typical alterations of senescence like hypertrophy and exacerbated β-galactosidase staining cannot be observed in U937 cells upon



Samples	Ctrl	Nimesulide	Celecoxib
Half life (min)	36,7 ± 4,24	39,49 ± 4,34	43,19 ± 4,46

Figure 5. Effects of COX-2 inhibitors on c-Myc mRNA. Quantification of c-Myc mRNA in U937 cells treated for 1 h with nimesulide (100 μM) or celecoxib (40 μM) (A) by Real-time PCR analysis. The expression of mRNA is represented as the ratio of c-Myc mRNA/β-actin mRNA. The data are the mean of three different experiments ± SD. Time-course analysis of c-Myc mRNA stability, studied by using the transcription inhibitor actinomycin D (ACTD; time 0). In U937 cells, untreated or pre-treated with nimesulide (100 μM) or celecoxib (40 μM) for 1 h (B). The corresponding half-life values ± SD are reported in the bottom part. The effect of nimesulide on ERK1/2 phosphorylation is shown in (C). As a negative control, the specific ERK inhibitor PD98059 was used (25 μM; 1 h). The data are the mean ± SD of three independent experiments.

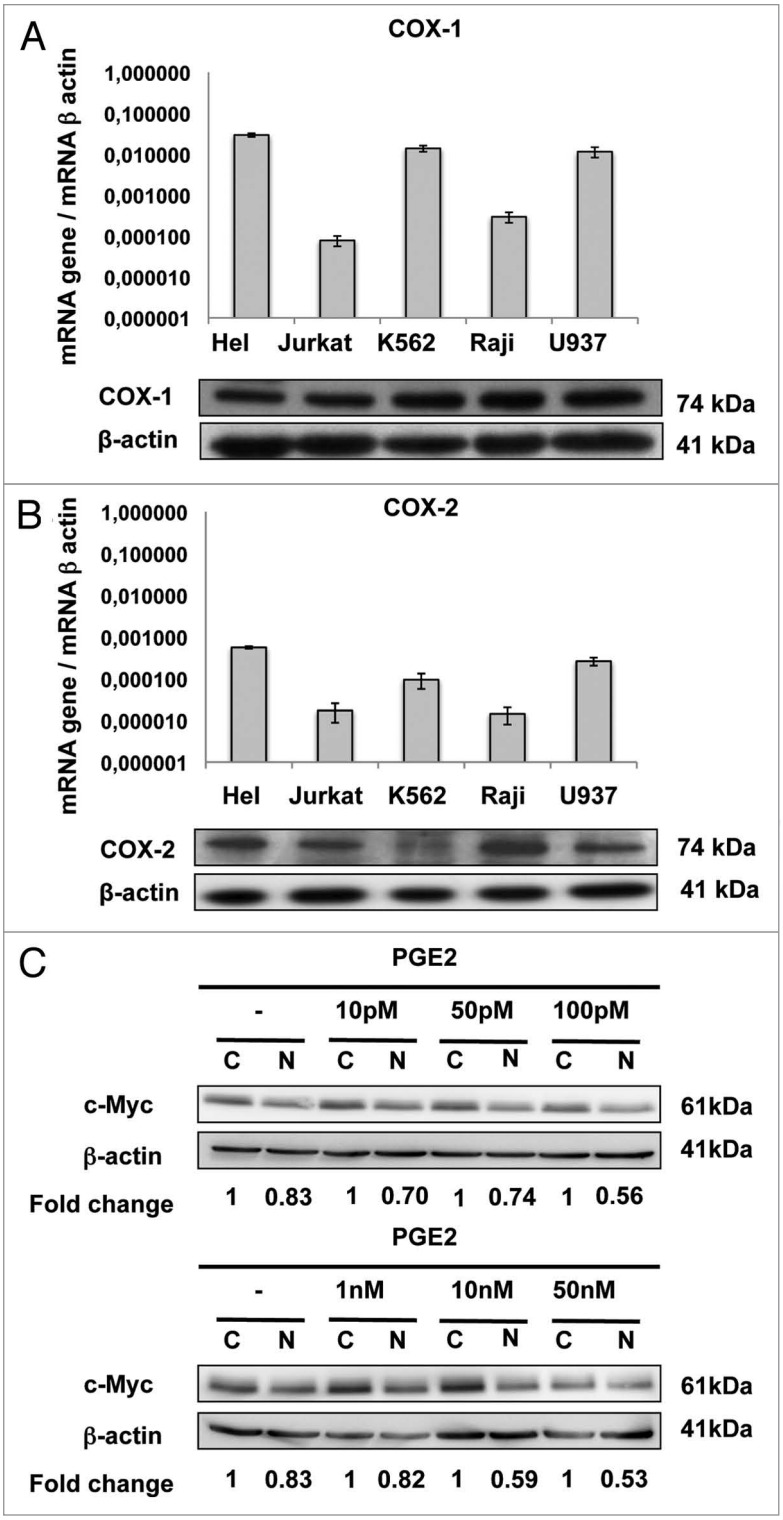


Figure 6. COX-1 and COX-2 expression in different hematopoietic cell lines. COX-1 (A) and COX-2 (B) expression at the level of mRNA (top), as determined by Real-time PCR analysis, and at the protein level (bottom), as determined by western blot analysis. β-actin (mRNA or protein) was used in both analyses as a reference. For mRNA quantification, the data are the means of three independent experiments ± SD. For western blot analysis, one of at least three independent experiments is shown. (C) The effect of PGE2 on nimesulide-induced c-Myc downregulation. U937 cells were incubated with the indicated concentrations of PGE2 for 1 h, then treated with nimesulide (100 μM) for 1 h. The expression of c-Myc was analyzed by western blot.

COX-2 inhibitor treatment. It has been suggested that the choice between quiescence and senescence depends on mTOR activation.[67,68] The inhibition of mTOR is frequently associated with quiescence and this inhibition can be driven by p53.[68] However, U937 cells do not express p53 and p73. Therefore, our data imply that the choice between quiescence/senescence should be p53-independent in U937 cells treated with COX-2 inhibitors.

In these latest years, an upsurge of interest concerns the ability of cancer cells to enter in a dormant state.[69] This condition can be interrupted even after years up to decades from the first diagnosis and successful treatment of cancer patients and is currently considered the main factor contributing to tumor relapse. A desirable condition is to find out therapeutic treatments that maintain these cells in dormancy. A comparison of the hallmarks described for cancer dormant cells to the pattern of alterations produced by COX-2 inhibitors reveals a number of similarities: the inhibition of cell proliferation and the enter in G_0 phase, and most-notably, c-Myc downregulation and the acquisition of a more differentiated state, by means of which the cells tend to behave like the normal counterpart.[70] Importantly, the re-activation of c-Myc protein expression is per se sufficient to reactivate the malignant phenotype.[71] We do not know yet what exactly maintains the cells so longer in dormancy. Conceivably, cells may cycle very much slower rather than completely stopping in G_0, to be maintained so long times. The fact that COX-2 inhibitors similarly trigger an early and stable downregulation c-Myc and affect cell proliferation may be thus an intriguing aspect to be further elucidated.

c-Myc is known to be overexpressed in many kinds of tumors, such as Burkitt's lymphomas and different leukemia,[72] where it contributes to their aggressiveness and poor prognosis. Thus, much effort is being placed on targeting this oncogene.[13,14] Our data identify a novel biological property of COX-2 inhibitors and suggests their possible use to target this oncoprotein. Moreover, it would be interesting to check a possible effect on other members of the MYC family such as MYCN, which is amplified in pediatric tumors of the nervous system.[73] Thus, investigating whether COX-2 inhibitors lead to similar effects in these tumors may have important clinical implications. Besides, the dysregulation of differentiation is frequently associated with acute myeloid leukemia. The use of pro-differentiating agents like retinoic acid, RXR agonist (i.e., bexarotene) or valproic acid, has already shown an ability to overcome this defect in leukemia[74] and is associated with a better prognosis. Similarly, 1,25-dihydroxyvitamin D_3 (1,25D) displays an ability to induce differentiation in leukemic cells.[75] However, the clinical use of this drug bears the risk of severe side effects, such as hyperkalemia.[76] Thus, efforts are being placed on a combination

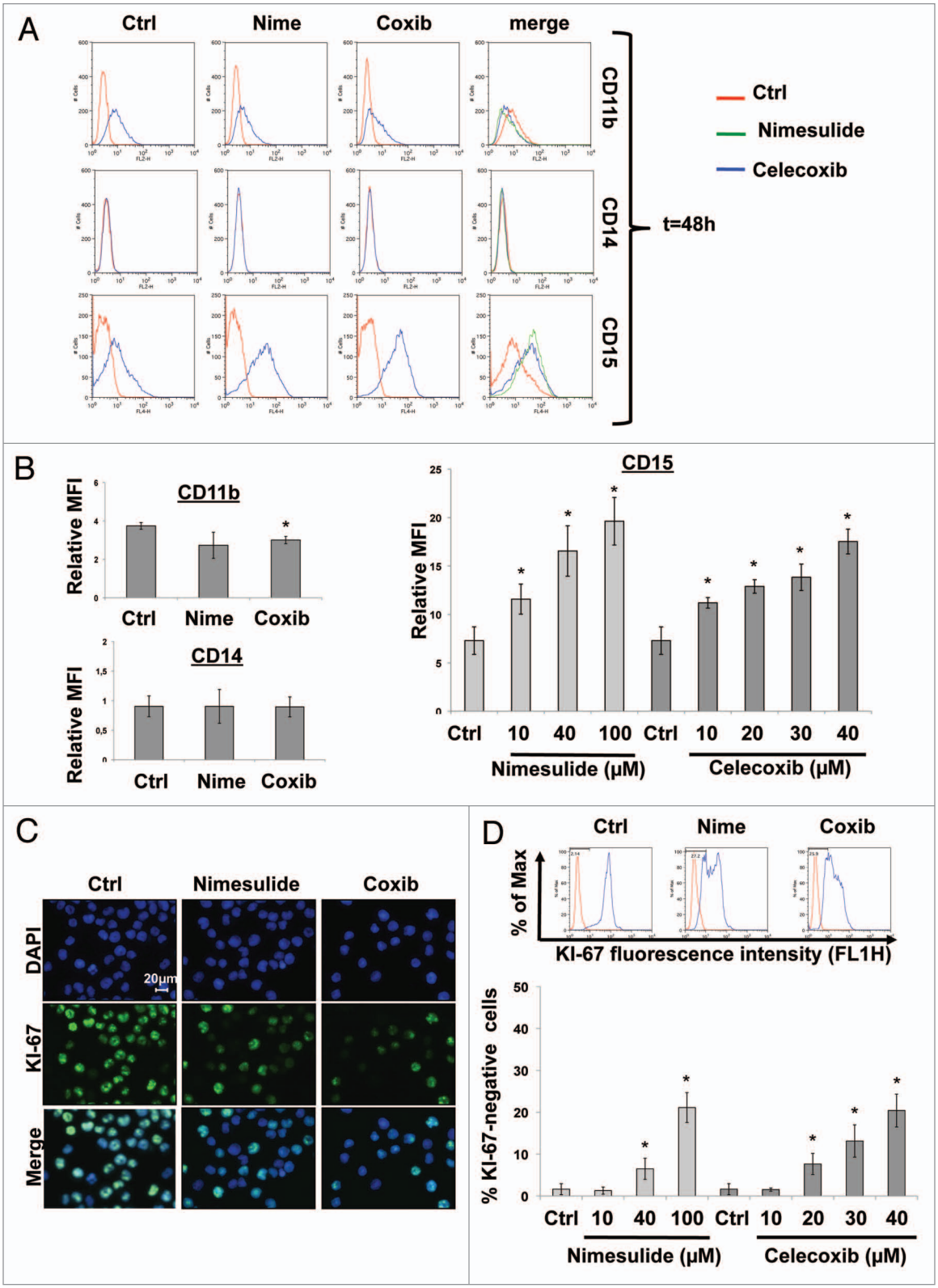


Figure 7. COX-2 inhibitors induce CD15 expression in U937, cells. Flow cytometric analysis of CD11b, CD14 and CD15 in U937 cells treated for 48 h with nimesulide or celecoxib (A and B). Analysis of K_i-67 protein expression by fluorescence microscopy (C) and flow cytometry (D) in U937 cells treated for 48 h with nimesulide or celecoxib.

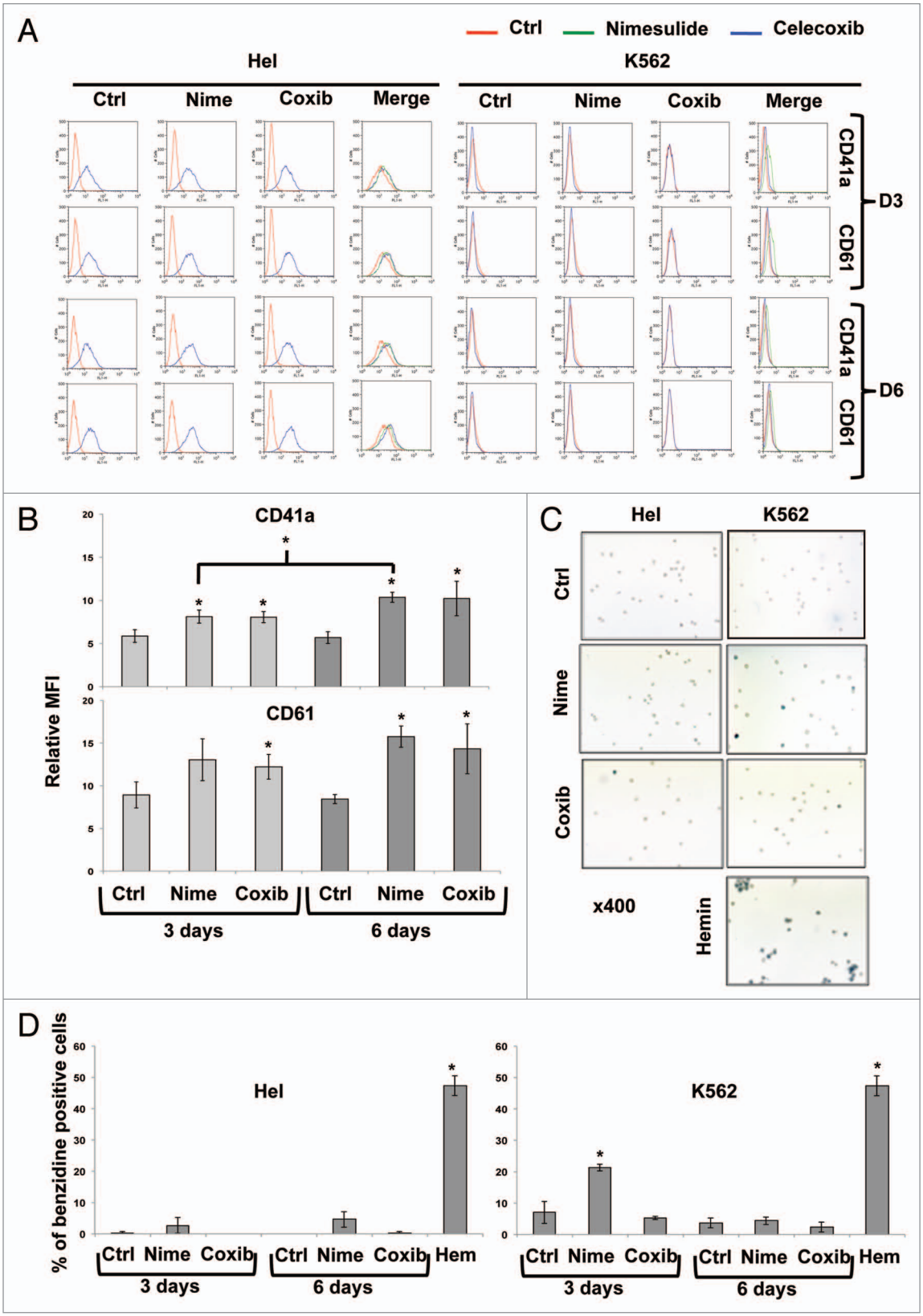


Figure 8. Differential effects of COX-2 inhibitors on megakaryocytic differentiation markers in Hel and K562 cells. (A) Flow cytometric analysis of CD41a and CD61 in Hel and K562 cells treated for 3 d and 6 d with nimesulide or celecoxib. The corresponding quantifications of CD41a and CD61 for Hel are represented in (B). (C and D) Benzidine staining of Hel and K562 cells after 3 and 6 d of treatment with COX-2 inhibitors. As a positive control, K562 cells were treated for 48 h with hemin (30 μM).

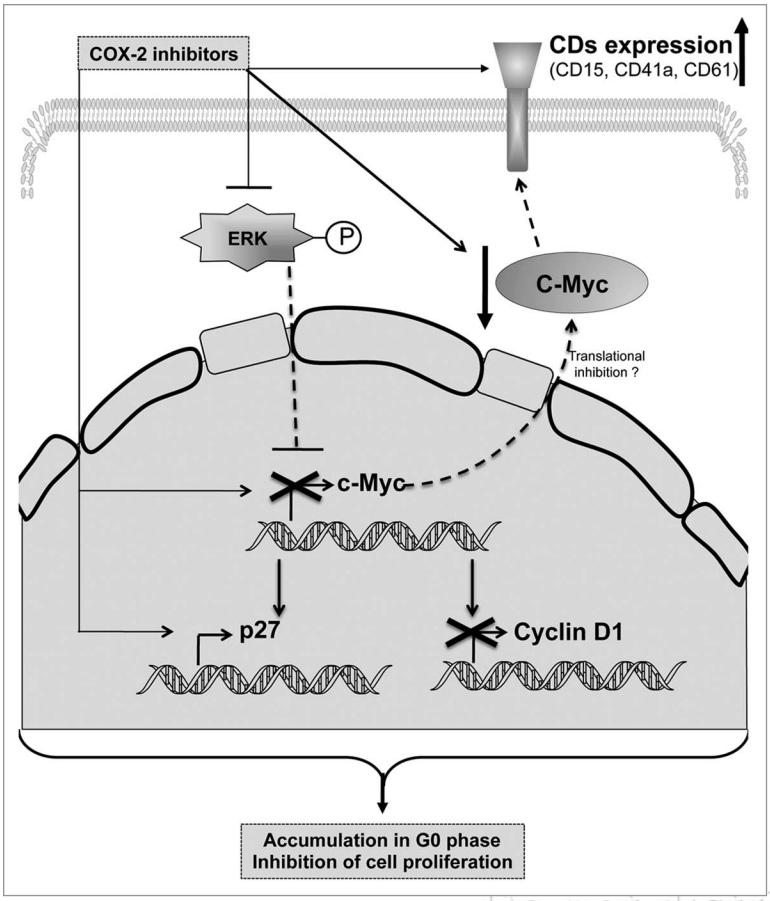


Figure 9. Action of COX-2 inhibitors on cell proliferation modulation of hematopoietic cancer cells: a model. COX-2 inhibitors early downregulate c-Myc. COX-2 inhibitors lead to a reduction of cyclin D1 and an upregulation of p27, which are, respectively, positively and negatively transcriptionally regulated by c-Myc. Consequently, the inhibition of G_1/S transition and accumulation of cells in G_0 phase take place. This is accompanied by the expression of cell differentiation markers and the reduction of cell proliferation. COX-2 inhibitors inhibit ERK1/2 phosphorylation, which may be implicated in c-Myc expression inhibition.

of lower concentrations of 1,25D with other drugs. In this context, an enhancement of 1,25D-induced differentiation has been obtained with NSAIDs (indomethacin) and COX-2 inhibitors (DUP-697).[75] In this study, this effect was associated with an enhancement of Raf1 phosphorylation and a decrease of ERK1/2 phosphorylation in HL-60 and U937 cells. Moreover, in another study the abrogation of Cot1 oncogene, associated to an inhibition of its downstream target ERK5, sensitizes AML cell models to 1,25D-induced differentiation.[77] Our study demonstrates an ability of COX-2 inhibitors to increase myeloid differentiation markers and to inhibit ERK1/2 phosphorylation, thus showing interesting similarities. Altogether, our data suggest a beneficial effect of these drugs in pro-differentiating therapy; besides, conceivably, COX-2 inhibitors might sensitize our cell models to differentiation inducers.

In summary, we have shown for the first time that COX-2 inhibitors inhibit c-Myc expression at very early times. The data are in favor of an early effect on transcription, where the early inhibition of ERK-mediated pathway may play a role. The subsequent upregulation of p27, together with the downregulation of cyclin D1 and PCNA, contributes to stop the cells in G_0 and impairs the G_1/S phase transition of the cell cycle. These events are associated with a more advanced differentiated state. **Figure 9** gives a hypothetical scheme of the molecular events implicated in the action of COX-2 inhibitors.

Materials and Methods

Cell culture and reagents. U937 (human histiocytic lymphoma), Jurkat (acute lymphoid leukemic T cells), K562 (chronic myeloid leukemia), Raji (Burkitt's lymphoma) and Hel (human megakaryocytic acute myeloid leukemia) cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ) were cultured in RPMI 1640 medium (Bio-Whittaker) containing 10% (v/v) fetal calf serum (FCS; Lonza, Verviers, Belgium), 1% (v/v) antibiotic-antimycotic solution (Lonza) and 2 mM L-glutamine (Lonza) at 37°C in a 5% CO_2 humidified atmosphere. The experiments were performed on cells in the logarithmic phase of growth and in culture medium containing 10% FCS. Nimesulide and NS-398 were purchased from Cayman Chemicals. Celecoxib was purchased from Merck. SB203580 (inhibitor of $p38^{MAPK}$) and PD98059 (inhibitor of ERK1/2) were purchased from Promega.

Cell viability assays. Cell viability assays were performed by using the Trypan blue exclusion assay. The fraction of Trypan blue-positive cells was estimated by using a Malassez chamber and confirmed by using the cell counter Cedex (Innovatis AG).

Evaluation of apoptosis. The percentage of apoptotic cells was quantified as the fraction of cells showing apoptotic, fragmented nuclei[78] as assessed by counting at least 100 cells in at least three random fields by fluorescence microscopy (Leica-DM IRB microscope) after staining with the DNA-specific dye Hoechst 33342 (Sigma). Alternatively, cleavage of caspase-3 (Santa Cruz Biotechnology) was assessed by western blot (WB) analysis. Etoposide (VP16; 100 μM for 5 h in U937 cells) was used as a positive control.

Immunofluorescence analysis. *Carboxyfluorescein succimidyl ester (CFSE) cell tracker assay.* CFSE cell tracker assay was performed according to the manufacturer's instructions (Sigma). Briefly, cells were washed twice in PBS and then resuspended (1×10^6 cells/ml) in a solution of PBS containing 0.2 μM CFSE for 10 min at 37°C. After two washes in PBS, cell were resuspended in culture medium (2×10^5 cells/ml). The fluorescence intensity was measured by flow cytometry (FACSCalibur, Becton Dickinson Biosciences) at 0, 24 and 48 h after the treatment with the COX-2 inhibitors. Data were recorded (10,000 events/sample) using the CellQuest software (www.bdbiosciences.com/features/products/display_product.php?keyID = 92) and further analyzed with FlowJo 8.8.5 software (Tree Star Inc.).

Cell cycle analysis. Cell cycle distribution was analyzed according to standard procedures based on DNA staining with

propidium iodide (1 μg/ml, Sigma-Aldrich) and RNase A (100 μg/ml; Roche) in PBS before analysis by FACS. Events were recorded statistically (10,000 events/sample) using the CellQuest software. Data were further analyzed by using Flow-Jo 8.8.5 software.

Analysis of cell differentiation markers expression. Cell differentiation was analyzed by quantification of cell surface makers. 1 x 10^6 cells were washed twice with PBS and then incubated during 1 h with fluorescein isothiocyanate (FITC)-conjugated antibodies against CD11b, CD14 and APC-conjugated antibody against CD15 for the myeloid differentiation. For the megakaryocytic markers, FITC-conjugated antibodies against CD41a (BD Biosciences) and CD61 (DAKO) were used. Isotype immunoglobulins (BD Biosciences) were used as negative controls. Finally, cells were washed twice in PBS and analyzed by flow cytometry. Erythroid differentiation was monitored by benzidine staining as previously described in reference 79.

K_i-67 nuclear antigen expression. U937 cells were fixed, permeabilized and immunostained with an antibody against K_i-67 (BD Biosciences) during 1 h at room temperature. The cells were washed twice in PBS and immunostained with a FITC-conjugated secondary antibody during 1 h at 4°C. After two washes with PBS, the cells were analyzed by flow cytometry and by fluorescence microscopy.

Senescence-associated β-galactosidase (SA-β-gal) histochemical analysis. The staining of SA-β-gal was performed at pH = 6.0 according to the manufacturer's protocol (Sigma Aldrich). Briefly, cells were washed twice with PBS 1x and then fixed in a solution containing 2% paraformaldehyde and 0.2% glutaraldehyde. Fixed cells were stained for 10 min at 37°C with an acidic solution (pH = 6.0) containing X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside). After overnight incubation, the cells were washed with PBS 1X and the staining pattern was analyzed by microscopy (Leica, DM 2000). As a positive control, K562 cells treated up to 4 d with 100 nM of doxorubicine (Sigma Aldrich) were used.[80]

RNA extraction and real-time PCR analysis. Total RNA from U937 and K562 cells treated with COX-2 inhibitors was extracted using the Nucleospin RNA II Kit (Macherey-Nagel), according to the manufacturer's protocol. To study mRNA stability, the cells were pre-treated with 100 μM nimesulide or 40 μM celecoxib for the indicated times; cells where then incubated with the transcription inhibitor actinomycin D (5 μg/ml) and total mRNA was extracted after 20, 40 or 60 min. cDNA was synthesized using the Superscript™ First-strand Synthesis System for RT-PCR (Invitrogen). One μg RNA was used for reverse transcription, using oligo(dT) primers. cDNA products were used for PCR amplification using the Platinum® High Fidelity *Taq* DNA Polymerase and gene-specific primers for COX-1 (sense: 5'-TCA TCC GAG AGA TGC TCA TGC G-3'; antisense: 5'-AGA CCA GGC ACC AGA CCA AAG ACC-3'), COX-2 (sense: 5'-GCC CAG CAC TTC ACG CAT CAG-3'; antisense: 5'-AGA CCA GGC ACC AGA CCA AAG ACC-3'), c-Myc (sense: 5'-TGC TCC ATG AGG AGA CAC C-3'; antisense: 5'-TCG ATT TCT TCC TCA TCT TCT TG-3'), cyclin D1 (sense: 5'-CGT GGC CTC TAA GAT GAA GG-3'; antisense: 5'-CCA CTT GAG CTT GTT CAC CA-3'), PCNA (sense: 5'-GGC GTG AAC CTC ACC AGT AT-3'; antisense: 5'-AGG TAT CCG CGT TAT CTT CG-3'), and p27 (sense: 5'-AAT AAG GAA GCG ACC TGC AA-3'; antisense: 5'-GGG GAA CCG TCT GAA ACA TT-3') and Rb1 (sense: 5'-TGC TAT GTC AAG ACT GTT GAA GAA-3'; antisense: 5'-AAC TGC TGG GTT GTG TCA AA-3'). β-actin was used as a control (sense: 5'-CTG GAA CGG TGA AGG TGA CA-3'; antisense: 5'-AAG GGA CTT CCT GTA ACA ATG CA-3'). All the primers were from Eurogentec. cDNA amplification was performed for 40 cycles with the following settings: 94°C for 2 min, 60°C for 1 min and 68°C for 2 min. Results were expressed as a ratio: mRNA of target gene/β-actin mRNA.

Total protein extraction. Total cell extracts were prepared using Mammalian Protein Extraction Reagent (M-PER, Pierce) according to the manufacturer's instructions. Briefly, 1 x 10^6 cells per sample were washed with PBS and the pellet was re-suspended in 500 μl of M-PER® supplemented with protease inhibitor cocktail (Complete, Roche) and phosphatase inhibitor cocktail (PhosphoStop®, Roche). The suspension was put on a shaker with vertical agitation for 15 min at 4°C and then centrifuged at 15,000 g for 15 min at 4°C. Supernatants were removed, aliquoted and stored at -80°C until use. For protein stability studies, cells were treated with 10 μg/ml cycloheximide for 5, 10, 20, 40 or 60 min.

Immunoblot analysis. Total protein extract (20 μg, unless otherwise specified) was separated by size using sodium dodecyl sulfate PAGE (SDS-PAGE; 10%). Proteins were then transferred to nitrocellulose membranes. β-actin was used as a loading control. Subsequent immunoblotting was performed by blocking non-specific binding regions of the membranes with 5% non-fat milk in PBS-Tween (1 h). Membranes were then incubated with 0.5–1 μg/ml of the following primary antibodies: 1 h for anti-β-actin (Sigma) or overnight for anti-COX-1, anti-COX-2, anti-p27, anti-cyclin D1, anti-PCNA (Santa Cruz Biotechnology), anti-cyclin D1 and anti-c-Myc (Cell Signaling), anti-phospho ERK1/2, anti-ERK1/2, anti-phospho-$p38^{MAPK}$, anti-$p38^{MAPK}$ (BD Biosciences). The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). All antibodies were diluted in a PBS-Tween solution containing 5% of bovine serum albumin (BSA) or 5% of milk. The protein bands were revealed using the ECL Plus western Blotting Detection System Kit (GE Healthcare). Densitometric quantification of the bands was performed using ImageJ 1.43 u software (www3.imperial.ac.uk/portal/page/portallive/imagingfacility/links/imaging_software).

Statistical analysis. Statistical analyses were performed using the Student's t-test for unpaired data. p values ≤0.05 were considered significant. To confirm the dose-dependent effect of some treatments, ANOVA parametric analysis was used.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at:
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