Flavopiridol Inhibits NF-κB Activation Induced by Various Carcinogens and Inflammatory Agents through Inhibition of IκBα Kinase and p65 Phosphorylation

ABROGATION OF CYCLIN D1, CYCLOOXYGENASE-2, AND MATRIX METALLOPROTEASE-9*

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Flavopiridol, a synthetic flavone closely related to a compound originally isolated from the stem bark of the native Indian plant *Dysoxylum binectariferum*, has been found to inhibit cyclin-dependent kinases, induce apoptosis, suppress inflammation, and modulate the immune response. Because several genes in which expression is altered by flavopiridol are regulated by NF-κB, we propose that this flavone must affect the activation of NF-κB. For this report, we investigated the effect of flavopiridol on NF-κB activation by various carcinogens and inflammatory agents. Flavopiridol suppressed tumor necrosis factor (TNF)-activation of NF-κB in a dose- and time-dependent manner in several cell types, with optimum inhibition occurring upon treatment of cells with 100 nM flavopiridol for 6 h. This effect was mediated through inhibition of IκBα kinase, phosphorylation, ubiquitination, and degradation of IκBα (an inhibitor of NF-κB), and suppression of phosphorylation, acetylation, and nuclear translocation of the p65 subunit of NF-κB activation. Besides TNF, flavopiridol also suppressed NF-κB activated by a carcinogen (cigarette smoke condensate), tumor promoters (phorbol myristate acetate and okadaic acid), and an inflammatory agent (H2O2). TNF-induced NF-κB-dependent reporter gene transcription was also suppressed by this flavone. NF-κB reporter activity induced by TNF receptor 1, TNF receptor-associated death domain, TNF receptor-associated factor-2, NF-κB-inducing kinase, and IκBα kinase, were all blocked by flavopiridol but not that activated by p65. Furthermore, flavopiridol suppressed TNF-induced activation of Akt. Flavopiridol also inhibited the expression of the TNF-induced NF-κB-regulated gene products cyclin D1, cyclooxygenase-2, and matrix metalloproteinase-9. Overall, our results indicated that flavopiridol inhibits activation of NF-κB and NF-κB-regulated gene expression, which may explain the ability of flavopiridol to suppress inflammation, modulate the immune response, and regulate cell growth.

Flavopiridol is a semisynthetic flavonoid closely related to a compound originally isolated from the stem bark of *Dysoxylum binectariferum* (also called rohitukine), a plant indigenous to India. The parent compound is identical to flavopiridol except that a methyl group replaces the chlorophenyl moiety at position 2 (1). Flavopiridol has been shown to be a potent inhibitor of cyclin-dependent kinase (CDK)1, CDK 2, CDK 4, and CDK 7 (2). It inhibits CDKs by competing with ATP at the nucleotide-binding site on CDKs as indicated by kinetics studies (3) and x-ray crystallography of the CDK 2-flavopiridol complex (4). The tyrosine phosphorylation of CDK 2 is also inhibited by this flavone (5). Through inhibition of CDKs, flavopiridol induces arrest of cell growth at the G1 and G2 phases of the cell cycle (2, 6). Because of its ability to suppress the growth of breast carcinoma (2), lung carcinoma (7), chronic B cell leukemia and lymphoma (8–10), multiple myeloma (11), and head and neck squamous cell carcinoma (12), flavopiridol is currently in clinical trials for the treatment of different cancers (13–15). Flavopiridol has also been shown to enhance the activity of other growth-suppressing agents, such as tumor necrosis factor (TNF), doxorubicin, and etoposide (16–21).

Research in the last few years has indicated that besides inhibiting CDK activity, the expression of anti-apoptotic proteins, such as Bcl-2 (8), Mcl-1 (11, 22, 23), cyclin D1 (19, 24), and vascular endothelial growth factor (25, 26) has been shown to be suppressed by flavopiridol. All of the genes for these proteins are known to be regulated by the nuclear transcription factor NF-κB (27–33). Flavopiridol has also been shown to suppress inflammation and regulate the immune system (9), and again, NF-κB has been shown to control the expression of immunosuppressive and anti-inflammatory molecules (34).

These lines of evidence indicated that flavopiridol may regulate the activity of NF-κB. Thus in the present report we investigated the effect of flavopiridol on NF-κB activation induced by various carcinogens, inflammatory agents, and immune modulators. We also investigated the mechanism by which flavopiridol suppresses NF-κB activation, and we found this inhibition was mediated through the suppression of Akt-induced IκBα kinase activation. Flavopiridol also suppressed...
the induced NF-κB-regulated gene products such as COX-2, cyclin D1, and MMP-9.

MATERIALS AND METHODS

Reagents—Flavopiridol was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI, National Institutes of Health (Bethesda, MD). A 1× solution of flavopiridol was prepared in dimethyl sulfoxide (MeSO) and then diluted in the cell culture medium. Bacteria-derived human recombinant TNF, purified to homogeneity with a specific activity of 5 × 10⁷ units/mg, was kindly provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640 medium, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were obtained from Invitrogen. Cigarette smoke condensate was kindly provided by Dr. C. Gary Gariola (Toxicology Research Institute of the University of Kentucky, Lexington). The antibodies anti-p65, anti-p50, anti-1×Ba, anti-cyclin D1, anti-poly-(ADP)ribose polymerase (PARP), and anti-Akt antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific anti-IκB-α and anti-IκB-β antibodies were kindly provided by Imgenex (San Diego, CA). The antibody that recognizes the serine 529-phosphorylated form of p65 was obtained from Rockland Laboratories (Gilbertsville, PA).

Cell Lines—We used Jurkat (human T-cell line), A293 (human kidney cell line), and HL60 (human myeloid leukemia) cells, and all cells were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Electrophoretic Mobility Shift Assays (EMSA)—To measure NF-κB activation, we performed EMSA as described previously (35). Briefly, nuclear extracts prepared from TNF-treated cells (2 × 10⁶/ml) were incubated with 5 ng end-labeled double-stranded NF-κB oligonucleotide (10 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5′-TTCTTACAAACCTCGCTGG-GACTTTCACGGAGGCGTGG-3′ (boldface indicates NF-κB binding sites), for 30 min at 37 °C before the complex was analyzed by EMSA. Antibodies against cyclin D1 and preimmune serum were included as negative controls. The dried gels were visualized, analyzed by EMSA. Antibodies against cyclin D1 and preimmune serum were included as negative controls. After overnight incubation at 4 °C the slides were washed, incubated with goat anti-rabbit IgG-Alexa 594 (Molecular Probe, Eugene, OR) at 1:100 dilutions for 1 h, and counter-stained for nuclei with Hoechst 33342 (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium purchased from Sigma and analyzed under a fluorescence microscope (Laborphot-2, Nikon, Tokyo, Japan). Pictures were captured using Photometrics Coolsnap CF color camera (Nikon, Tokyo, Japan) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA).

Western Blot Analysis—To determine the levels of protein expression in cytoplasm or nuclear extracts, we prepared each extract (36) from TNF-treated cells and fractionated it by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by ECL reagent (Amersham Biosciences). The density of the bands was measured using NIH Image.

1×Ba Kinase Assay—The IKK assay was performed by a method described previously (37). Briefly, the IKK complex from whole-cell extracts was precipitated with antibody against IKK-α, followed by treatment with protein A/G-Sepharose beads (Pierce). After a 2 h incubation, the beads were washed with lysis buffer and then assayed in kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM MgCl₂, 2 mM dithiothreitol, 20 μCi of [γ-32P]ATP, 10 μM unlabelled ATP, and 2 μg of substrate glutathione S-transferase-1×Ba (1–54). After the immunocomplex was incubated at 30 °C for 30 min, it was boiled with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized by PhosphorImager. To determine the total amounts of IKK-α and -β in each sample, the IKK immunoprecipitate was resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK-α or anti-IKK-β antibodies.

Nuclear Localization of p65 NF-κB by Immunocytochemistry—The nuclear translocation of the p65 subunit of NF-κB was examined by an immunocytochemical method as described previously (38). Briefly, treated cells were plated on a poly-L-lysine-coated glass slide by centrifugation using a cytopsin 4 (Thermoshend, Pittsburg, PA), air dried, fixed with cold acetone, and permeabilized with 0.2% Triton X-100. After being washed in phosphate-buffered saline, the slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human p65 or 1×Ba antibodies at 1:100 dilutions. After overnight incubation at 4 °C the slides were washed, incubated with goat anti-rabbit IgG-Alexa 594 (Molecular Probe, Eugene, OR) at 1:100 dilutions for 1 h, and counter-stained for nuclei with Hoechst 33342 (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium purchased from Sigma and analyzed under a fluorescence microscope (Laborphot-2, Nikon, Tokyo, Japan). Pictures were captured using Photometrics Coolsnap CF color camera (Nikon, Tokyo, Japan) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA).

Secretory Alkaline Phosphatase (SEAP) Assay—The effect of flavopiridol on TNF, TNFR 1-3, TRADD, TRAF 2, NIK, IKK-α, IKK-β, and p65-induced NF-κB-dependent reporter gene transcription was analyzed by SEAP assay as described previously (37). To examine TNF-induced reporter gene expression, A293 cells (0.5 million cells/well) were plated in 6-well plates and transiently transfected by the calcium phosphate method with pNF-κB-SEAP (0.5 μg) and the control plasmid pcMV-FLAG1 DNA (2 μg). After 24 h, cells were washed and then treated with 100 nM flavopiridol for 8 h. Cells were then exposed to 1 μM TNF for 24 h, harvested from the cell culture medium, and analyzed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech, Palo Alto, CA), using a 96-well fluorescence plate reader (Fluoroscan II, Labsystems, Chicago, IL) with excitation set at 360 nm and emission at 460 nm.

To examine TNFR 1, TRADD, TRAF 2, NIK, IKK-α, IKK-β, and p65-induced NF-κB-dependent reporter gene transcription, cells (0.5 million cells/well) were plated in six-well plates and transiently transfected by the calcium phosphate method with pNF-κB-SEAP (0.5 μg), the inducer plasmid (1 μg), and the control plasmid pcMVFLAG1 (1 μg) DNA. After 24 h, cells were washed and then treated with 100 nM flavopiridol. After 32 h, the cell culture medium was harvested and analyzed for SEAP activity as described above.

RESULTS

In the present study, we investigated the effect of flavopiridol on NF-κB activation induced by various carcinogens and inflammatory stimuli including PMA, okadaic acid, H₂O₂, cigarette smoke condensate, and TNF. The effect of flavopiridol on the TNF-induced NF-κB activation was studied in detail, because the pathway has been well characterized. The structure of flavopiridol, (−)-cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)piperidinyl]-4H-1-benzoypyranyl-4-one, is shown in Fig. 1. The concentration of flavone, the NF-κB activator, used and the time of exposure had minimal effect on the viability of these cells (data not shown).

Flavopiridol Inhibits TNF-dependent NF-κB Activation—To determine the effect of flavopiridol on NF-κB activation, Jur-
kat, A293, and HL60 cells were incubated for 8 h with different concentrations of flavopiridol followed by 0.1 nM TNF for 30 min. Then nuclear extracts were prepared and analyzed for NF-κB activation by EMSA. As shown in Fig. 2, flavopiridol by itself did not induce NF-κB activation but rather inhibited TNF-mediated NF-κB activation in a dose-dependent manner in all cell lines. Treatment with 150 nM flavopiridol suppressed TNF-induced NF-κB activation by almost 100% in all cell lines. During all subsequent experiments, we used HL60 cells and employed 150 nM flavopiridol.

We next tested the length of incubation required for flavopiridol to suppress TNF-induced NF-κB activation. Cells were incubated with the flavone for different times before treatment with TNF for 30 min. TNF-induced NF-κB activation was inhibited by flavopiridol in a time-dependent manner (Fig. 3A). When we incubated cells with flavopiridol for 6 h, NF-κB activation by TNF was completely inhibited.

Previous studies from our laboratory have shown that 10 nM TNF can activate NF-κB within 5 min and that this induction is higher in its intensity than that obtained with cells using 100-fold lower concentrations of TNF for longer times (39). To determine the effect of flavopiridol on NF-κB activation at high concentrations of TNF, cells were pretreated with flavopiridol, exposed to various concentrations of TNF for 30 min, and analyzed for NF-κB activation by EMSA (Fig. 3B). NF-κB activated by 10 nM TNF was completely suppressed by flavopiridol; the latter alone did not activate NF-κB. These results showed that flavopiridol is a very potent inhibitor of TNF-induced NF-κB activation.

Because NF-κB is a complex of proteins, various combinations of Rel/NF-κB protein can constitute an active NF-κB heterodimer that binds to a specific sequence in DNA (40). To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated nuclear extracts from TNF-stimulated cells with antibodies to either the p50 (NF-κB1) or the p65 (RelA) subunit of NF-κB. Both shifted the band to a higher molecular mass (Fig. 3C) thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor antibody against anti-cyclin D1 had any effect. Excess unlabeled NF-κB (100-fold) caused complete disappearance of the band, and a mutant oligonucleotide of NF-κB did not affect NF-κB binding activity.

To determine whether flavopiridol directly modifies the NF-κB complex, we incubated nuclear extracts from TNF-treated cells with various concentrations of flavopiridol and then analyzed for DNA binding activity by EMSA. Our results, in Fig. 3D, show that flavopiridol did not modify the DNA binding ability of NF-κB complex. Therefore, flavopiridol inhib-
its NF-κB activation not by preventing DNA binding directly but by an indirect mechanism.

Flavopiridol Inhibits TNF-dependent IκBα Phosphorylation—The activation of NF-κB is known to require the phosphorylation and degradation of IκBα, the natural inhibitor of NF-κB (40). To determine whether inhibition of TNF-induced NF-κB activation was because of inhibition of IκBα degradation, we pretreated cells with flavopiridol for 8 h and then exposed them to 0.1 nM TNF for the indicated times. Then we examined the cells for NF-κB activation in the nucleus by EMSA and for IκBα status in the cytoplasm by Western blot analysis. TNF-induced activation of NF-κB increased with time but did not occur at all in flavopiridol-pretreated cells even after up to 60 min of TNF stimulation (Fig. 4A). TNF induced IκBα degradation in control cells as early as 10 min, but in flavopiridol-pretreated cells, TNF failed to induce degradation of IκBα (Fig. 4B). These results indicate that flavopiridol inhibits both TNF-induced NF-κB activation and IκBα degradation.

To determine whether flavopiridol affects TNF-induced IκBα phosphorylation, we examined the TNF-induced phosphorylated form of IκBα by Western blot analysis using an antibody that is specific for the serine-phosphorylated form of IκBα. Because TNF-induced phosphorylation of IκBα leads to its
rapid degradation (40), we blocked phosphorylation and degradation of IκBα using the proteasome inhibitor N-Ac-Leu-Leu-Leu-norleucinal (ALLN) (41). TNF alone slightly induced phosphorylation of IκBα, but when cells were pretreated with the inhibitor, TNF-induced phosphorylation of IκBα was more pronounced (Fig. 4C). Flavopiridol almost completely suppressed the IκBα phosphorylation induced by TNF, even in the presence of the proteasome inhibitor.

Phosphorylation of IκBα by TNF leads to ubiquitination and degradation of IκBα (40). To investigate whether flavopiridol affects TNF-induced IκBα ubiquitination, we performed Western blot analysis. We used proteasome inhibitor ALLN to block the degradation of IκBα (41). TNF treatment alone slightly induced ubiquitination of IκBα, but when cells were pretreated with ALLN, TNF-induced ubiquitination of IκBα was enhanced (Fig. 4D). Flavopiridol almost completely suppressed the IκBα ubiquitination induced by TNF, even in the presence of the proteasome inhibitor.

Flavopiridol Inhibits TNF-induced IKK Activation—It has been shown that IKK is required for TNF-induced phosphorylation of IκBα (40). Because flavopiridol inhibits the phosphorylation of IκBα, we determined the effect of flavopiridol on TNF-induced IKK activation. As shown in Fig. 5A, in immune complex kinase assays TNF activated IKK as early as 2 min after TNF treatment. Flavopiridol treatment completely suppressed the TNF-induced activation of IKK. Neither TNF nor flavopiridol had any effect on the expression of IKK-α or -β proteins.

To evaluate whether flavopiridol directly interacts with IKK proteins, we incubated cytoplasmic extracts from TNF-treated cells with various concentrations of flavopiridol and then performed the kinase assay. The results in Fig. 5B show that flavopiridol did not directly affect the activity of IKK, suggesting that flavopiridol inhibits TNF-induced IKK activity through an indirect mechanism.

Flavopiridol Inhibits TNF-induced Akt Activation—Our results until now indicated that flavopiridol inhibits TNF-induced NF-κB activation through inhibition of IKK activation. However, we show that flavopiridol does not directly inhibit IKK. It is possible that this inhibition is because of inhibition of
an upstream kinase. Previous studies (42) have reported that Akt can associate with and activate IKK-α. Thus it is possible that flavopiridol suppresses TNF-induced Akt activation. To determine the effect of flavopiridol on the activation of Akt induced by TNF, we treated the cells with flavopiridol (150 nM) for 8 h and then exposed the cells to TNF (1 nM) for different times. Thereafter whole-cell extracts were prepared, and we performed Western blot analysis using phosphospecific anti-Akt and anti-Akt antibodies. Results showed that TNF induced Akt activation in a time-dependent manner (Fig. 5C), and pretreatment with flavopiridol completely suppressed the activation. Thus these results indicate that flavopiridol inhibits IKK activation through suppression of Akt.

Flavopiridol Inhibits TNF-induced Nuclear Translocation of p65—TNF induces the phosphorylation of p65, which is required for NF-κB transcriptional activity. The phosphorylation of the p65 subunit is known to be mediated through IKK (43). Western blot analysis showed that TNF induced the phosphorylation of the cytoplasmic pool of p65 in a time-dependent manner, and p65 phosphorylation could be seen as early as 5 min and increased up to 30 min (Fig. 6A). Pretreatment with flavopiridol abolished the TNF-induced phosphorylation of cytoplasmic p65. Flavopiridol blocked TNF-induced translocation of p65 to the nucleus almost completely (Fig. 6B).

The acetylation of p65 plays a key role in IκBα-mediated activation of NF-κB transcriptional activity (44). To examine the effect of flavopiridol on the acetylation of p65 by TNF, cells were pretreated with flavopiridol for 8 h and then exposed to TNF for the indicated times. Whole-cell extracts were prepared and immunoprecipitated with anti-p65 antibody, and Western blot analysis was performed using anti-acetyllysine antibody. Results showed that TNF induced acetylation of p65 in a time-dependent manner, and flavopiridol completely suppressed the TNF-induced acetylation of p65 (Fig. 6C).

As further confirmation of these results, immunocytochemistry showed that p65 localized in the cytoplasm of untreated cells. It was found to be translocated to the nucleus when treated with TNF, but pre-treatment with flavopiridol clearly suppressed this TNF-induced translocation (Fig. 6D). Flavopiridol Blocks NF-κB Activation Induced by PMA, Okadaic Acid, H2O2, and Cigarette Smoke Condensate—Previous studies have shown that PMA, okadaic acid, H2O2, and cigarette smoke condensate are potent activators of NF-κB (45–47). However, they activate NF-κB through a pathway with overlapping and non-overlapping steps. We therefore examined the effect of flavopiridol on the activation of NF-κB by these agents. As shown in Fig. 7, flavopiridol suppressed the activation of NF-κB induced by all of these agents, suggesting that flavopiridol acts at a step in the NF-κB activation pathway that is common to the activity of all of these agents.

Flavopiridol Represses TNF-induced NF-κB-dependent Reporter Gene Expression—Although we have shown that flavopiridol blocks the DNA binding step in NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps (48). To determine the effect of flavopiridol on TNF-induced NF-κB-dependent reporter gene expression, we transiently transfected the cells with the NF-κB-regulated SEAP reporter construct, incubated them with TNF, and then stimulated the cells with TNF. An almost 4.5-fold increase in SEAP activity over the vector control was observed upon stimulation with 1 nM TNF (Fig. 8A). Flavopiridol repressed NF-κB-dependent reporter gene expression induced by TNF.

TNF-induced NF-κB activation is mediated through sequential interaction of the TNF receptor with TNFR1, TRADD, TRAF2, NIK, and IKK resulting in phosphorylation of IκBα (49, 50). To delineate the site of action of flavopiridol in the
TNF-signaling pathway leading to NF-κB activation, cells were transfected with TNFR 1-, TRADD-, TRAF 2-, NIK-, IKK-, and p65-expressing plasmids and then NF-κB-dependent SEAP expression was monitored. As shown in Fig. 8A, cells transfected with TNFR 1-, TRADD-, TRAF 2-, NIK-, IKK-, and p65-expressing plasmids induced NF-κB-dependent expression of SEAP. Pretreatment with flavopiridol suppressed TNFR 1-, TRADD-, TRAF 2-, NIK- and IKK-induced but not p65-induced NF-κB reporter gene expression. These results suggest that flavopiridol affects a step downstream from IKK and upstream from p65.

Because TNF treatment induces cyclin D1, COX-2, and MMP-9, which have NF-κB binding sites in their promoters (31, 32, 51, 52), we next examined whether flavopiridol inhibits TNF-induced cyclin D1, COX-2, and MMP-9. Cells were pretreated with flavopiridol for 8 h and then treated with TNF for the indicated times, and whole-cell extracts were prepared and analyzed by Western blot analysis for the expression of cyclin D1, COX-2, and MMP-9 (Fig. 8B). TNF induced cyclin D1, COX-2, and MMP-9 expressions in a time-dependent manner, and flavopiridol blocked TNF-induced expression of these gene products.
Flavopiridol Suppresses TNF-mediated NF-κB Activation

DISCUSSION

The apoptotic, anti-inflammatory, and immunomodulatory effects of flavopiridol and the associated gene expression pattern suggested that flavopiridol likely suppresses NF-κB activation. In the present report we demonstrate that flavopiridol does indeed inhibit NF-κB activated by a variety of agents and in a variety of cell lines. The inhibition of NF-κB activity was found to be because of suppression of Akt-induced IKK activation, thus resulting in inhibition of IκB phosphorylation, ubiquitination, and degradation. Consequently, flavopiridol also blocked the p65 phosphorylation and acetylation, p65 nuclear translocation, and NF-κB-dependent reporter gene transcription. Furthermore flavopiridol suppressed NF-κB activation induced by TNFR 1, TRADD, TRAF 2, NIK, and IKK but not that induced by p65. Flavopiridol also inhibited the TNF-induced expression of cyclin D1, COX-2, and MMP-9 proteins, all of which have a NF-κB binding site in their promoters, and regulated their transcription.

Our results here indicate that flavopiridol strongly inhibits NF-κB activation. Because NF-κB activation is found by highly diverse stimuli, including TNF, PMA, H2O2, okadaic acid, and cigarette smoke condensate, was inhibited, flavopiridol must suppress activation at a step common to all of these activations. For most of these stimuli, NF-κB activation requires sequential phosphorylation at serines 32 and 36 of IκB. IκB undergoes phosphorylation by activation of a kinase complex consisting of IKK-α and -β (53, 54), which leads to ubiquitination at lysines 21 and 22 and degradation of IκB (55). Because flavopiridol blocked this entire cascade, it must act upstream of IκB phosphorylation. TNF-induced NF-κB activation involves the sequential interaction of the TNF receptor with TRADD, TRAF 2, and NIK, which then activates IKK (49, 50). We found that flavopiridol inhibited NF-κB-dependent reporter gene expression induced by TNFR 1, TRADD, TRAF 2, NIK, and IKK, but not by p65, which also suggests that flavopiridol acts at a step downstream from IKK and upstream from p65.

In the present studies, we also found that flavopiridol inhibited TNF-induced activation of IKK. The antibody that specifically detected the phosphorylated form of IκBα showed that flavopiridol blocked TNF-induced phosphorylation of IκBα. Although the phosphorylation of IκBα is regulated by IKK (consisting of IKK-α, -β, and -γ), a large number of kinases regulate IKK, including NIK, TAK 1, Akt, and MEKK 1 (40, 46). Akt and NIK are primarily known to activate IKK-α, whereas MEKK 1 and atypical protein kinase C activate IKK-β. Flavopiridol inhibited IKK activity with direct interfering with the IKK protein, thereby blocking IκBα phosphorylation, ubiquitination, and degradation. Thus it may be possible that flavopiridol blocked IKK activation by inhibiting one or many of the upstream kinases responsible for IKK activation. It has been shown that TNF-induced IKK activation requires the activation of Akt (42). Our results demonstrate that flavopiridol can indeed suppress the TNF-induced Akt activation. Akt has been shown to induce IKK-α phosphorylation at threonine 23. Mutation of this amino acid blocks phosphorylation by Akt or TNF and activation of NF-κB (42). We show that flavopiridol does not affect the binding of NF-κB protein to the DNA (Fig. 3B) but blocks the ubiquitination of IκBα and the acylation of p65. Our results thus show that flavopiridol is inhibiting IKK, which leads to the inhibition of phosphorylation, ubiquitination, and degradation of IκBα. It could also explain the inhibition of phosphorylation, acetylation, and nuclear translocation of p65. We also demonstrate that purified IKK is not inhibited by flavopiridol (Fig. 5B), but the activation of IKK is suppressed (Fig. 5A). The activation of IKK is in part dependent on an upstream kinase, Akt, which is inhibited by flavopiridol (Fig. 5C). Thus these results clearly show that suppression of Akt leads to inhibition of IKK activation, which in turn blocks phosphorylation, ubiquitination, and degradation of IκBα, suggesting that the IKK is the rate-limiting step. Furthermore, IKK has also been implicated in the p65 phosphorylation (43).

Activation of NF-κB has been linked with the regulation of the cell cycle (56). Flavopiridol is a potent inhibitor of CDKs (2), and NF-κB activation has been shown to be regulated by CDKs (57). The CDKs were found to regulate transcriptional activation by NF-κB through interactions with the coactivator p300. The transcriptional activation domain of RelA (p65) interacted with an amino-terminal region of p300, distinct from a carboxy-terminal region of p300 required for binding to the coactivator CDK 2 complex. The CDK inhibitor p21 or a dominant negative CDK 2, which inhibited p300-associated cyclin E-CDK 2 activity, stimulated NF-κB-dependent gene expression, which was also enhanced by expression of p300 in the presence of p21. The interaction of NF-κB and CDKs through the p300 and CBP coactivators provides a mechanism for the coordination of transcriptional activation with cell cycle progression. Besides IKK activation, this may be another mechanism of suppression of NF-κB by flavopiridol.

Besides activating NF-κB, TNF is also a potent inducer of apoptosis. NF-κB activation has been shown to mediate the suppression of apoptosis induced by TNF (58–60). Flavopiridol has shown to enhance the apoptosis induced by TNF and the TNF-related cytokine TNF-related apoptosis-inducing ligand (17). It is possible that the flavopiridol potentiates the apoptotic effects of TNF and TNF-related apoptosis-inducing ligand through suppression of NF-κB as described here. Flavopiridol has been shown to down-regulate the expression of anti-apoptotic genes (8, 11, 23). Because these genes are regulated by NF-κB (27–30), these genes must be down-regulated through inhibition of NF-κB.
In our study, flavopiridol down-regulated the expression of cyclin D1, COX-2, and MMP-9, all known to be regulated by NF-κB (31, 32, 51, 52). The down-regulation of cyclin D1 by flavopiridol has been reported previously (24), but the down-regulation of COX-2 and MMP-9 by flavopiridol has not. Both COX-2 and MMP-9 play an important role in angiogenesis and the invasion of tumors. Vascular endothelial growth factor, another growth factor known to mediate angiogenesis, has also been shown to be down-regulated by flavopiridol (25, 26). Because vascular endothelial growth factor is an NF-κB-regulated gene (33), it is possible that flavopiridol suppresses vascular endothelial growth factor expression by down-regulating NF-κB. Besides anti-apoptosis, NF-κB has been implicated in inflammation and immune regulation (34). Thus these activities are also most likely mediated through the suppression of NF-κB.

Overall, our results demonstrate that flavopiridol is a potent inhibitor of NF-κB activation, which may explain its proapoptotic, anti-angiogenic, anti-inflammatory, and immunomodulatory effects.

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