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R-Roscovitine simultaneously targets both the p53 and NF-κB pathways and causes potentiation of apoptosis: implications in cancer therapy

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Seliciclib (CYC202, R-Roscovitine) is a 2, 6, 9-substituted purine analog that is currently in phase II clinical trials as an anticancer agent. We show in this study that R-Roscovitine can downregulate nuclear factor-kappa B (NF-κB) activation in response to tumor necrosis factor (TNF)α and interleukin 1. Activation of p53-dependent transcription is not compromised when R-Roscovitine is combined with TNFα. We characterize the molecular mechanism governing NF-κB repression and show that R-Roscovitine inhibits the IκB kinase (IKK) kinase activity, which leads to defective IκBα phosphorylation, degradation and hence nuclear function of NF-κB. We further show that the downregulation of the NF-κB pathway is also at the level of p65 modification and that the phosphorylation of p65 at Ser 536 is repressed by R-Roscovitine. Consistent with repression of canonical IKK signaling pathway, the induction of NF-κB target genes monocyte chemoattractant protein, intercellular adhesion molecule-1, cyclooxygenase-2 and IL-8 is also inhibited by R-Roscovitine. We further show that treatment of cells with TNFα and R-Roscovitine causes potentiation of cell death. Based on these results, we suggest the potential use of R-Roscovitine as a bitargeted anticancer drug that functions by simultaneously causing p53 activation and NF-κB suppression. This study also provides mechanistic insight into the molecular mechanism of action of R-Roscovitine, thereby possibly explaining its anti-inflammatory properties.
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The p53 and nuclear factor-kappa B (NF-κB) pathways are two critical transcriptional regulatory networks deregulated in various human ailments including cancer and there has been a lot of evidence of cross-talk between these two pathways.[1] Activation of p53 is associated with cell cycle arrest and apoptosis while NF-κB activation is associated with cell survival. Hence, the cross-talk between these pathways must be finely tuned and regulated.

p53 is one of the most extensively studied tumor suppressor proteins.[2] Loss or mutation of p53 function is correlated with increased cancer susceptibility. Hence, activating p53 has been a goal of several small molecule inhibitors currently being evaluated in the clinic.

NF-κB is a transcription factor critical for the control of inflammation, apoptosis and cell proliferation.[3,4] Chronic inflammation by constitutively active NF-κB has been shown to contribute to the development of many cancers.[5] It is becoming apparent that deregulated activity of NF-κB is observed and causally linked to the development of several diseases that have an inflammatory component.[6,7] Hence, identification of NF-κB inhibitors has been the focus of several academic and pharmaceutical establishments.[6,7] Since p53 promotes cell death and NF-κB prevents cell death, an anticancer agent that simultaneously activates p53 and inhibits NF-κB would offer greater potential to target two cancer targets positively.

One of the ways in which the p53 pathway has been successfully targeted is by using cyclin-dependent kinase (CDK) inhibitors for treating various cancers, either as single agents or in combination with other drugs. Seliciclib (CYC202, R-Roscovitine) is a 2, 6, 9-substituted purine analogue that is currently in phase II clinical trials as an anticancer agent. It competes with ATP to bind to the active site on CDKs and it was recently shown that R-Roscovitine inhibits RNA polymerase-II-dependent transcription and downregulates Mcl-1, leading to apoptosis.[8] R-Roscovitine has been shown to repress inhibition of p53 by Mdm2 and thereby activate p53 by blocking its degradation.[9] Disruption of the nucleolus to stabilize p53 has also been proposed as another alternate mechanism to activate p53.[10] R-Roscovitine has also been shown to inhibit retinoblastoma protein phosphorylation, decrease levels of cyclin D1 protein and activate the mitogen-activated protein kinase pathway.[11] These studies provide information on the cellular pharmacology of the drug and are a good source of pharmacodynamic markers in the development of the drug in the clinic. R-Roscovitine has been

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Abbreviations: CDK, cyclin-dependent kinase; Cox, cyclooxygenase; FLIP, FLICE-inhibitory protein; IκB, inhibitory subunit of NF-κB; ICAM-1, intercellular adhesion molecule-1; IKK, IκB kinase; IL1, interleukin 1; MCP-1, monocyte chemoattractant protein; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-kappa B; PUMA, p53-upregulated modulator of apoptosis; TNF, tumor necrosis factor
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proposed for combination chemotherapy by affecting DNA repair, apoptosis and having a chemoprotective effect.[12] It has been reported to have antitumor activity against several cancer cell lines.[11,13,14] It can also induce apoptosis in multiple myeloma cells,[15] primary B-cell chronic lymphatic leukemia cells[16] and also maturing cerebellar granule neurons.[17] Another study demonstrated the ability of R-Roscovitine to promote resolution of inflammation and inflammatory cell apoptosis associated with the loss of expression of Mcl-1.[18] This was further confirmed in mouse models of bleomycin-induced lung injury; carrageenan elicited acute pleurisy and passively induced arthritis, thereby suggesting its potential use for treating various inflammatory diseases. R-Roscovitine was also reported to effectively arrest cystic disease in mouse models of polycystic kidney disease (PKD), thereby suggesting its potential use in the treatment of PKD.[19] Thus, there is considerable interest in elucidating the mechanism of action of R-Roscovitine to help in its evaluation as a potential drug for use in the clinic.

In this study, we show that R-Roscovitine can inhibit activation of NF-κB. While the NF-κB pathway is repressed, activation of p53 is not compromised, suggesting its potential to target both these critical cancer targets simultaneously. We characterize the molecular mechanism underlying its action. Our results provide deeper insight into the cellular pharmacology of R-Roscovitine and could also help to explain its anti-inflammatory effects.

Results

R-Roscovitine represses TNFα-induced activation of NF-κB in a dose-dependent manner. Since the NF-κB pathway has been shown to be a target for anti-inflammatory molecules,[20] we checked whether R-Roscovitine, which has anti-inflammatory properties,[18] has any effect on the NF-κB pathway. For this, A549-NF-κB-luc (A549) and 293-NF-κB-luc (293) cells were treated with different concentrations of R-Roscovitine (5, 10, 15 and 30 μM). Both the cell lines used are stable cell lines derived from human A549 and 293 cells, respectively, with chromosomal integration of a luciferase reporter construct regulated by six copies of the NF-κB response element (Panomics). A549 cells show optimum NF-κB activation when incubated with 50 ng/ml tumor necrosis factor (TNF)α for 6 h, while 293 cells show optimum activation when treated with 20 ng/ml TNFα for 8 h before luciferase activity is read (Panomics).

We used a concentration range of R-Roscovitine that has been previously shown to increase p53-dependent transcription,[8] and found that R-Roscovitine inhibits TNFα-induced activation of NF-κB reporter in a dose-dependent manner (Figure 1a, lanes 6–10). The results were further confirmed in 293 cells (Figure 1b, lanes 6–10). We conclude that R-Roscovitine inhibits the activation of NF-κB in a dose-dependent manner.

p53 activation is not compromised upon combination of R-Roscovitine with TNFα. It is known that R-Roscovitine activates p53-dependent transcription.[8,9] We next tested whether the suppression of TNFα-induced activation of NF-κB after treatment with R-Roscovitine was at the expense of p53 pathway activation. As shown in Figure 2a (lanes 3,4), treating cells with R-Roscovitine or its combination with TNFα showed a significant increase in p53 protein levels, while treatment of A549 cells with TNFα alone did not show any increase (Figure 2a, lane 2). We also tested if the treatment had any effect on p53 modifications. As shown in Figure 2a (lanes 3–4), simultaneous treatment with R-Roscovitine and TNFα for 12 h showed an increase in phosphorylation of both Ser392 and Ser15 and also acetylation of p53.

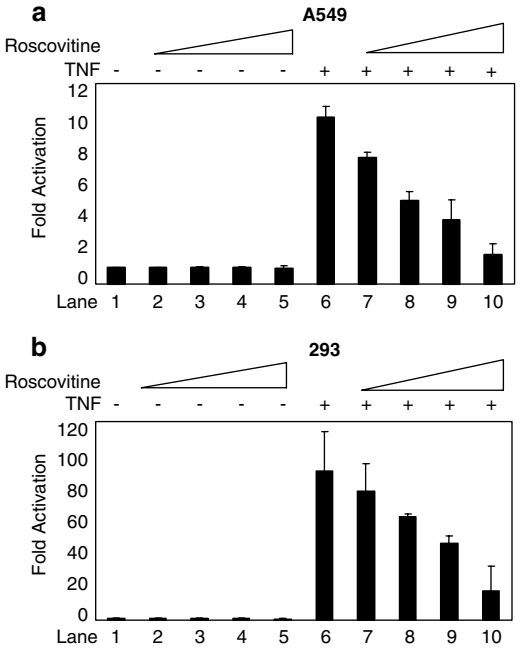

Figure 1 R-Roscovitine inhibits TNFα-induced activation of the NF-κB pathway in a dose-dependent manner. (**a**, **b**) A549-NF-κB-Luc (A549) cells were treated with R-Roscovitine and TNFα (50 ng/ml) and reporter activity was measured 6 h after treatment. 293-NF-κB-Luc (293) cells were treated with R-Roscovitine and TNFα (20 ng/ml), and reporter activity was measured 8 h after treatment. Increasing concentrations of R-Roscovitine used: 5, 10, 15 and 30 μM. The fold activation and repression represented is an average of triplicates of two independent data sets

p53 activation (Figure 2a) was further confirmed by using a cell-based reporter assay in ARN8 human melanoma cells which measures induction of p53 transcriptional activity.[21] Treatment of cells with R-Roscovitine alone induces p53-dependent transcription, while treatment of cells with TNFα alone shows no activation (Figure 2b). However, a similar degree of p53 activation is still maintained when the cells are simultaneously treated with R-Roscovitine and TNFα. This was further confirmed by quantitative RT-PCR for p53 target genes (Figure 2c). Treatment of cells with R-Roscovitine and TNFα showed an increase in mRNA levels of p53 target genes like p21 and p53-upregulated modulator of apoptosis (PUMA). We thus conclude that p53 activation is not compromised upon combination of R-Roscovitine and TNFα.

R-Roscovitine inhibits NF-κB target genes. TNFα has been shown to upregulate levels of NF-κB target genes like intercellular adhesion molecule-1 (ICAM-1), cyclooxygenase

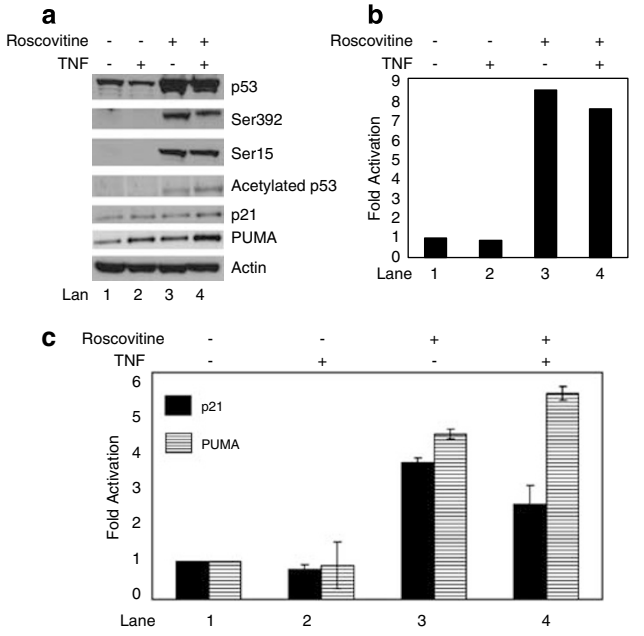

Figure 2 p53 activation is not compromised upon combination of R-Roscovitine with TNFα. (**a**) Combination of R-Roscovitine and TNFα activates levels of p53, phosphorylation of p53 at Ser392 and Ser15 and p53 target genes p21 and PUMA. A549 cells were treated with 30 μM R-Roscovitine and 50 ng/ml TNFα for 12 h. Cells were harvested and whole cells lysates prepared. Equal amounts of cell lysate were analyzed by western blotting with antibodies as mentioned, and actin levels were used to ensure equal loading. (**b**) Combination of R-Roscovitine and TNFα activate p53-dependent transcription in ARN8 reporter cell line. (**c**) p53 target genes like p21 and PUMA are activated at the RNA level upon combination of R-Roscovitine and TNFα as seen by quantitative RT-PCR

(Cox-2), cyclin D1, Bcl-xL, FLICE-inhibitory protein (FLIP), etc.[22–25] We assessed whether R-Roscovitine could downregulate these endogenous NF-κB target genes in a manner similar to its repression of the NF-κB reporter gene activity (Figure 1). Figure 3a and b (lanes 7–10) document that R-Roscovitine repressed the expression of NF-κB gene products ICAM-1, monocyte chemoattractant protein (MCP-1), Bcl-xL and FLIP in both A549 and H1299 cells. To validate if this repression occurred at the level of transcription, we carried out quantitative RT-PCRs. We show that downregulation of NF-κB targets MCP-1, Cox-2 and IL8 in both A549 and H1299 cells indeed occurred at the level of transcription (Figure 3c–e). Besides TNFα, NF-κB activity is also induced by inflammatory cytokines like interleukin 1 (IL1).[26] We found that R-Roscovitine strikingly inhibits NF-κB targets ICAM-1 and MCP-1 induced by IL1 as well (Figure 3f). Taken together, we believe that R-Roscovitine could be used as a potent inhibitor of NF-κB signaling.

R-Roscovitine inhibits TNFα-induced IKK kinase activity. We next studied the effect of R-Roscovitine on IκB kinase (IKK) activity. Treatment of cells with TNFα led to the increase of IKK autophosphorylation activity (Figure 4a, compare lanes 1 and 2) and consequently the IKK activity towards its substrate, GST-IκBα (Figure 4b, compare lanes 1 and 2). While R-Roscovitine had no effect on the basal IKK activity (Figure 4b, compare lanes 2 and 3), it inhibited TNFα-induced IKK activity towards GST-IκBα (Figure 4b, compare lanes 1 and 4). Overall protein levels of NF-κB essential modulator (NEMO) and IKK2 induced by TNFα are not affected in the presence of R-Roscovitine in A549 cells (Figure 4c, lanes 1–4). The autoactivaton of IKK as measured by a phospho-specific antibody demonstrated that while TNFα induced IKK autophosphorylation (Figure 4c, compare lanes 1 and 2), R-Roscovitine blocked this phosphorylation in the activation loop of IKKs (Figure 4c, compare lanes 1 and 4). We cannot, at this point, distinguish between the possibilities of whether R-Roscovitine is direct inhibitor of IKKs or does it inhibit an upstream kinase that activates IKK (and is pulled down along with them in the immunoprecipitations).

To further test which IKK subunit is the seat of action of R-Roscovitine, WT MEFs and IKK1 and IKK2 knockout MEFs were treated with R-Roscovitine and TNFα in a manner similar to A549 and H1299 (Figure 3a and b). As seen with A549 and H1299 cells, in WT MEFs, R-Roscovitine inhibits TNFα-induced activation of NF-κB target gene MCP-1 (Figure 4d, compare lanes 1–5 to lanes 6–10 in left panel). Compared to WT and IKK1−/− MEFs, MCP-1 is less inducible in IKK2−/− (Figure 4d, compare lanes 1–5 to lanes 6–10 in all panels). Furthermore, R-Roscovitine-mediated repression of MCP-1 is much less dramatic in IKK2−/− MEFs compared to WT and IKK1−/− MEFs (Figure 4d, compare lanes 1–5 to lanes 6–10 in all panels). We conclude that Roscovitine inhibits the IKK kinase activity and most likely mediates its effects by targeting an upstream IKK kinase or a critical component of the IKK complex. Overall, our biochemical, pharmacological and genetic data clearly indicate that R-Roscovitine is an inhibitor of canonical NF-κB signaling and this depends on its ability to block the kinase activity of the IKK complex.

R-Roscovitine inhibits TNFα-dependent IκBα phosphorylation and degradation. It is known that the degradation of IκBα is required for nuclear accumulation of NF-κB.[27] Since R-Roscovitine could inhibit IKK activity, we next investigated if it could block IKK-mediated IκBα phosphorylation and degradation. Treatment of cells with TNFα led to phosphorylation of IκBα (Figure 4e and f, lanes 1–5). Since IκBα is also a target of NF-κB, TNFα treatment also led to its degradation and re-synthesis (Figure 4e and f, lanes 1–5). However both phosphorylation of IκB as well as its degradation and re-synthesis in response to TNFα were blocked by R-Roscovitine (Figure 4e and f, compare lanes 1–5 to lanes 6–10). Thus, R-Roscovitine represses the NF-κB pathway by inhibiting IKK-induced phosphorylation and hence degradation of IκBα. Since protein expression levels of IκBα seemed lower upon treatment with R-Roscovitine alone (Figure 4e, compare lanes 1 and 6), we next determined if R-Roscovitine treatment alone causes loss of IκBα. For this, we treated cells with R-Roscovitine alone for various time intervals (Supplementary Figure 2a). R-Roscovitine treatment alone decreased levels of IκBα, 24 h after treatment. In all our time course studies, cells were pretreated with R-Roscovitine for 12 h followed by a time-dependent treatment with TNFα. Hence at the final time points, cells were exposed to R-Roscovitine for about

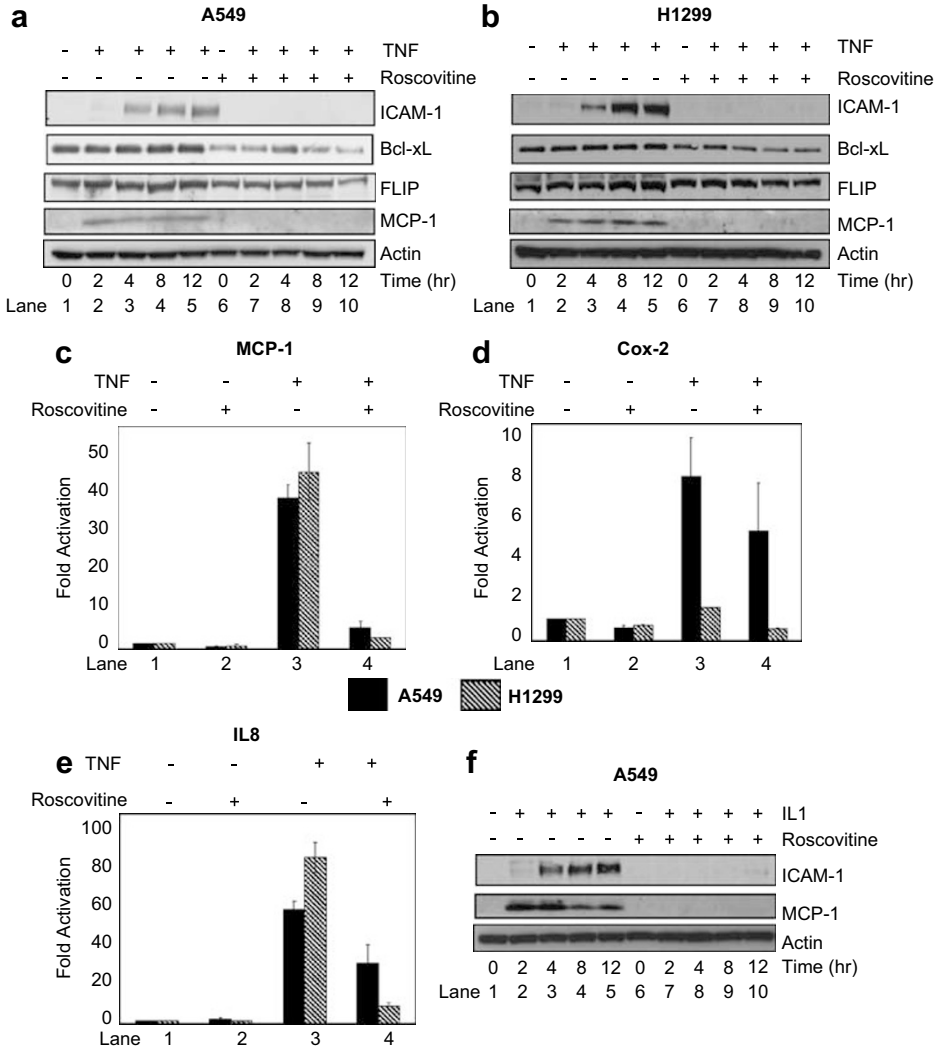

Figure 3 R-Roscovitine inhibits expression of NF-κB target genes. (**a**, **b**) R-Roscovitine inhibits expression of NF-κB-regulated gene products like ICAM-1, FLIP, MCP-1 and Bcl-xL in both A549 and H1299 cells. Cells were treated with 30 μM R-Roscovitine and 50 ng/ml TNFα for different time intervals as indicated. At different time points, cells were harvested and whole cells lysates prepared. Equal amounts of cell lysate were analyzed by western blotting with antibodies as mentioned, and actin levels were used to ensure equal loading. (**c**–**e**) Quantitative RT-PCR data showing downregulation of TNFα-induced NF-κB-regulated gene products MCP-1, Cox-2 and IL-8 by R-Roscovitine in both A549 and H1299 cells. Cells were treated with 30 μM R-Roscovitine and 50 ng/ml TNFα for 12 h before they were harvested and total RNA was isolated (refer to Materials and Methods for more details). (**f**) R-Roscovitine inhibits protein expression of ICAM-1 and MCP-1 induced by IL-1, another NF-κB-activating stimuli

16–24 h, thus leading us to wonder if the effects of R-Roscovitine on IκBα are merely reflective of a general downregulation of Poll II transcripts like IκBα. However, densitometric analysis of IκBα degradation and re-synthesis (Figure 4g and h, lanes 1–5) shows that treatment with TNFα alone decreases levels of IκBα after 30 min, which is restored to starting levels in a time-dependent manner. Upon treatment with R-Roscovitine, even though the starting levels of IκBα are lower, TNFα treatment did not cause degradation and re-synthesis of IκBα (Figure 4g and h, lanes 7–10). Furthermore, there was no effect on IκBα levels upon treatment with R-Roscovitine alone in either IKK1 or IKK2 knockout MEFs (Supplementary Figure 2b), indicating that R-Roscovitine-mediated lowering of IκBα levels could be cell type specific and is not the only cause of reduced IκBα levels seen in some of our assays. Taken together, our results demonstrate that apart from exerting its effect on other pathways, R-Roscovitine is a potent inhibitor of IKK activity and hence the NF-κB pathway.

R-Roscovitine inhibits TNFα-induced p65 Ser536 phosphorylation. TNFα-induced phosphorylation of p65 is critical for NF-κB transcription.[28] To further understand the mechanism of NF-κB repression by R-Roscovitine, we assessed the levels of p65 phosphorylation and other critical components of the NF-κB pathway. As shown in Figure 4e and f, R-Roscovitine significantly downregulates TNFα-induced activation of p65 phosphorylation at Ser536 (compare lanes 1–6 and 7–10). A similar decrease in phosphorylation is seen in p65 phosphorylation at Ser276; however, the effect is less pronounced than phosphorylation at Ser536. These effects are seen in both A549 and H1299 cells, further supporting our results that downregulation of the

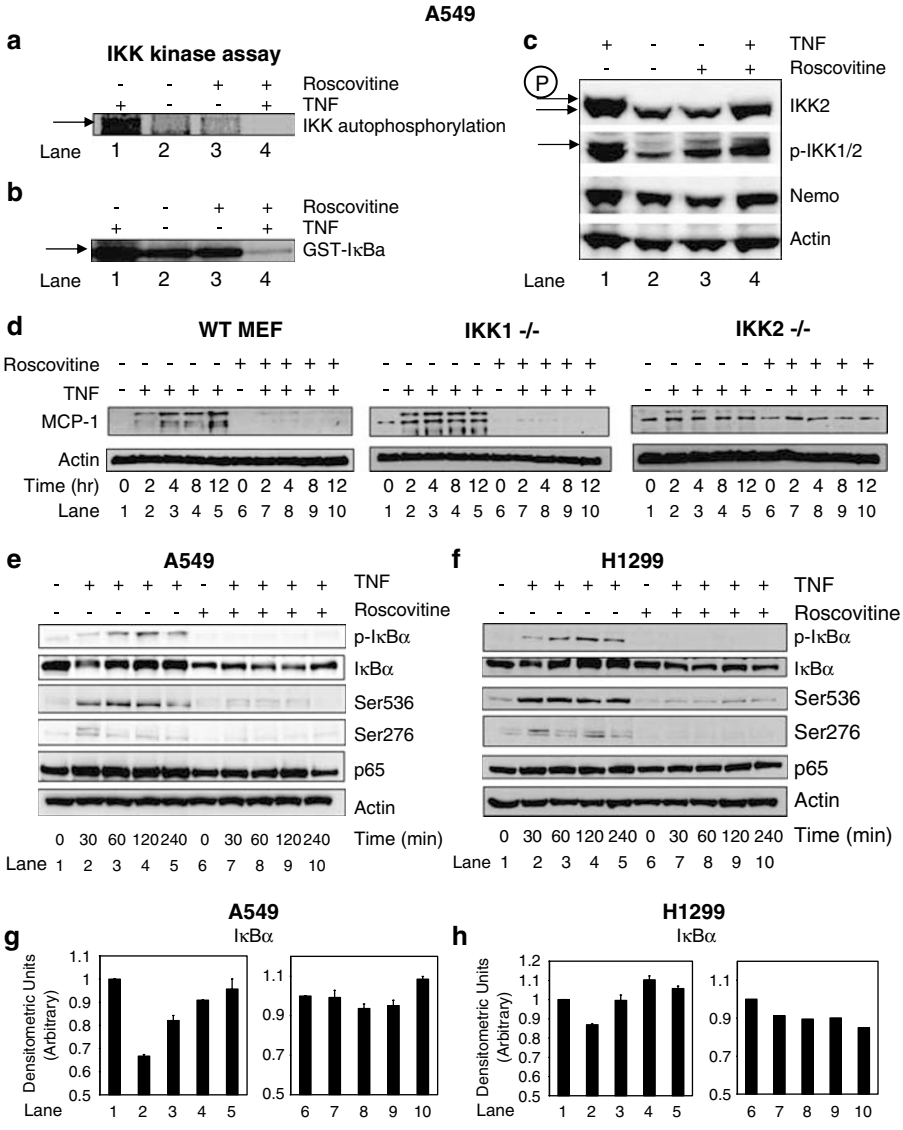

Figure 4 R-Roscovitine inhibits TNFα-dependent IKK kinase activity, IκBα phosphorylation, IκBα degradation and p65 phosphorylation. (**a**, **c**) R-Roscovitine inhibits IKK autophosphorylation in response to TNFα. A549 cells were pretreated with R-Roscovitine for 12 h, followed by treatment with TNFα for 7 min. The IP material was included in a kinase assay (**a**, **b**) and also run on a gel to probe with the various antibodies as indicated (**c**). IKK kinase assay was performed as described in the Materials and Methods. The IKK autophosphorylation band has been indicated by an arrow in (**a**). The two arrows in the top panel of Figure 4c point to the native and phospho form of IKK2. The phosphorylated IKK2 species is detected much clearly in p-IKK1/2 blot (second panel). (**b**) R-Roscovitine inhibits IKK kinase activity towards IκBα. Immunoprecipitated IKK complex from cells treated with the indicated reagents was assayed for its ability to phosphorylate GST-IκBα (1–54). Phosphorylated IκBα has been indicated by an arrow. (**d**) Effect of R-Roscovitine on TNFα-induced NF-κB target gene MCP-1 in WT MEFs and IKK1 −/− and IKK2 −/− MEFs. Cells were treated and harvested as mentioned earlier. Actin was used as a loading control. (**e**, **f**) R-Roscovitine inhibits TNFα-induced phosphorylation and degradation of IκBα and phosphorylation of Ser536 of p65 in both A549 and H1299 cells. (**g**, **h**) Densitometric analysis of IκBα levels upon TNFα treatment in the presence and absence of R-Roscovitine in both A549 and H1299 cells suggest that R-Roscovitine inhibits degradation of IκBα. The densitometric values have been derived by comparing the images shown in Figure 4e and f

NF-κB pathway by R-Roscovitine is independent of the status of p53 in the cells. Given that serine 536 is considered a *bona fide* IKK site, these results are consistent with R-Roscovitine being an inhibitor of canonical IKK signaling.

R-Roscovitine represses TNFα-induced p65 nuclear localization. Since nuclear localization of the p65 subunit is critical for NF-κB function, we next checked if R-Roscovitine affects this process. R-Roscovitine inhibits TNFα-induced nuclear accumulation of p65 at all the time points tested (Figures 5 and 6). This effect is seen in both A549 and H1299 cells. Taken together, the results presented thus far indicate that R-Roscovitine is an inhibitor of IKK kinase activity. Inhibition of this activity leads to loss of IκB degradation and consequently leads to loss of modification and nuclear accumulation of NF-κB.

R-Roscovitine potentiates cytotoxic effects of TNFα. It has been shown that TNFα induces apoptosis if NF-κB is repressed.[29] Since R-Roscovitine can inhibit NF-κB, we also

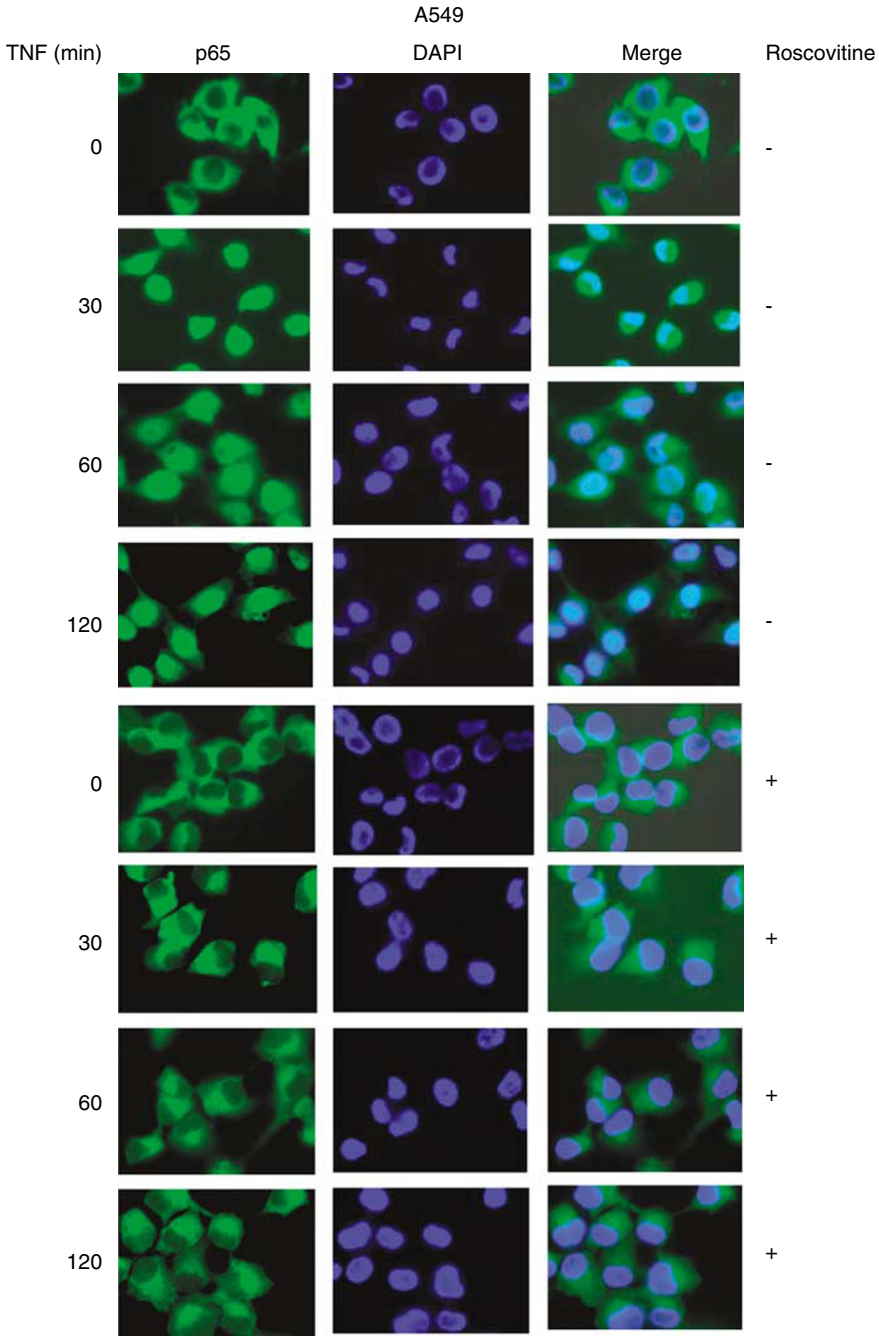

Figure 5 R-Roscovitine represses TNFα-induced p65 nuclear localization in A549 cells. p65 localization upon treatment of cells with TNFα alone or in combination with R-Roscovitine for the indicated times was assessed by immunocytochemistry (refer to Materials and Methods for more details)

investigated if combining R-Roscovitine and TNFα could lead to TNFα-induced cell death. Using annexin V staining as a marker for cell death, we observed that R-Roscovitine potentiated TNFα-induced cell death in a concentration- and time-dependent manner in both A549 and H1299 cells (Figure 7a and b, compare lanes 1–6). Furthermore, combination of R-Roscovitine and TNFα for 12 h caused a significant increase in PARP cleavage, another hallmark of apoptosis (Figure 7c, lane 4). R-Roscovitine alone has been shown to induce PARP cleavage.[8] Treatment of A549 cells with R-Roscovitine alone induces PARP cleavage only after 24 h (Figure 7d, lane 6), further supporting that R-Roscovitine potentiates cytotoxic effects of TNFα.

Thus, we believe that R-Roscovitine is a useful agent that can block critical physiological functions of NF-κB.

Discussion

R-Roscovitine is currently in clinical trials, both as a single agent for treatment of different cancers or in more limited

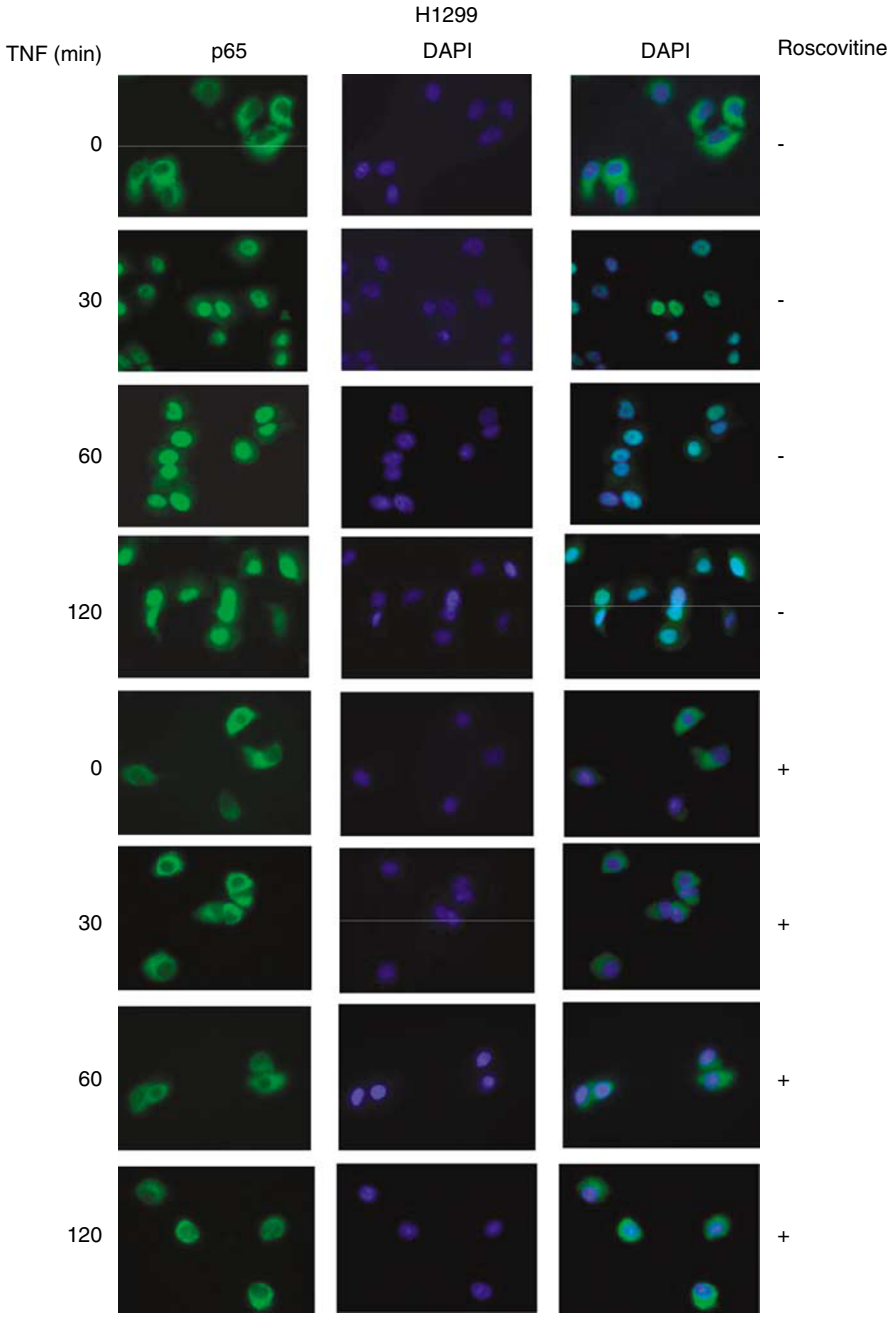

Figure 6 R-Roscovitine represses TNFα-induced p65 nuclear localization in H1299 cells. p65 localization upon treatment of cells with TNFα alone or in combination with R-Roscovitine for the indicated times was assessed by immunocytochemistry (refer to Materials and Methods for more details)

cases, in combination with nongenotoxic agents.[30] A model based on our study is presented in Figure 8. In this study, we have shown that R-Roscovitine inhibits TNFα-induced NF-κB pathway through inhibition of the IKK kinase activity and phosphorylation and degradation of IκBα. Furthermore, we show that it prevents nuclear accumulation of p65 and downregulates phosphorylation of p65 at a crucial serine 536 residue, which is required for chromatin remodeling. Consistent with being a potent inhibitor of NF-κB, R-Roscovitine also potentiates apoptosis induced by TNFα and downregulates expression of NF-κB target genes. Finally, we demonstrate that R-Roscovitine can also inhibit NF-κB-dependent gene activation in response to other stimuli such as IL1 (Figure 3f).

Clinical use of TNFα as a single agent has been extremely limited due to its severe side effects.[31] It is becoming increasingly evident that the best strategy to use in cancer chemotherapy is a combination of known drugs that synergistically act on various pathways and targets simultaneously. This is also emerging as an improved means of combating the problem of drug resistance and improving the efficiency of therapeutic response to tumors. Our studies suggest the

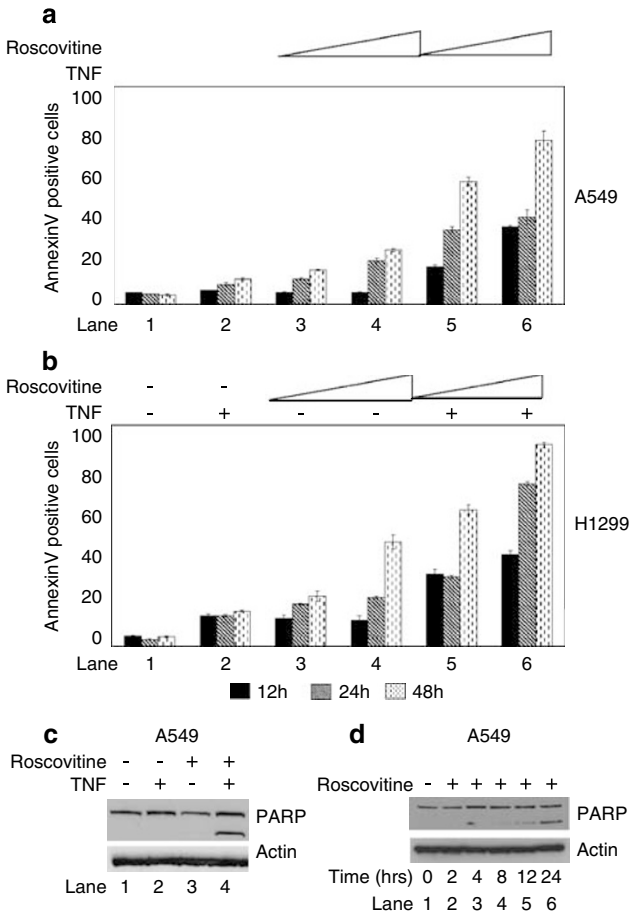

Figure 7 R-Roscovitine potentiates TNFα-induced apoptosis in A549 and H1299 cells in a time- and concentration-dependent manner. (**a**, **b**) Cells were treated with 15 and 30 μM R-Roscovitine alone, or in combination with 50 ng/ml TNFα for 12, 24 and 48 h, as indicated. Apoptosis was assayed by an increase in annexinV-positive cells. The % annexinV-positive cells represented are an average of triplicate data sets. (**c**) Combination treatment of R-Roscovitine and TNFα shows an increase in PARP cleavage in A549 cells, another hallmark of apoptosis (compare lanes 1 and 4). (**d**) Treatment of A549 cells with 30 μM R-Roscovitine alone induces PARP cleavage in a time-dependent manner. Cells were treated and harvested as mentioned earlier (refer to Materials and Methods for more details)

possibility of combining R-Roscovitine with TNFα or with other NF-κB inhibitors for treatment of various cancers.

Inhibition of transcription has been predicted to sensitize cancer cells to TNFα. This has been reported for flavopiridol,[32] a semisynthetic flavonoid and another potent inhibitor of cyclin-dependent kinases CDK1, CDK2, CDK4 and CDK7.[33] Previous studies showing very strong cytotoxicity of flavopiridol in combination with either TNFα or tumor necrosis factor-related apoptosis inducing ligand (TRAIL) have been very encouraging. While most efforts towards understanding the mechanism of action of flavopiridol have focused on the p53 pathway, work by Takada and Aggarwal[34] showed that flavopiridol has the additional property of inhibiting TNFα-induced activation of NF-κB in a dose-dependent manner. It does so by inhibiting IκBα kinase, a key NF-κB regulator, and affecting its critical post-translational modifications including its degradation by ubiquitination. Flavopiridol also affects nuclear localization of p65 and inhibits NF-κB target genes like cyclin D1, thereby inhibiting inflammation and modulating cell growth.[34] We see similar effects with R-Roscovitine on the NF-κB pathway. Both R-Roscovitine and flavopiridol are CDK inhibitors that suppress general transcription and paradoxically activate p53-dependent transcription. We show in this study that R-Roscovitine activates p53-dependent transcription even when combined with TNFα, and potentiates TNFα-induced apoptosis in a dose- and time-dependent manner. We also show in this study that the effect of R-Roscovitine on the NF-κB pathway is independent of p53, further suggesting its potential to target both these pathways simultaneously, regardless of the p53 status in tumors. It is possible that, since R-Roscovitine potentiates cell death independently of p53 (Figure 7a and b), the pathway involving the interaction of Fas-associated death domain (FADD) and the mitochondrial caspase cascade is involved.[31] Furthermore, our study is focused on the mechanism of action of R-Roscovitine in lung cancer cells, where current clinical trials are focused (Appraise trial, Cyclacel, http://www.cyclacel.com/cyc/rd/trials/).

A recent study[35] showed that nuclear expression of p65 was significantly increased in both small-cell and non-small cell lung cancers, thereby suggesting that inflammation could have a potential role in the early pathogenesis of lung cancer. Thus, R-Roscovitine could have therapeutic applications in this context as well. We have shown in this study that R-Roscovitine inhibits TNFα-induced activation of NF-κB target genes involved in antiapoptosis (Bcl-xL and FLIP), proliferation (Cox-2) and invasion of tumors (ICAM-1) (Figure 3a and b).

Earlier studies have shown that R-Roscovitine induces apoptosis by inhibiting RNA polymerase II (Pol II)-dependent transcription and downregulating genes involved in cell survival.[8,16] Consistent with their studies, we also see downregulation of total Pol II and Ser2 phosphorylation upon treatment with R-Roscovitine (Supplementary data 1). However, our data also support specific effects of R-Roscovitine on inhibiting TNFα-induced activation of the NF-κB pathway. Our reporter assay (Figure 1) shows that R-Roscovitine only inhibits TNFα-induced activation. While levels of IκBα are downregulated upon long exposure to R-Roscovitine, no effect is seen on levels of IκBα in other cells like the IKK1 and IKK2 knockout MEFs. Thus, while inhibition of Pol II transcription by R-Roscovitine could be a contributor to NF-κB inhibition, the effect we report cannot be completely explained by a general downregulation of Pol II-dependent transcription by R-Roscovitine.

It is interesting to note that the protein levels of both IκBα and Bcl-xL are decreased in A549 cells upon treatment with R-Roscovitine for 24 h (Supplementary data 2 and 3). This is not an effect of general transcription inhibition since a similar effect is not seen in MEFs or IKK knockout cells. A very interesting study by Junan Li *et al.*,[36] showed that IκBα binds CDK4 and inhibits its activity. They proposed a functional cross-talk between the NF-κB pathway and the p16-CDK4-Rb pathway. While our results show that R-Roscovitine inhibits the NF-κB pathway and IκBα protein levels at longer time points, R-Roscovitine has also been shown to inhibit Rb protein phosphorylation.[11] This further suggests the cross-talk between these signaling pathways and suggests that R-Roscovitine could be targeting both these pathways by

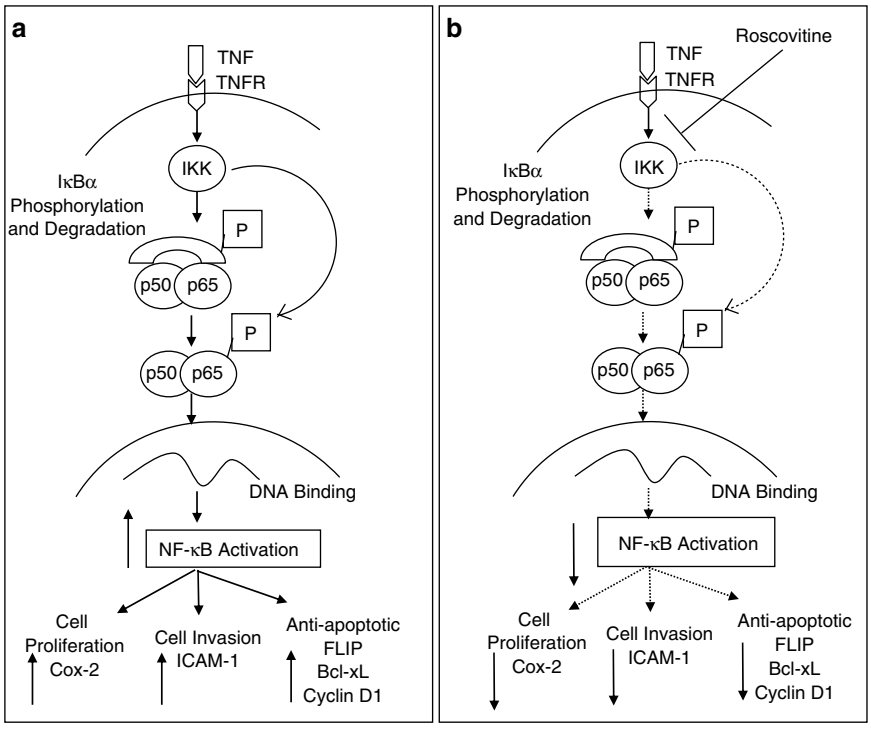

Figure 8 Model depicting mechanism of action of R-Roscovitine and its inhibition of the NF-κB pathway. (**a**) The canonical TNFα-induced NF-κB activation pathway. (**b**) R-Roscovitine inhibits TNFα-induced phosphorylation and hence degradation of IκBα, as depicted by dashed lines. Since IκBα bound p65 is not modified and since R-Roscovitine-mediated inhibition of IKK blocks its activity, the phosphorylation of p65 at serine 536 is also affected by this treatment (since it is an IKK site). R-Roscovitine treatment eventually prevents the nuclear localization of p65 and thereby downregulates the NF-κB pathway and its various gene products involved in cell invasion, cell proliferation and antiapoptosis

affecting IκBα. Further studies would be aimed at better understanding the mechanism of this functional cross-talk and its molecular pharmacology to aid development of R-Roscovitine in the clinic.

Another recent work by Komarova *et.al.*[37] showed p53 as a suppressor of inflammatory response in mice and proposed a general 'buffering' role of these reciprocally controlled pathways. This 'pharmacological tuning', as proposed by them, would offer the potential of targeting both the pathways in one attempt. Quinacrine, an anti-malarial drug, and flavopiridol have also been shown to have both these desirable properties.[38]

Finally, our study shows that R-Roscovitine inhibits the NF-κB pathway, thereby possibly explaining its anti-inflammatory properties. We show that it downregulates NF-κB gene targets involved in antiapoptosis, cell invasion and proliferation (Figure 8). Current studies are underway to further delineate the targets of R-Roscovitine and their effects on various signaling pathways. This study provides the scientific rationale for combination therapy with R-Roscovitine and also suggests biomarkers for effects of R-Roscovitine and possibly other CDK inhibitors currently in the clinic for the management of lung cancer. It would also be worthwhile to get a deeper insight into the mechanism of action of R-Roscovitine in combination with NF-κB inhibitors in the clinic. Thus, R-Roscovitine has the potential to simultaneously benefit two critical targets in cancer in a desirable way: activation of p53 and suppression of NF-κB. This would be particularly useful in cancers where NF-κB is constitutively active, thereby maintaining p53 in a repressed state. We further propose a thorough clinical evaluation of this approach.

Materials and Methods

Cell culture, cell lines and reagents. A549-NF-κB-luc (A549) and 293-NF-κB-luc (293) were purchased from Panomics Inc. Both cell lines were cultured at 37°C and 5% CO_2 in DMEM medium supplemented with 10% FBS, L-glutamine, penicillin and streptomycin and were maintained in selection medium containing HygromycinB. ARN8 human melanoma cells and H1299 (p53-null) human non-small-cell lung carcinoma cells were cultured in DMEM medium supplemented with 10% FBS, penicillin and streptomycin. R-Roscovitine was purchased from Calbiochem and TNFα was purchased from Sigma.

NF-κB reporter activity assay. A549-NF-κB-luc (A549) and 293-NF-κB-luc (293) cells were seeded in 96-well plates at 10 000 cells/well. Cells were treated with different concentrations of the drugs as mentioned for different time intervals. The luciferase activity was measured using the Bright-Glo™ luciferase assay system (Promega) and the assay was performed as per the manufacturer's instructions. The fold activation or repression was calculated relative to the control sample and all measurements were done by calculating the average of triplicate samples of two independent experiments.

p53 reporter activity assay. The p53 reporter activity assay was a cell-based reporter assay developed in ARN8 cells. The details of the reporter system have been described elsewhere.[21] The assay was performed in 96-well plates and the cells were seeded and treated as described above. The FluoReporter LacZ/Galactosidase quantitation kit was used to measure fluorescence with excitation at 390 nm and emission at 460 nm. SpectraMAX (Molecular Probes) was used to measure fluorescence readings.

AnnexinV staining. Cells were collected by trypsinization, washed twice in PBS and harvested in PBS. Apoptosis was evaluated using the annexin V (FITC)-

propidium iodide binding assay (Roche). The extent of apoptosis was quantified as a percentage of annexin V-positive cells over the total cell population. Flow cytometric analysis was performed on an LSR II system (BD Biosciences).

Western blot analysis. Cells were seeded at 1×10^6 cells/ml and treated with 30 μM R-Roscovitine and 50 ng/ml TNFα. Before harvesting, cells were washed twice with PBS. The pellet was split for use for western blot analysis and quantitative RT-PCR. Cells were lysed on ice in RIPA buffer with 2 $\times$ protease inhibitor cocktail (Roche) and the protein concentration was determined using the Bradford reagent (Bio-Rad). In total, 30 μg of lysate in SDS gel loading buffer and 10 mM DTT was separated on 4–12% Bis-Tris gels (Invitrogen). Proteins were transferred onto nitrocellulose membrane (Bio-Rad) and equal loading confirmed by Ponceau S staining (Sigma). The membrane was blocked in 5% milk in PBS with 0.1% Tween-20 (PBST) for 1 h and incubated overnight with primary antibody at 4°C. Membranes were washed with PBST and then incubated in horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Membranes were washed again for 1 h with PBST and developed using ECL detection reagents (Amersham). Antibodies were used to detect p53 (DO-1), p21, PUMA (Calbiochem), p65, IκBα, ICAM-1, MCP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), Acetylated p53, p65-Ser536, p65-ser276, p-IκBα, PARP, p53-Ser15, p53-Ser392, Bcl-xL, FLIP (Cell Signaling Technology) and Beta-actin (Sigma).

IKK kinase activity assay. IKK kinase activity was performed as described previously.[39] The cDNA coding for the first 54 amino acids of IκBα was isolated by PCR from total MEF cDNA and cloned into pOPTG expression vector as a GST fusion gene. The GST-IκBα (1–54aa) fusion protein was expressed in RIPL bacteria, purified on glutathione sepharose beads (GE Healthcare) and used in the kinase assay.

Quantitative RT-PCR. Cells were treated and harvested in the same way as for western blot studies. Total RNA was isolated using the RNeasy kits (Qiagen). The RNA was quantitated by spectrophotometric analysis and used for quantitative RT-PCR. The primers used for each target analyzed are available upon request. The power SYBR green PCR master mix (Applied Biosystems) was used for quantitation of mRNA levels.

Immunostaining and fluorescence microscopy. Indirect immunofluorescence was carried out as described previously.[40] DO-1 (anti-p53), p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse Alexa-488-coupled secondary antibody (Molecular Probes) were used. Immunofluorescence was visualized using AxioImager Z1 (Zeiss).

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)