Nuclear factor κB as a target for new drug development in myeloid malignancies

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ABSTRACT

The transcription nuclear factor κ B (NF-κB) can intervene in oncogenesis through to its capacity to regulate the expression of a large number of genes that regulate apoptosis, cell proliferation and differentiation as well as inflammation, angiogenesis and tumor migration. Impaired NF-κB activity has been demonstrated not only in solid cancers but also in various types of hematologic malignancies including acute myeloid leukemia, chronic myelogenous leukemia and in a subset of myelodysplastic syndromes. The underlying mechanisms, illustrated in the text and although quite diverse in different diseases, provide the rationale for new therapeutic strategies combining different NF-κB or proteasome inhibitors. It has, therefore, been proposed that inhibition of NF-κB could be an adjuvant therapy for cancer and many phase I/II clinical studies are ongoing with different inhibitors. This review highlights the *in vitro* and *in vivo* results of NF-κB inhibition in myeloid malignancies.

Key words: NF-κB, proteasome inhibitors, acute myeloid leukemia, chronic myelogenous leukemia.

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The transcription nuclear factor κ B (NF-κB) can intervene in oncogenesis through to its capacity to regulate the expression of a large number of genes that regulate apoptosis, cell proliferation and differentiation as well as inflammation, angiogenesis and tumor migration.[1]

NF-κB proteins are a small group of related and evolutionarily conserved proteins which in mammals consists of five members: Rel (c-Rel), RelA/p65, RelB, p50, and p52.[2,3] In resting cells, NF-κB proteins are predominantly cytoplasmic, associating with members of the inhibitory IκB family such as IκBα, IκBβ and Iκβε.[2] These interact with NF-κB through multiple ankyrin repeats and also inhibit its DNA binding activity. IκB proteins were originally thought to sequester NF-κB in the cytoplasm by masking its nuclear localization sequences (NLS). However, IκBα can only mask one NLS in the dimer; so NF-κB complexes undergo constitutive nuclear translocation.[4] Importantly, a nuclear export signal (NES) in IκBα precludes high steady state levels of these complexes in the nucleus.[5]

Two NF-κB activation pathways exist; the first is normally triggered in response to infections or exposure to pro-inflammatory cytokines that activate the IKK complex leading to phosphorylation-induced IκB degradation, the other pathway leads to selective activation of p52: RelB dimers. This pathway is triggered by certain members of the tumor necrosis factor (TNF) cytokine family through selective activation of IKKα by the upstream kinase NIK.[6]

The IKK complex consists of two kinase subunits, IKKα and IKK, and a non-enzymatic regulatory component, IKKλ/NEMO. In response to many stimuli such as inflammatory cytokines, bacterial lipopolysaccharide, phorbol esters, viral infection or stress, IκB are phosphorylated on two critical serine residues.[6] This modification triggers IκB ubiquitination and destruction via the 26S proteasome degradation machinery. As a consequence, free NF-κB enters the nucleus

and regulates transcription of over 150 genes encoding cell adhesion molecules, cytokines, growth factors, components of the immune systems and anti-apoptotic genes such as FLIP, cIAPs, Bcl-2 and Bcl-XL.[1] It is also implicated in the regulation of cell proliferation by controlling D-type cyclins.[7] Since NF-κB regulates the expression of a variety of proteins that inhibit apoptosis and promote cell survival and proliferation, it is strongly implicated in carcinogenesis.[8,9]

Finally, proteasomes are key regulators of cells, being responsible for the degradation of many intracellular proteins, thereby helping to maintain cellular homeostasis during biological processes, such as cell cycling, signal transduction, response to stress and gene transcription. Deregulation of proteasomal function may induce oncogenesis because the proteasome is responsible for the consequent constitutive activation of NF-κB.[10]

NF-κB and cancer

The NF-κB family of transcription factors can potently suppress apoptosis and is, thereby, considered to be a key survival factor for several types of cancer.[9,11] The association of NF-κB activation with tumor promotion, progression and metastasis is well documented and has been demonstrated in several mouse models.[12-13] The IKK-dependent NF-κB activation pathway is a critical molecular event for the generation and progression of colon cancer.[14] IKK, activation in enterocytes suppresses apoptosis of pre-neoplastic cells. Conversely, inactivation of IKK, results in a dramatic decrease in tumor cell number due to increased apoptosis, but has no effects on proliferation.[14]

The oncogenic role of NF-κB was also demonstrated in Mdr2-deficient mice, which develop cholestatic hepatitis followed by hepatocellular carcinoma.[13] NF-κB activation also plays a critical role in inflammation-driven tumor progression as demonstrated in a syngeneic colon and mammary cancer xenograft mouse model.[12]

The role of NF-κB has been demonstrated not only in solid cancers, but also in various types of hematologic malignancies.[9] The underlying mechanisms could be quite diverse, spanning from amplifications or rearrangements of genes coding for some NF-κB family members detected in various leukemias and lymphomas, to mutations or deletions that invalidate the inhibitory function of IκB in Hodgkin's lymphoma and to oncogenic activation of the IKK complex mainly found in leukemias and lymphomas or paracrine or autocrine secretion of NF-κB activators in Hodgkin's lymphoma.[15] Finally, acute myeloid and lymphoblastic leukemias and chronic myeloid leukemia, express abnormally high levels of both mRNA and protein of the proteasome compared with normal cells.[16] Based on the knowledge available at present, it is difficult to reach a definitive conclusion regarding the possibility that NF-κB could represent a primary or a secondary event in the leukemic process. Much evidence supports the theory that, in the majority of cases, this increased activation could be dependent on the presence of abnormal upstream pathways regulated by the phosphorylation of oncogenic tyrosine kinases such as Bcr-Abl or from the activation of Ras.[17] In multiple myeloma, NF-ÎB promotes cell survival mainly through the expression of genes coding for anti-apoptotic proteins, named cellular inhibitor of apoptosis protein (c-IAP),18 Bcl-XL, and bfl-1/A1.[9] Finally, NF-κB could be activated in an autocrine manner, because several NF-κB-inducing cytokines including tumor necrosis factor (TNF)α, interleukin (IL)-1 and RANKL are produced by multiple myeloma cells.[19]

NF-κB is also known to be able to stimulate cell proliferation via induction of growth factors such as IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF) or cell cycle regulators (cyclin D1, c-myc).[7] Moreover, NF-κB could participate in the chemoresistance of tumor cells mediated by the expression of the multidrug resistance protein. NF-κB could promote metastasis through induction of the extracellular matrix-degrading enzymes matrix metalloproteinase 9 and urokinase-type plasminogen activator.[20,21] In addition, many commonly used antineoplastic drugs also activate NF-κB, an event that interferes with treatment.

NF-κB and acute leukemia

Acute myeloid leukemia (AML) is typically a disease of stem/progenitor cell origin. Although conventional chemotherapy regimens often ablate actively cycling leukemic blast cells, the primitive leukemic stem cell population is likely to be drug-resistant. Moreover, currently available drugs may not effectively distinguish between malignant stem cells and normal hematopoietic stem cells. Leukemic stem cells, which are quiescent or slowly cycling and therefore less sensitive to chemotherapy, are responsible for disease relapse and represent the target for future innovative therapies.[22-24] A future goal could be to exploit the unique properties of leukemic cells to induce apoptosis in the leukemic stem cells population while sparing normal stem cells. One interesting difference is constitutive activation of NF-κB. Recently, NF-κB has been found to be activated in $CD34^{+}/CD38^{-}$ blast cells derived from patients with *de novo* AML.[25,26] In line with this study, Frelin *et al.*[27] reported an increase of NF-κB activity which strongly correlated with blast cell count. This correlation was not present in the study by Bueso-Ramos *et al.*,[28] although activation of NF-κB in leukemia patients has been well documented.

Although further studies based on a highly sensitive method for the detection of NF-κB activity are required to better characterize the subset of patients showing impaired activity of this protein, the reported data provide evidence that NF-κB activation is not uniform among AML patients. In accordance with published

data, in our cohort of 80 AML patients evaluated at diagnosis by EMSA assay, about 40 percent presented increased NF-κB DNA binding activity (*unpublished data*). In our study, these patients are characterized by increased white cell counts at diagnosis and increased blast percentages in the bone marrow compared to those patients presenting with undetectable levels of NF-κB activity, suggesting a link between NF-κB and cell proliferation. In particular, cyclin D1, whose expression is regulated by NF-κB and overexpressed in several human cancers could be a candidate to mediate the NF-κB effect on blast proliferation. Alternatively, NF-κB action could be due to the induction of genes coding for AML growth factors such as GM-CSF or granulocyte colony-stimulating factor (G-CSF). Finally, it has already been shown that human leukemic cells express abnormally high levels of proteasomes compared with normal cells. Both mRNA and protein proteasomes were higher in leukemic cell lines and in leukemic cells from patients affected by acute myeloid and lymphoblastic leukemias and chronic myeloid and lymphocytic leukemias.[16]

Recently published studies have reported the activation of NF-κB in a percentage of high risk myelodysplastic patients.[29] Interestingly, although the activation of NF-κB is not generally observed in patients with myelodysplastic syndromes, when it is, it is restricted to the population of myelodysplastic cells carrying cytogenetic alterations, demonstrating the specificity of this abnormality for the clonal population. Finally, the same study demonstrated a correlation between the degree of DNA binding activity and the risk of progression.

At present, NF-κB has been observed to be activated in AML blasts rather than in their normal counterparts.[25]Limited information is available on the role of NF-κB in regulating normal hematopoiesis. Its role in primary hematopoietic cells has been investigated mainly by using chemical inhibitors and overexpression of dominant negative constructs.[30,31]

Two studies have explored the consequences of aberrant activity of NF-κB in normal hematopoiesis. Schepers *et al*.[32] investigated the effects in cord blood-derived CD34$^+$ cells. The authors concluded that constitutive NF-κB activity as a single hit is not sufficient to induce changes in steady state hematopoiesis with regard to proliferation, differentiation or self-renewal. Similar conclusions were derived from a study by Romano *et al*.[33] showing that NF-κB is not relevant for basal cell survival of CD34$^+$ cells, but only for cells triggered with a stress response such as exposure to chemotherapy.

Although the molecular mechanism of NF-κB activation in leukemic stem cells or AML blasts remains elusive at present, NF-κB and its unique role in the apoptotic and proliferation pathways and in drug resistance could represent an attractive candidate to be targeted by selective drugs. It has, therefore, been proposed that inhibition of NF-κB could be an adjuvant therapy for cancer.

As shown in many studies,[25,27] the in vitro treatment of blast cells with NF-κB or proteasome inhibitors leads to apoptosis of blast cells. Interestingly, these effects are more selective on leukemic cells, producing minor effects on normal stem cell populations. The proteasome inhibitor that blocks NF-κB but also other signaling pathways has shown selective toxicity for leukemic stem cells rather than for hematopoiesis stem cells *in vitro*. These studies provide the rational for Phase I/II clinical trials for the treatment of refractory or relapsed AML patients with proteasome inhibitors.[34]

Chronic myelogenous leukemia

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by the t (9;22) translocation resulting in expression of a fusion oncoprotein, Bcr-Abl, which exhibits constitutive tyrosine kinase activity.[35-37] Constitutive activation of Bcr-Abl kinase signals to a variety of downstream survival pathways, including the mitogen-activated protein kinase/extracellular signal-regulating kinase cascade, Akt, signal transducers and activators of transcription (STAT), cell adhesion molecules such as E-selectin, intercellular adhesion molecule-1 and NF-κB, among others.[38]

The activation of NF-κB by this oncoprotein mediates proliferation, transformation, and resistance to apoptosis in Bcr-Abl-positive cells.[39,17] Importantly, studies in nude mice and primary bone marrow transformation assays reveal a requirement for NF-κB in tumorigenesis and transformation by Bcr-Abl.[39,17] In addition, studies using IκBα-SR-infected cells demonstrate that the activation of NF-κB is necessary for Bcr-Abl-induced transformation.

NF-κB has been identified as a downstream component of the Bcr-Abl-initiated signaling pathway, although the pathway employed by Bcr-Abl to activate NF-κB remains largely undetrmined and is currently under investigation. It has been demonstrated that Bcr-Abl activates NF-κB-dependent gene expression, at least partially, in a Ras-dependent manner. Bcr-Abl activates PI3K and INK, both of which can function downstream of Ras and may be capable of stimulating NF-κB transcriptional activity by inducing the transactivation function of p65.[40] Bcr-Abl activation of NF-κB may, therefore, be caused by an increase in nuclear translocation as well as an increase in transactivation potential. This, it is assumed that Bcr-Abl may signal via multiple pathways to activate NF-κB, which is required for Bcr-Abl-mediated transformation.[39] Therefore, it is likely that the products of NF-κB responsive genes play a role in Bcr-Abl-mediated leukemogenesis. Once activated, NF-κB regulates the transcription of many key genes such as *c-myc*, necessary for Bcr-Abl transformation as well as many surface molecules necessary for mediating cell adhesion and cell-cell interactions.

Different studies demonstrated an intrinsic activity of NF-κB in Bcr-Abl-positive cells,[17,40-42] with contrasting data, while the same studies, showed a detectable level of NF-κB activity in chronic and blast phases of disease[17,42] with one showing binding activity only in advanced stages.[28]

In spite of these apparent discrepancies, most likely due to the sensitivity of the method used, there is common agreement on progressively increasing activity during disease progression. Based on these findings, different types of NF-κB inhibitors have been tested in vitro. Among them, proteasome inhibitors have been widely used, although it might be considered that their distinct effects on cellular properties are not fully to be ascribed to the modulation of NF-κB, but also to a number of proteins that could, potentially, be affected.

PS341 (bortezomib), a specific proteasome inhibitor, has been evaluated in cell lines sensitive and resistant to imatinib treatment.[41] Proteasome inhibition induces proliferation arrest and apoptosis also in imatinib-resistant cells providing a rationale for the use of this drug in the subset of patients resistant to imatinib.

Dai *et al*.[43] explored the antiproliferative effect of the combination of bortezomib and flavopiridol, a cyclin-dependent kinase inhibitor, in CML cell lines. They found that the two drugs interact synergistically to induce apoptosis in CML cells resistant to imatinib through both Bcr-Abl-dependent and -independent mechanisms. The combination treatment resulted in a significant rate of apoptosis associated with a marked reduction of NF-κB DNA binding activity. Interestingly, in imatinib-resistant K562 cells displaying increased bcr-abl expression, bortezomib/flavopiridol treatment markedly increased apoptosis in association with down-regulation of Bcr-Abl and BclxL, and diminished phosphorylation of Lyn, Hck, CrkL, and Akt. Taken together, these findings suggest that a strategy combining bortezomib and other drugs including flavopiridol warrants further examination in CML.

Recently, we reported the in vitro effects induced by the IKK inhibitor PS1145 (Millennium, Cambridge, USA) in CML cells both sensitive and resistant to imatinib.[42] In our study, we demonstrated that PS1145 is able to induce growth arrest and apoptosis in cell lines and in bone marrow cells from CML patients. This effect was more profound in imatinib-resistant cells treated with the association of imatinib and PS1145 as a compared to the effects obtained by incubation with PS1145 as single agent or to the effects obtained in sensitive cells. This is probably due to the fact that PS1145 may act in this setting with different mechanisms of action, and not only by blocking phosphorylation of the 32/36 serine residues of IκBα in the cytoplasm. These synergistic effects of the two drugs in resistant cells can be, at least partially, explained by the PS1145-dependent increased amount of nuclear IκB that plays an important apoptotic role within the nucleus. Nuclear IκB is generally phosphorylated by Abl and once phosphorylated it promotes the nuclear export of NF-κB, therefore blocking its activity. The fact that PS1145 alone, without the activity of imatinib, is unable to induce the same apoptotic effect prompted us to explore the role of imatinib in this particular context. Using western blot assays, we clearly demonstrated that after the incubation with both compounds the amount of the nuclear Bcr-Abl increases and becomes detectable. It is well known that nuclear Bcr-Abl acts as a potent apoptosis inducer, probably through blocking nuclear IκBα. It remains to be elucidated whether imatinib plays a role in nuclear import of Bcr-Abl-resistant cells by itself or through synergistic effects with PS1145. Finally, it remains to be explored whether this action of PS1145 is unique or could be shared by other NF-κB or proteasome inhibitors. The combination of imatinib plus the IKK inhibitors could, therefore, represent a valid approach to be tested in vivo for the treatment of CML patients resistant to imatinib therapy.

Clinical studies with bortezomib in CML

Based on striking phase II data[44,45] PS-341 (Bortezomib, Velcade) has been approved for the treatment of multiple myeloma by the U.S. Food and Drug Administration. A number of phase I trials have been done with varying schedules of PS-341. PS-341 is generally well tolerated, although non-hematologic toxicities such as fatigue, diarrhea, nausea, vomiting, and sensory neuropathy were observed in all studies. Thrombocytopenia was dose-limiting in the phase I trials that employed the twice-weekly schedule for 4 of 6 weeks,[34,46] These studies, conducted among patients with hematologic malignancies, established a lower maximum tolerated dose compared that determited in studies conducted in a mixed population comprised mainly of patients with solid tumors. PS-341 given at the dose of 1.5 mg/m^2 twice weekly for 2 of every 3 weeks is well tolerated and should be tested in CML patients in combination with imatinib. Recently, a phase II study on the treatment of CML patients resistant to imatinib with a combination of imatinib plus bortezomib was approved and is ongoing in many Italian institutions. Patients will receive a twice-weekly intravenous bolus dose of bortezomib 1.3 mg/m^2 for the first 2 weeks of each 3 week cycle (days 1, 4, 8, and 11). Treatment with bortezomib may be repeated every 3 weeks for up to 12 courses. During the interval between bortezomib infusions, patients will receive imatinib at the dose each patient was receiving at enrollment for the study duration.

Bortezomib in combination with conventional or investigational chemotherapy

Histone deacetylase inhibitors (HDI) constitute a diverse group of compounds that promote histone acetylation, chromatin uncoiling, and transcription of a variety of genes involved in multiple cellular processes,

including differentiation.[47] Their role in the treatment of CML remains to be defined. Previous studies have indicated that exposure of tumor cells to HDI such as phenylbutyrate leads to inactivation of NF-B.[48] Such findings raise the possibility that co-administration of HDI with proteasome inhibitors, which interrupt the same pathway,[41] might be associated with enhanced antitumor activity. Currently, no information is available concerning interactions between clinically relevant HDI and proteasome inhibitors in leukemia cells in general, and Bcr-Abl-positive leukemic cells in particular. To address this issue, Yu *et al.*[49] examined the effects of treatment of Bcr-Abl-positive cells (K562 and LAMA 84), including those resistant to imatinib, with HDI in combination with the proteasome inhibitor bortezomib. They reported that these agents interact in a highly synergistic manner to induce mitochondrial injury, caspase activation, and apoptosis in Bcr-Abl-positive cells, and that these events are associated with multiple perturbations in signaling and survival pathways, including inhibition of p21CIP1 induction, potentiation of Jun kinase (JNK) phosphorylation, and interference with NF-κB DNA binding. Moreover, the HDI/bortezomib regimen potently induces apoptosis in continuously cultured and primary Bcr-Abl-positive cells that are resistant to imatinib, as well as in Bcr-Abl leukemic cells. Collectively, these findings suggest that an approach combining clinically relevant HDI with proteasome inhibitors warrants further investigation as a therapeutic strategy for both Bcr-Abl- negative leukemias as well as those that are Bcr-Abl-positive and otherwise resistant to standard cytotoxic agents.

Authors' Contributions

DC and GM drafted the article, FM provided the in vitro data, GS and MB were responsible for the clinical trials and for the design of the article.

Conflict of Interest

The authors reported no potential conflicts of interest.

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