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# Inhibition of Human Immunodeficiency Virus Type 1 Replication by Blocking IκB Kinase with Noraristeromycin

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**Nuclear factor κB (NF-κB) is one of the critical transcription factors in inflammatory responses and replication of viruses such as human immunodeficiency virus (HIV). In fact, it has been demonstrated that various NF-κB inhibitors could block HIV replication. To explore more potent NF-κB inhibitors, we focused on carbocyclic adenine nucleosides that had been reported to have anti-inflammatory effects. We synthesized 15 carbocyclic adenine nucleoside compounds and examined their effects on the NF-κB-dependent gene expression using HEK293 cell line. Among these compounds, noraristeromycin (NAM) exhibited the most potent inhibitory effect on the NF-κB activity under the non-cytotoxic concentrations. NAM-inhibited IκBα phosphorylation and degradation upon stimulation of cells with tumour necrosis factor-α (TNF-α). In addition, NAM prevented p65 phoshorylation. These findings suggested that both IκB kinase-α (IKK-α) and -β were targeted by NAM. Interestingly, *in vitro* kinase assay revealed that NAM inhibited the kinase activity of IKK-α more potently than that of IKK-β. When we treated the cell lines, OM10.1 and Molt4/IIIB, in which HIV-1 is latently and chronically infected, we found a strong suppressive effect of NAM on HIV-1 viral replication upon stimulation with TNF-α.**

**Key words: NF-κB, IKK, noraristreromycin, phosphorylation, transcription.**

Abbreviations: ACHP, 2-amino-3-cyano-4-alkyl-6-(2-hydroxyphenyl)pyridine; AZT, 3′-azido-3′-deoxythymidine; cPA, 9-[(1*S*,3*R*)-*cis*-cyclopentan-3-ol]adenine; CREB, cAMP-responsive element binding protein; HIV, human immunodeficiency virus; IKK, IκB kinase; NAM, noraristeromycin; NF-κB, nuclear factor κB; TNF-α, tumour necrosis factor α.

Nuclear factor κB (NF-κB) is an inducible cellular transcription factor that regulates a wide variety of cellular genes involved in the control of the inflammatory and immune response, cellular proliferation, apoptosis and cell-cycle progression (*1–3*). In addition to these kinds of genes, NF-κB is also a potent cellular activator of human immunodeficiency virus type 1 (HIV-1) gene expression (*4*, *5*) and thus positively controls viral replication in the infected cells. In cells chronically infected with HIV-1, activation of NF-κB together with constitutive active Sp1 could trigger the transcription of viral genes including the transactivator Tat that mediates transcriptional elongation of viral expression, culminating in the explosive replication of HIV-1. Thus, down-regulation of NF-κB activity is expected to have beneficial effects on the clinical development of AIDS in HIV infected individuals (*6*).

NF-κB is a hetero- or homo-dimer consisting of Rel family proteins, p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100, and normally present in the cytoplasm in association with its inhibitor protein IκB (*1–3*). Stimulation by the inflammatory cytokines such as TNF-α and IL-1β activates IκB kinase (IKK) complex by upstream kinases such as mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/3 (MEKK1/3) and NF-κB-inducing kinase (NIK) (*7*). IKK is a large molecular weight complex consisting of at least two catalytic subunits, IKK-α, IKK-β and the associated regulatory subunit IKK-γ/NEMO. Activated IKK complex phosphorylates IκB on two specific serine residues (Ser32/36 in IκBα, and this phosphorylation leads to polyubiquitination of IκB, its subsequent degradation by 26S proteasome and thus nuclear translocation of NF-κB (*7*). In addition, we and others have demonstrated that IKK-α and IKK-β could also phosphorylate NF-κB p65 subunit at Ser536, which is crucial for the NF-κB-mediated transactivation of target genes (*8*, *9*).

A number of low-molecular weight compounds have been reported to block NF-κB activity through inhibiting various steps of NF-κB signalling. For example, dehydroxymethylepoxyquinomycin and magnolol inhibit nuclear translocation of NF-κB (*10*, *11*). A serine/threonine kinase inhibitor fasudil hydrochloride blocks HIV-1 replication from latently infected cells through its

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inhibitory effect on the signal transduction of NF-κB by presumably inhibiting IKK (*12*). Moreover, monovalent gold salt compounds, such as aurothioglucose and aurothiomalate, inhibit the DNA binding of NF-κB dimer through its redox mechanism and are thus considered to exhibit clinical benefit in the treatment of rheumatoid arthritis (*13*, *14*). In addition, inhibitors of 26S proteasome such as bortezomib were shown to inhibit IκB degradation and NF-κB activation (*3*, *15*). However, clinical applicability of these compounds is limited due to their relatively poor specificity and/or low bioavailability.

IKK is thought to be a focal target of drug development and several IKK inhibitors have been reported (*16–19*). Carbocyclic adenine nucleosides have been reported to show anti-inflammatory effects by inhibiting TNF-α production from macrophage (*20–22*). One of such compounds, 9-[(1*S*,3*R*)-*cis*-cyclopentan-3-ol]adenine (cPA) was previously reported to inhibit LPS-induced NF-κB activation (*23*) although its effect on IKK has not been explored. In this study, the effects of newly synthesized 15 carbocyclic adenine nucleosides including cPA have been examined on NF-κB-dependent gene expression and IKK. We demonstrate that noraristeromycin (NAM), among these compounds, exhibited the most potent inhibitory effect on the NF-κB activity by blocking the IKK activity at non-cytotoxic concentrations. Furthermore, we also demonstrate a strong suppressive effect of NAM on HIV-1 viral replication from the latently infected OM10.1 and the chronically infected Molt4/IIIB cells.

## MATERIALS AND METHODS

*Synthetic Carbocyclic Adenine Nucleosides (Fig. 1)*—cPA (compound **1**) (*22*), NAM (compound **2**) (*24*) and carbocyclic adenine nucleosides such as compounds **3** (*25*), **4**–**7** (*24*), **8** (*22*), **9** (*26*) and **15** (*27*) were synthesized as previously described. Compound **10** was easily prepared by osmium-oxidation of 9-[(1′*R*, 4′*R*)-4′-hydroxy-2′-cyclopenten-1′-yl]-9-*H*-2-fluoroadenine (*25*), which was obtained by the Mitunobu reaction of 2-fluoroadenine with ((1*S*, 4*R*)-*cis*-4-acetoxy-2-cyclopenten-1-ol. On the other hand, compound **11** was obtained by osmium oxidation of 9-[(1′*R*, 4′*R*)-4′-hydroxy-2′-cyclopenten-1′-yl]-9-*H*-2,6-diaminopurine, which was prepared by the palladium-coupling reaction of 2-amino-6-chloropurine with (1*S*, 4*R*)-*cis*-4-acetoxy-2-cyclopenten-1-ol and subsequent ammonolysis. Analogous palladium-coupling reaction of 8-azaadenine with (1*S*, 4*R*)-*cis*-4-acetoxy-2-cyclopenten-1-ol and subsequent osmium oxidation gave compound **12**. Ammonolysis of 9-[(1′*R*, 4′*S*)-4′-hydroxy-2′-cyclopenten-1′-yl]-9-*H*-$N^6$-benzoyladenine (*24*, *27*) and 9-[(1′*R*, 4′*R*)-4′-acetoxy-2′-cyclopenten-1′-yl]-9-*H*-adenine (*27*) afforded the corresponding compounds **13** and **14**, respectively.

*Other Reagents and Plasmids*—Recombinant human TNF-α, forskolin and 3′-azidodeoxythimidine were purchased from Roche (Penzberg, Germany), Wako (Tokyo, Japan) and Sigma (St.Louis, MO, USA), respectively. Antibodies against p65 (C20), IκBα (C21), IKK-α (H744) and α-tubulin (H300) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against the phosphorylated form of p65 at Ser536 and the phosphorylated form of IκBα at Ser32 and Ser36 were obtained from cell signalling. Horseradish peroxidase-conjugated secondary antibodies against mouse IgG and rabbit IgG were purchased from Amersham Biosciences (Buckinhamshire, UK). Anti-Flag M2 affinity gel was purchased from Sigma.

The reporter plasmid expressing firefly luciferase under the control of NF-κB (pGL3-κB luc) was described previously (*8*). pCRE-luc, expressing firefly luciferase under the control of cAMP-responsive element binding protein (CREB), and pRL-TK were purchased from Stratagene (La Jolla, CA, USA) and Promega (Madison, WI, USA), respectively. Flag-IKK-α and Flag-IKK-β expression vectors (*9*) were kindly provided from Dr H. Nakano. pGST-IκBα(*1-54*) (*9*) was a gift from Dr H. Sakurai.

*Cell Culture and Transfection*—HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% $CO_2$ humidified air. Cells were transfected using Fugene-6 transfection reagent (Roche) according to the manufacture's instructions as described previously (*8*). OM10.1, latently infected macrophage/monocyto cell line with HIV-1, and Molt4/IIIB cells, chronically infected with HIV-1, were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% $CO_2$ humidified air. To maintain the latency of the HIV-1 in OM10.1, 20μM 3′-azido-3′-deoxythymidine (AZT) was added in the culture medium and was excluded prior to experiments (*28*).

*Luciferase Assay*—Transient luciferase assays were performed as described previously (*8*). Briefly, HEK293 cells were transfected with a total of 0.5 μg of plasmid DNA using Fugene-6 transfection reagent (Roche). Blank control plasmid pUC19 was used to equalize the amount of DNA for each transfection. After 24 h of transfection, cells were pre-treated with compounds of indicated concentrations for 30 min, and stimulated with or without TNF-α for additional 24 h. To stimulate protein kinase A and eventually activate the transcriptional activity of CREB, pCRE-luc plasmid was transfected and treated with forskolin for 24 h. The cells were then harvested with 500 μl of lysis buffer (Promega) and the same amount of lysates (∼3 μg) was prepared for the luciferase assay. Luciferase activity was measured by the Luciferase Assay System (Promega) as previously described (*8*). Transfection efficiency was monitored by *Renilla* luciferase activity with pRL-TK plasmid (Promega) containing TK promoter as an internal control. The data are presented as the fold increase in luciferase activities (means ± SD) relative to control of three independent transfections.

*Measurement of Cell Viability*—In order to assess the cytotoxicity of NAM and its derivatives, WST-1 assay (Roche) was performed as described previously (*28*). HEK293 cells were seeded at a density of $1 \times 10^5$ cells/ml in a 96-well plate (in 100 μl/well) and were cultured for 24 h at 37°C. The cytotoxicities of NAM to OM10.1 and Molt4/IIIB cells were similarly evaluated. NAM was added with indicated concentrations and cells were treated for additional 24 h. After 48 h incubation, 10 μl of WST-1 reagent were added, the cells were further incubated for 4 h and absorbance at 450 nm was

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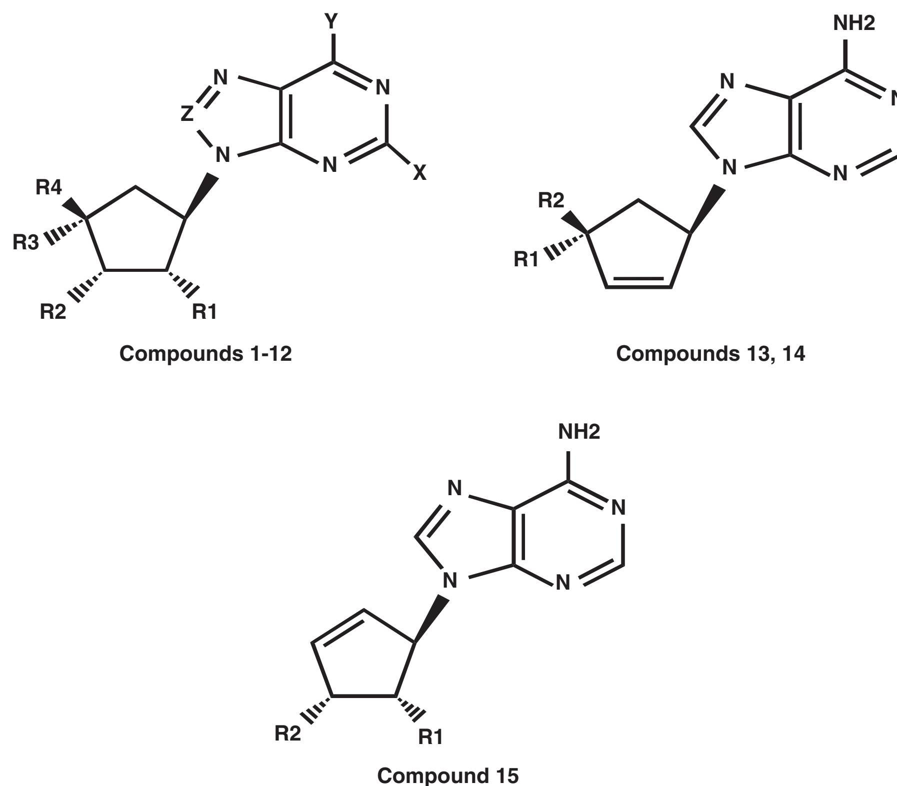


Fig. 1. **Structural formulae of synthetic carbocyclic adenine nucleosides.**

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measured with the aid of multi-plate reader using plain medium as blank.

*Immunoblot Analysis*—Immunoblot analysis was performed as described previously (*8*, *18*). Briefly, HEK293 cells were pre-treated with indicated concentrations of NAM for 24 h, and then stimulated with 1 ng/ml TNF-α for indicated time. After washing with ice cold PBS, cells were harvested with ice cold lysis buffer (25 mM HEPES–KOH, pH 7.5, 200 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Nonidet P-40, 1 μM dithiothreitol, 20 mM β-glycerophosphate, 0.1 mM $Na_3VO_4$ and 20 mM NaF) supplemented with protease inhibitor cocktail (Roche). Equal amounts of samples (30 μg) were resolved by 10% SDS–PAGE and transferred on PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA). The membranes were probed with the indicated primary antibodies, and horseradish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL, USA).

In Vitro *Kinase Assay*—The enzymatic activity of the endogenous IKK complex was assessed as following ('endogenous' assay): approximately $6 \times 10^5$ HEK293 cells were either stimulated or unstimulated with 20 ng/ml TNF-α, washed with PBS after 15 min, cells were harvested for preparation of cell lysate and the IKK complex was immunoprecipitated with anti-IKK-α antibody. The cell lysates from both TNF-α-treated or non-treated control were prepared and the IKK activity was measured with one-tenth of either cell lysate *in vitro* with the IKK substrate GST-IκBα(1-54) (*9*, *29*). In 'exogenous' assay, the enzymatic activities of IKK-α and IKK-β were measured according to Fiorentino *et al.* (*29*). Briefly, HEK293 cells ($6 \times 10^5$) were transfected with 2 μg of Flag-IKK-α or Flag-IKK-β expression vectors by using Fugene-6 transfection reagent (Roche) according to the manufacture's instructions. At 24 h after transfection, cells were left untreated or treated with 20 ng/ml TNF-α for 15 min to stimulate the IKK activity. The transfected cells were harvested and resuspended in kinase lysis buffer [50 mM Tris–KOH (pH 7.5), 200 mM NaCl, 2 mM EDTA, 1% Brij97, 10% glycerol, 0.5% TritonX-100] supplemented with a protease inhibitor cocktail (Roche), 2 mM phenylmethanesulphonyl fluoride-α-toluenesulphonyl fluoride, 50 μM dithiothreitol and 1 mM $Na_3VO_4$. These cell lysates were immunoprecipitated either with anti-Flag or anti-IKK-α antibody for 3 h at 4°C. The immunoprecipitates were washed three times with kinase lysis buffer and then washed once with kinase reaction buffer [20 mM HEPES–KOH (pH 7.5), 5 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 0.1 mM $Na_3VO_4$, 20 mM NaF and 10 mM β-glycerophosphate). The IKK substrate GST-IκBα(*1-54*) protein was purified from *Escherichia coli* expressing this protein according to the method previously described (*9*). Kinase assays were performed in 30 μl of kinase reaction buffer containing 5 μM ATP, 5 μCi of [γ-$^{32}$P]ATP and 1.0 μg of GST-IκBα(*1-54*) in the presence or absence of various concentrations of NAM. After incubation at 30°C for 20 min, reactions were terminated by adding 6 μl of the SDS sample buffer and boiling for 5 min. Equal amounts of samples were resolved by 10% SDS–PAGE and gels

Table 1. **Effects of carbocyclic nucleoside compounds on NF-κB dependent transcription.**

| Compound No. | X | Y | Z | $R_1$ | $R_2$ | $R_3$ | $R_4$ | (%) inhibition | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Control | TNF-α |
| 1 (cPA) | H | $NH_2$ | C | H | H | H | OH | $-7\pm12$ | $30\pm6$ |
| 2 (NAM) | H | $NH_2$ | C | OH | OH | H | OH | $-5\pm5$ | $56\pm4$ |
| 3 | F | $NH_2$ | C | OH | OH | H | OH | $19\pm8$ | $56\pm3$ |
| 4 | Br | $NH_2$ | C | OH | OH | H | OH | $31\pm2$ | $60\pm0$ |
| 5 | $NH_2$ | $NH_2$ | C | OH | OH | H | OH | $-1\pm6$ | $39\pm3$ |
| 6 | $CH_3$ | $NH_2$ | C | OH | OH | H | OH | $17\pm7$ | $37\pm5$ |
| 7 | $NH_2$ | Cl | C | OH | OH | H | OH | $-8\pm6$ | $10\pm5$ |
| 8 | H | $NH_2$ | C | H | H | OH | H | $14\pm11$ | $23\pm17$ |
| 9 | H | $NH_2$ | C | OH | OH | OH | H | $13\pm11$ | $47\pm4$ |
| 10 | F | $NH_2$ | C | OH | OH | OH | H | $-15\pm10$ | $-11\pm3$ |
| 11 | $NH_2$ | $NH_2$ | C | OH | OH | OH | H | $2\pm9$ | $15\pm4$ |
| 12 | H | $NH_2$ | N | OH | OH | OH | H | $17\pm5$ | $20\pm7$ |
| 13 | – | – | – | H | OH | – | – | $-6\pm18$ | $7\pm4$ |
| 14 | – | – | – | OH | H | – | – | $3\pm8$ | $19\pm2$ |
| 15 | – | – | – | OH | OH | – | – | $14\pm21$ | $55\pm2$ |

HEK293 cells were transfected with 4×κB-luc reporter plasmid. Twenty-four hours after transfection, cells were pre-treated with 100 μM of each synthetic carbocyclic adenine nucleoside compound for 30 min and stimulated with 1 ng/ml of TNFα. Forty-eight hours after transfection, cells were lysed and luciferase activity was determined. The data are presented as the percentage of inhibition in luciferase activities relative to control transfection. Values are the means ± SD of three independent transfections.

were vacuum dried. Radioactive signals were detected with BAS-1800 II (Fuji film, Tokyo, Japan) and quantified with Image Gauge V 4.0 (Fuji film) software.

*Measurement of Viral p24 Antigen*—The viral replication levels of HIV-1 from latently infected cells were measured as previously described (*28*). Briefly, OM10.1 cells were seeded at a density of $2\times10^5$ cells/ml in 12-well plates and maintained at 37°C in 5% $CO_2$ humidified air. Thirty minutes after treatment with NAM, cells were stimulated by 0.2 ng/ml TNF-α, incubated for 24 h and culture supernatants were collected to measure the HIV-1 p24 antigen level using a commercial kit (RETRO-TEK HIV-1 p24 Antigen ELISA kit; Zepto Metrix Corp., Buffalo, NY, USA) as previously reported (*28*).

## RESULTS

*Effects of Carbocyclic Adenine Nucleosides on NF-κB Transactivation*—Since it is known that high concentrations of cPA could inhibit NF-κB activation, we have synthesized 15 carbocyclic adenine nucleoside derivatives with modifications at the second position of adenine and the 2′, 3′ and 4′ positions of carbocyclic moiety. In order to examine the effects of these compounds on the NF-κB-mediated transcription, HEK293 cells were pre-treated with each compound at 100 μM for 30 min and stimulated with TNF-α for 24 h. The effects of these compounds were evaluated by performing transient luciferase assay with NF-κB-dependent luciferase reporter plasmid. We assessed the non-specific effects, mostly the cytotoxic effect, of each compound by measuring the effect of each compound on basal transcription without TNF-α stimulation ('Control' in Table 1). As shown in Table 1, whereas cPA at 100 μM exhibited ~30% inhibition of NF-κB-mediated transcription, greater inhibitory effects were demonstrated with eight compounds (compounds **2**–**6**, **8**, **9** and **15**). The dose-dependent inhibition of the basal as well as the TNF-α-stimulated and NF-κB-mediated transcription by compounds **1**, **8**, **9**, **13**–**15** were observed (data not shown). Among these compounds, we dropped six compounds (compounds **3**, **4**, **6**, **8**, **9** and **15**) because of their strong effects on the basal transcription level, reflecting the non-specific cytotoxicity. Two strong inhibitors compound **2** (NAM) and compound **5** (2-amino-NAM), shared structural characteristics including (*1*) OH modifications of three cyclic carbons (trihydroxylation in $R_1$, $R_2$ and $R_4$) and (*2*) H- or $NH_2$-modification of adenine nucleoside at the second position (X). However, NAM exhibited higher anti-NF-κB effect than 2-amino-NAM and similar non-specific effect (Table 1). It is also noted that halogen [F (compound **3**) or Br (compound **4**)] modification of adenine nucleoside at the second position (X) showed strong non-specific inhibitory effects as well as the inhibitory effects on the NF-κB-mediated transactivation even when cyclic carbons are equally trihydroxylated. On the other hand, methylation or amination at the second position (X) did not enhance the inhibitory effect on NF-κB-mediated transcription.

*The NF-κB-specific Inhibition by NAM*—Thus, our initial screening elucidated NAM as a potential anti-NF-κB inhibitor. Figure 2A shows the dose-dependent inhibitory effect of NAM on the NF-κB-mediated transcription. The 50% inhibition was obtained at the concentration of 2.7 μM ($IC_{50}$) with NAM; whereas, 50% cytotoxicity of NAM was 730 μM ($CC_{50}$) (Fig. 2C), thus therapeutic window of NAM for NF-κB inhibition was ~270-fold. The apparent lack of inhibitory effect of NAM on the basal transcription level (Fig. 2A, see the experiments with no TNF-α) suggested that the inhibitory effect of NAM is specific for NF-κB. In order to further examine the specificity of NAM, we tested whether it could inhibit gene expression by another transcription factor CREB, whose activity is controlled by protein kinase A pathway. As shown in Fig. 2B, NAM exhibited no significant effect on CREB-dependent transcription induced by forskolin. In addition, NAM



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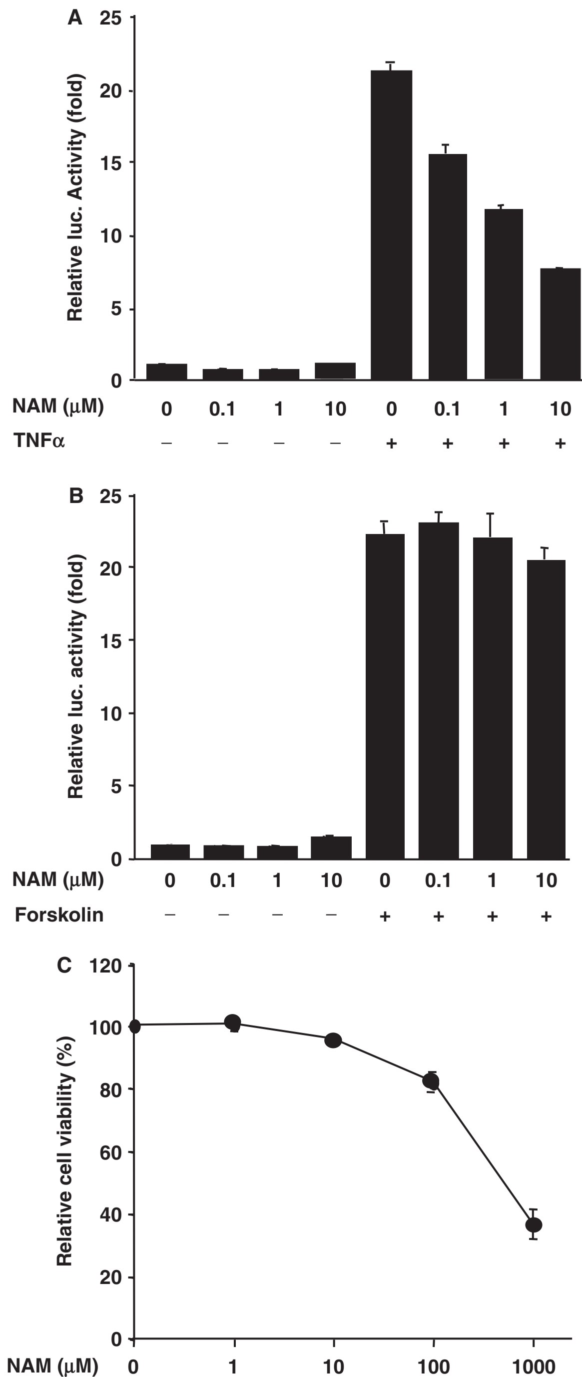


Fig. 2. **Inhibition of NF-κB-dependent transcription by NAM.** (A and B) HEK293 cells were transfected with 4 × κB-luc (A) or 4 × CRE-luc (B) reporter plasmids. Twenty-four hours after transfection, cells were pre-treated with the indicated concentrations of NAM for 30 min and stimulated with 1.0 ng/ml of TNF-α (A) or 10 μM of forskolin (B). Forty-eight hours after transfection, cells were lysed and luciferase activity was determined. The data are presented as the fold increase in luciferase activities relative to control transfection (no stimulation). Values are the means ± SD of three independent transfections. Insets show expanded ranges of NAM effects on the basal transcription from 4 × κB-luc and CRE-luc reporter plasmids. (C) The cytotoxic effects of NAM. The HEK293 cell viability was determined by WST assay. This experiment was performed in triplicates and the means ± SD are shown.

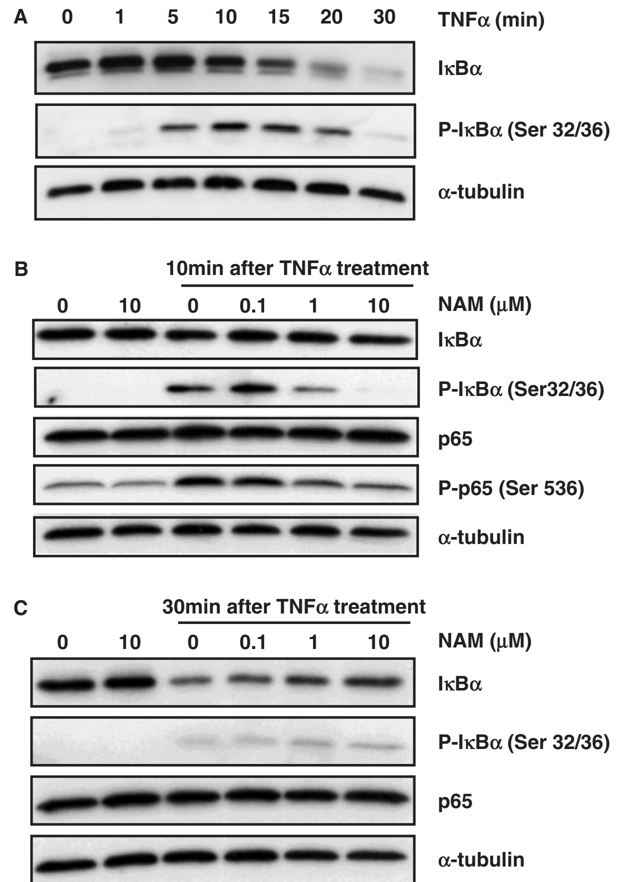


Fig. 3. **Inhibition of IκBα and p65 phosphorylation and IκBα degradation by NAM *in vivo*.** (A) Time course of IκBα phosphorylation and its degradation following TNF-αstimulation. HEK293 cells were stimulated with 1.0 ng/ml of TNF-α for indicated periods (min). It was noted that maximum IκBα phosphorylation and degradation were observed after 10 and 30 min of TNF-α stimulation, respectively. (B and C) Inhibition of IκBα phosphorylation by NAM. Cells were cultured in the presence of the indicated concentrations of NAM for 24 h, stimulated with TNF-α (1.0 ng/ml) for 10 (B) or 30 min (C), and harvested for western blot analyses. Equal amounts of lysate (30 μg) were immunoblotted with specific antibodies against IκBα, phospho-IκBα (at Ser32/36), p65 and phospho-p65 (at Ser536). Anti-α-tubulin antibody was used as an internal control.

did not inhibit TNF-α-induced AP1 transactivation (data not shown). Collectively, these results indicate that NAM specifically inhibits the signalling pathway that leads to NF-κB activation under non-cytotoxic concentrations.

*Inhibition of IκBα and p65 Phosphorylation and IκBα Degradation by NAM* In Vivo—In order to investigate the mechanism by which NAM inhibits NF-κB activation pathway, we first examined the effect of NAM on the level of phosphorylation of IκBα at Ser32/36 residues, known to be crucial in the NF-κB activation pathway (*2*). As shown in Fig. 3A, the phosphorylation of IκBα was observed as early as 1 min after TNF-α stimulation and reached a peak at 10 min of stimulation. IκBα was subsequently degraded and almost disappeared after 30 min of stimulation by TNF-α. In Fig. 3B and C, the effect of NAM on the IκBα phosphorylation was examined at 10 and

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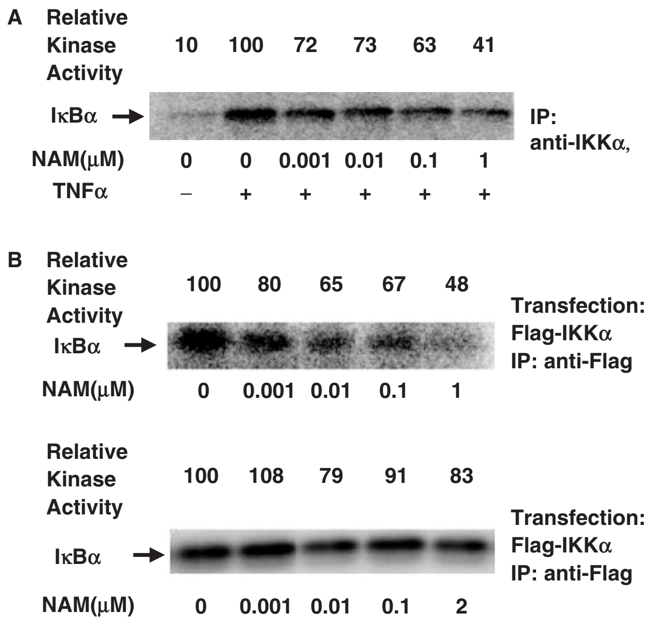


Fig. 4. **Inhibition of IKK by NAM *in vitro*.** (A) Effects of NAM on IKK complex *in vitro*. HEK293 cells were either stimulated or unstimulated with TNF-α (20 ng/ml) and the endogenous IKK complex was immunoprecipitated from the whole cell lysate by anti-IKK-α antibody. The kinase activity of the immunoprecipitated IKK complex was evaluated *in vitro* with purified recombinant GST-IκBα(1-54) protein as a substrate in the presence of various concentrations of NAM. (B) Effects of NAM on IKK-α and IKK-β *in vitro*. HEK 293 cells were transfected with expression vectors for Flag-IKK-α (upper panel) or IKK-β (lower panel). Twenty-four hours after transfection, cells were stimulated with 20 ng/ml of TNF-α for 15 min. Whole cell lysates were immunoprecipitated with anti-Flag antibody, and kinase activities of IKK-α or IKK-β were determined *in vitro* similarly as in A. *In vitro* kinase assays were performed in 30 μl of kinase reaction buffer containing 5 μM ATP, 5 μCi of [γ-$^{32}$P]ATP and 1.0 μg of GST-IκBα(1-54) for 20 min at 30°C. The relative kinase activities were quantified by densitometric measurement and are indicated below each gel band. These experiments were performed more than three times and the representative results are shown.

30 min after TNF-α stimulation. A dose-dependent inhibition of the IκBα phosphorylation by NAM is shown in Fig. 3B. In addition, Fig. 3C shows a dose-dependent suppression of IκBα degradation by NAM (at 30 min after TNF-α stimulation). We also found the inhibitory effect of NAM on the phosphorylation of NF-κB p65 subunit at Ser536 residue that is known to stimulate the transcriptional competence of NF-κB via 'non-canonical pathway' (*8*, *9*, *30*) (Fig. 3B). These results suggest that NAM suppresses the NF-κB-mediated transcription by inhibiting the phosphorylation of IκBα and p65.

*Inhibitory Effects of NAM on IKK Activity* In Vitro—Since the kinase molecules responsible for the phosphorylation of IκBα and p65 are IKK subunits, especially IKK-α and IKK-β, we performed *in vitro* kinase assay and asked which IKK subunit is responsible for the inhibitory effect of NAM. In Fig. 4A, the endogenous IKK complex was immunoprecipitated from HEK293 cells by incubation with anti-IKK-α antibody and the kinase activity was determined with GST-IκBα(*1-54*) as a substrate and the effects of NAM was examined. NAM inhibited the endogenous IKK activity following the TNF-α stimulation in a dose-dependent manner. The 50% inhibition thus obtained *in vitro* was ~0.64 μM. We then examined the effect of NAM on the kinase activities of IKK-α and IKK-β. In Fig. 4B, HEK293 cells were transfected with the expression vectors for Flag-IKK-α or Flag-IKK-β and stimulated with TNF-α to stimulate the upstream kinase cascade. The cell lysates were immunoprecipitated with anti-Flag antibody. Each one-tenth of the immunoprecipetate, containing the TNF-α-activated IKK, was dissolved in the kinase buffer, various amounts of NAM were added together with the same amount of GST-IκBα substrate. The effects of NAM on the kinase activities of immunoprecipitated IKK-α and IKK-β were then determined. As shown in Fig. 4B, NAM strongly inhibited the kinase activity of IKK-α but not IKK-β. The $IC_{50}$ value of NAM for IKK-α was 0.37 μM and was similar to that with the endogenous IKK complex (Fig. 4A). On the other hand, the effect of NAM on the IKK-β activity was very weak and NAM inhibited not >70% even at 100 μM.

*Suppression of HIV-1 Replication from the Latently Infected Cells by NAM*—Since the HIV-1 replication from the latently infected cells highly depends on NF-κB activity (*5*), we examined the effect of NAM on the NF-κB-dependent HIV-1 replication from one such cell line, OM10.1 (*28*). In Fig. 5A, OM10.1 cells were pre-incubated with various concentrations of NAM for 30 min, HIV-1 replication was induced by TNF-α, and the amounts of viral production were determined. The inhibitory effect of NAM was thus evaluated by measuring the viral p24 antigen levels in the cell culture supernatants of OM10.1 cells. As shown in Fig. 5A, NAM strongly suppressed the expression level of viral p24 protein induced by TNF-α in a dose dependent manner; whereas, no significant effect was observed on the basal level of HIV-1 production (without TNF-α stimulation). Similarly, the effect of NAM on chronic HIV production was examined in Fig. 5B. When Molt4/IIIB cells, chronically infected with HIV-1 and continuously producing the virions, were stimulated with TNF-α, the level of viral production was significantly augmented as previously reported (*28*). When Molt4/IIIB cells were pretreated with NAM at various concentrations, a significant reduction of HIV production was observed. Under these concentrations of NAM, no significant cytotoxicity was observed (Fig. 5A and B, right panels). Thus, NAM was effective in repressing the HIV-1 viral replication in chronically infected cells.

## DISCUSSION

In this study, we demonstrated that NAM showed the most potent inhibitory effect on the NF-κB-mediated transactivation under non-cytotoxic concentrations among newly synthesized 15 carbocyclic adenine nucleosides. Moreover, NAM strongly suppressed the HIV-1 replication in latently infected cells, implicating its efficacy during the maintenance therapy of HIV-1 infection (*4*, *5*). It is likely that this anti-HIV activity of NAM is mediated by inhibiting the NF-κB activation cascade. We found that NAM inhibited the TNF-α-induced phosphorylation and

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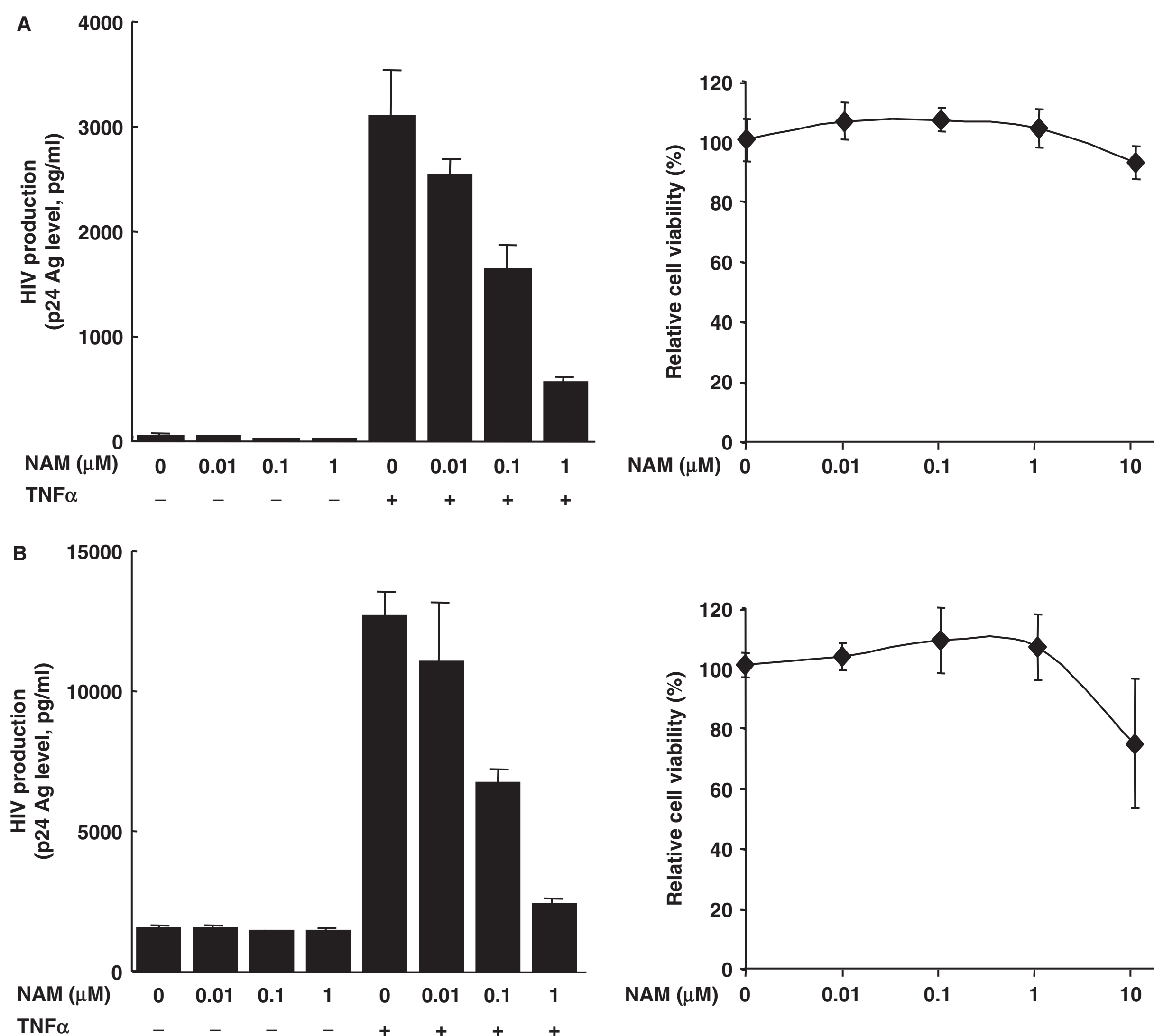


Fig. 5. **The effect of NAM on HIV-1 replication from chronically infected cells.** (A) Effects of NAM on the viral production from OM10.1, latently infected with HIV-1. (B) Effects of NAM on the viral production of Molt4/IIIB cells, chronically infected with HIV-1. Cells were seeded at a density of $5 \times 10^5$ cells/ml in 12-well plates, pre-treated with the indicated concentrations of NAM for 1 h, and were then stimulated with or without TNF-α (0.2 ng/ml). After further 24 h incubation, culture supernatants were collected and HIV-1 p24 antigen levels were measured using a commercial ELISA kit. The data are presented as the fold increase in p24 antigen levels relative to the untreated control. The cell viability in the presence of various concentrations of NAM was determined by WST-1 assay. These experiments were performed in triplicates and the means ± SD are shown.

subsequent degradation of IκBα. In addition, NAM also inhibited the phosphorylation of p65 at Ser536 that is a hallmark of IKK-α action via 'non-canonical pathway' (*8*, *30*). We further explored the NAM action and found that the inhibitory effects of NAM are through inhibition of the kinase activity of IKK. The *in vitro* kinase assay revealed that whereas IKK-β was not responsive to the NAM-mediated inhibition, NAM inhibited the kinase activity of IKK-α subunit at a concentration similar to its effect for the endogenous IKK complex. These findings suggest that NAM is a novel inhibitor of IKK-α and is a potential drug for the treatment of AIDS and other diseases in which NF-κB plays a major role (*3*, *31*).

IKK inhibitors previously reported are classified into two categories with their mechanisms of action; (i) ATP analogues and (ii) allosteric inhibitors. For example, 2-amino-3-cyano-4-alkyl-6-(2-hydroxyphenyl)pyridine (ACHP) and its derivatives act as ATP analogues and inhibit IKK (*17*, *18*). We have previously demonstrated that ACHP shows a strong inhibitory effect on the constitutive NF-κB activation in multiple myeloma (*18*) and adult T-cell leukaemia (*19*) cells and effectively induces apoptotic cell death. Similarly, ACHP was effective in suppression of the cytokine-mediated HIV-1 replication from the latently infected cells, which is dependent on NF-κB (*28*). It was demonstrated that ACHP shows greater inhibition on IKK-β than IKK-α (*17*). However, no compound except NAM has been demonstrated to specifically inhibit IKK-α. NAM appears to inhibit IKK activity as an ATP analogue because of its structural similarity although detailed studies are needed. Other ATP analogue IKK inhibitors such as AS602868 (*32*) and



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herbimycin A (*33*) were reported to be more specific for IKK-β than IKK-α. Whereas, these IKK inhibitors are competitive with ATP, other agents including BMS-345541 (*34*) and aspirin (*35*) are known to inhibit IKK-β through allosteric effects.

As far as we examined, the inhibitory action of carbonic adenine nucleosides on NF-κB correlated with the trihydroxylation of the carbonic ring and hydrogenation or amination of adenine nucleoside at position 2 ('X' in Fig. 1). Increase in non-specific and cytotoxic effects were observed upon halogenation of the position 2, suggesting that a strong negative charge in this position may induce interaction with many other kinases.

NAM showed a preferred inhibitory action on IKK-α activity over IKK-β. Cumulative evidences suggest that IKK-α has distinct and independent roles from IKK-β (*30*, *31*). Whereas, IKK-β gene knock-out induced fetal cell death because of massive apoptosis in liver tissues (*36*), IKK-α gene is dispensable for normal embryonic development although IKK-α-null mice have impaired secondary lymphoid tissues such as lymph nodes and Payer's patch in the intestine (*37*, *38*). Recent studies have revealed the involvement of IKK-α in the 'non-canonical' NF-κB activation pathway that lead to p65 and p100 phosphorylation (*8*, *30*). Whereas, p100 phosphorylation leads to its proteolytic processing to p52 NF-κB subunit, p65 phosphorylation is considered crucial for the transcriptional competence of NF-κB-mediated transactivation (*8*, *17*). IKK-α is also known to be involved in the phosphorylation of histone H3 that is involved in the chromatin remodelling by converting the repressive histone to transcriptionally active status (*39*, *40*). In this pathway, IKK-α is solely involved in the extracellular signalling elicited by lymphotoxin β, Blys/BAFF, RANK and CD40, independently of IKK-β (*41–44*). Moreover, constitutive NF-κB activation in some tumour cells, such as adult T-cell leukemia and multiple myeloma, appears to depend on the non-canonical pathway (*18*, *45*).

Thus, specific inhibition of IKK-α would lead to selective suppression of chronic immuno-inflammatory stimulation and carcinogenesis, in addition to the inhibition of HIV replication. These findings should foster the synthesis of improved analogues ameable to pharmacological development of effective agents for the treatment of pathological conditions where IKK-α plays a major role.

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## CONFLICT OF INTEREST

None declared.

## REFERENCES


1. Baeuerle, P.A. and Baltimore, D. (1996) NF-κB: ten years after. *Cell* **87**, 13–20
2. Ghosh, S. and Karin, M. (2002) Missing pieces in the NF-κB puzzle. *Cell* **109**(Suppl), S81–S96
3. Okamoto, T., Sanda, T., and Asamitsu, K. (2007) NF-κB signaling and carcinogenesis. *Curr. Pharm. Des.* **13**, 447–462
4. Okamoto, T. (1995) *Anti-AIDS Drug Development: Challenge Strategies and Prospects Regulatory Proteins of Human Immunodeficiency Virus and Therapy*, pp. 117–127, Harwood Publishers Inc., Chur, Switzerland
5. Hiscott, J., Kwon, H., and Genin, P. (2001) Hostile takeovers: viral appropriation of the NF-κB pathway. *J. Clin. Invest.* **107**, 143–151
6. Williams, S.A., Chen, L.F., Kwon, H., Fenard, D., Bisgrove, D., Verdin, E., and Greene, W.C. (2004) Prostratin antagonizes HIV latency by activating NF-κB. *J. Biol. Chem.* **279**, 42008–42017
7. Karin, M. and Ben-Neriah, Y. (2000) Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annu. Rev. Immunol.* **18**, 621–663
8. Jiang, X., Takahashi, N., Matsui, N., Tetsuka, T., and Okamoto, T. The NF-κB activation in lymphotoxinβ receptor signaling depends on the phosphorylation of p65 at serine 536. *J. Biol. Chem*. 278, 919–926.
9. Sakurai, H., Suzuki, S., Kawasaki, N., Nakano, H., Okazaki, T., Chino, A., Doi, T., and Saiki, I. (2003) Tumor necrosis factor-α-induced IKK phosphorylation of NF-κB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *J. Biol. Chem.* **278**, 36916–36923
10. Ariga, A., Namekawa, J., Matsumoto, N., Inoue, J., and Umezawa, K. (2002) Inhibition of tumor necrosis factor-α-induced nuclear translocation and activation of NF-κB by dehydroxymethylepoxyquinomicin. *J. Biol. Chem.* **277**, 24625–24630
11. Tanaka, K., Hasegawa, J., Asamitsu, K., and Okamoto, T. (2007) Magnolia ovovata extract and its active component magnolol prevent skin photoaging via inhibition of nuclear factor kappa B. *Eur. J. Pharmacol.* **22**, 212–219
12. Sato, T., Asamitsu, K., Yang, J.P., Takahashi, N., Tetsuka, T., Yoneyama, A., Kanagawa, A., and Okamoto, T. (1998) Inhibition of human immunodeficiency virus type 1 replication by a bioavailable serine/threonine kinase inhibitor, fasudil hydrochloride. *AIDS Res. Hum. Retroviruses* 14, 293–298.
13. Yang, J.P., Merin, J.P., Nakano, T., Kato, T., Kitade, Y., and Okamoto, T. (1995) Inhibition of the DNA-binding activity of NF-κB by gold compounds in vitro. *FEBS Lett.* **361**, 89–96
14. Yoshida, S., Kato, T., Sakurada, S., Kurono, C., Yang, J.P., Matsui, N., Soji, T., and Okamoto, T. (1999) Inhibition of IL-6 and IL-8 induction from cultured rheumatoid synovial fibroblasts by treatment with aurothioglucose. *Int. Immunol.* **11**, 151–158
15. Lenz, H.J. (2003) Clinical update: proteasome inhibitors in solid tumors. *Cancer Treat Rev.* **29**(Suppl 1), 41–48
16. Kishore, N., Sommers, C., Mathialagan, S., Guzova, J., Yao, M., Hauser, S., Huynh, K., Bonar, S., Mielke, C., Albee, L., Weier, R., Graneto, M., Hanau, C., Perry, T., and Tripp, C.S. (2003) A selective IKK-2 inhibitor blocks NF-κB-dependent gene expression in interleukin-1 beta-stimulated synovial fibroblasts. *J. Biol. Chem.* **278**, 32861–32871
17. Murata, T., Shimada, M., Sakakibara, S., Yoshino, T., Kadono, H., Masuda, T., Shimazaki, M., Shintani, T., Fuchikami, K., Sakai, K., Inbe, H., Takeshita, K., Niki, T., Umeda, M., Bacon, K.B., Ziegelbauer, K.B., and Lowinger, T.B. (2003) Discovery of novel and selective


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IKK-β serine-threonine protein kinase inhibitors. *Part 1. Bioorg. Med. Chem. Lett.* **13**, 913–918
18. Sanda, T., Iida, S., Ogura, H., Asamitsu, K., Murata, T., Bacon, K.B., Ueda, R., and Okamoto, T. (2005) Growth inhibition of multiple myeloma cells by a novel IκB kinase inhibitor. *Clin. Cancer Res.* **11**, 1974–1982
19. Sanda, T., Asamitsu, K., Ogura, H., Iida, S., Utsunomiya, A., Ueda, R., and Okamoto, T. (2006) Induction of cell death in adult T-cell leukemia cells by a novel IκB kinase inhibitor. *Leukemia* **20**, 590–598
20. Parmely, M.J., Zhou, W.W., Edwards, C.K. III, Borcherding, D.R., Silverstein, R., and Morrison, D.C. (1993) Adenosine and a related carbocyclic nucleoside analogue selectively inhibit tumor necrosis factor-alpha production and protect mice against endotoxin challenge. *J. Immunol.* **151**, 389–396
21. Bradshaw, M., Rutherford, M.S., Hoeper, B.J., McWhinney, C.D., Borcherding, D.R., Schook, L.B., and Edwards, C.K. III (1995) Specific transcriptional inhibition of bone marrow-derived macrophage tumor necrosis factor-alpha gene expression and protein production using novel enantiomeric carbocyclic nucleoside analogues. *J. Pharmacol. Exp. Ther.* **273**, 1506–1518
22. Borcherding, D.R., Peet, N.P., Munson, H.R., Zhang, H., Hoffman, P.F., Bowlin, T.L., and Edwards, C.K. III (1996) Carbocyclic nucleosides as inhibitors of human tumor necrosis factor-α production: effects of the stereoisomers of (3-hydroxycyclopentyl) adenines. *J. Med. Chem.* **39**, 2615–2620
23. Xia, D., Wang, F., and Parmely, M.J. (2000) Inhibition of nuclear factor-κb activation in mouse macrophages and the RAW 264.7 cell line by a synthetic adenyl carbocyclic nucleoside. *Biochem. Pharmaco.* **60**, 717–727
24. Kitade, Y., Kozaki, A., and Miwa, K. (2002) Synthesis of base-modified noraristeromycin derivatives and their inhibitory activity against human and Plasmodium falciparum recombinant S-adenosyl-L-homocysteine hydrolase. *Tetrahedron* **58**, 1271–1277
25. Kitade, Y., Kojima, H., Zulfiqar, F., Kim, H.-S., and Watanabe, Y. (2003) Synthesis of 2-fluoronoraristeromycin and its Inhibitory activity against Plasmodium falciparum S-adenosyl-L-homocysteine hydrolase. *Bioorg. Med. Chem. Lett.* **13**, 3963–3965
26. Suhaib, M., Siddiqi, S.M., Oertel, F.P., Chen, X., and Schneller, S.W. (1993) Enantiospecific synthesis of the fluoro and epimeric derivatives of 5′-Noraristeromycin. *J. Chem. Soc. Chem. Commun.* **8**, 707–708
27. Kitade, Y., Kozaki, A., and Yatome, C. (2001) Facile synthesis of 9-[(1′R, 2′S)-2′-hydroxy-3′-oxocyclopenten-1′-yl]-9-H-adenine possessing inhibitory activity against human recombinant S-adenosyl-L-homocysteine hydrolase. *Tetrahedron Lett.* **42**, 433–435
28. Victoriano, A.F.B., Asamitsu, K., Hibi, Y., Imai, K., Barzaga, N.G., and Okamoto, T. (2006) Inhibition of human immunodeficiency virus type 1 (HIV-1) replication in latently infected cells by a novel IKK inhibitor. (2006) *Antimicrob. Agents Chemother.* **50**, 547–555
29. Fiorentino, L., Stehlik, C., Oliveira, V., Ariza, M.E., Godzik, A., and Reed, J.C. (2002) A novel PAAD-containing protein that modulates NF-κB induction by cytokines tumor necrosis factor-alpha and interleukin-1beta. *J. Biol. Chem.* **277**, 35333–35340
30. Pomerantz, J.L. and Baltimore, D. (2002) Two pathways to NF-κB. *Mol. Cell* **10**, 693–695
31. Okamoto, T. (2006) NF-κB and rheumatic diseases. *Endocr. Metab. Immune Disord. Drug Targets* **6**, 359–372
32. Frelin, C., Imbert, V., Griessinger, E., Loubat, A., Dreano, M., and Peyron, J.F. (2003) AS602868, a pharmacological inhibitor of IKK2, reveals the apoptotic potential of TNF-α in Jurkat leukemic cells. *Oncogene* **22**, 8187–8194
33. Ogino, S., Tsuruma, K., Uehara, T., and Nomura, Y. (2004) Herbimycin A abrogates nuclear factor-κB activation by interacting preferentially with the IκB kinase beta subunit. *Mol. Pharmacol.* **65**, 1344–1351
34. Burke, J.R., Pattoli, M.A., Gregor, K.R., Brassil, P.J., MacMaster, J.F., McIntyre, K.W., Yang, X., Iotzova, V.S., Clarke, W., Strnad, J., Qiu, Y., and Zusi, F.C. (2003) BMS-345541 is a highly selective inhibitor of IκB kinase that binds at an allosteric site of the enzyme and blocks NF-κB-dependent transcription in mice. *J. Biol. Chem.* **278**, 1450–1456
35. Yin, M.J., Yamamoto, Y., and Gaynor, R.B. (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature* **396**, 77–80
36. Tanaka, M., Fuentes, M.E., Yamaguchi, K., Durnin, M.H., Dalrymple, S.A., Hardy, K.L., and Goeddel, D.V. (1999) Embryonic lethality, liver degeneration, and impaired NF-κB activation in IKKβ-deficient mice. *Immunity* **10**, 421–429
37. Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKKα subunit of IκB kinase. *Science* **284**, 316–320
38. Takeda, K., Takeuchi, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., Sanjo, H., Yoshikawa, K., Terada, N., and Akira, S. (1999) Limb and skin abnormalities in mice lacking IKKα. *Science* **284**, 313–316
39. Anest, V., Hanson, J.L., Cogswell, P.C., Steinbrecher, K.A., Strahl, B.D., and Baldwin, A.S. (2003) A nucleosomal function for IκB kinase-α in NF-κB-dependent gene expression. *Nature* **423**, 659–663
40. Yamamoto, Y., Verma, U.N., Prajapati, S., Kwak, Y.T., and Gaynor, R.B. (2003) Histone H3 phosphorylation by IKK-α is critical for cytokine-induced gene expression. *Nature* **423**, 655–659
41. Cao, Y., Bonizzi, G., Seagroves, T.N., Greten, F.R., Johnson, R., Schmidt, E.V., and Karin, M. (2001) IKKα provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. *Cell* **107**, 763–775
42. Claudio, E., Brown, K., Park, S., Wang, H., and Siebenlist, U. (2002) BAFF-induced NEMO-independent processing of NF-κB2 in maturing B cells. *Nat. Immunol.* **3**, 958–965
43. Coope, H.J., Atkinson, P.G., Huhse, B., Belich, M., Janzen, J., Holman, M.J., Klaus, G.G., Johnston, L.H., and Ley, S.C. (2002) CD40 regulates the processing of NF-κB2 p100 to p52. *EMBO J.* **21**, 5375–5385
44. Dejardin, E., Droin, N.M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z.W., Karin, M., Ware, C.F., and Green, D.R. (2002) The lymphotoxin-β receptor induces different patterns of gene expression via two NF-κB pathways. *Immunity* **17**, 525–535
45. Hironaka, N., Mochida, K., Mori, N., Maeda, M., Yamamoto, N., and Yamaoka, S. (2004) Tax-independent constitutive IκB kinase activation in adult T-cell leukemia cells. *Neoplasia* **6**, 266–278

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