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Abstract

Adult T-cell leukemia (ATL) is caused by human T-cell leukemia virus type I (HTLV-I) and remains incurable. NIK-333, a novel synthetic retinoid, prevents the recurrence of human hepatoma after surgical resection of primary tumors. We explored the effects of NIK-333 on HTLV-I-infected T-cell lines and ATL cells. NIK-333 inhibited cell proliferation, induced G1 arrest, and resulted in massive apoptosis in all tested HTLV-I-infected T-cell lines and ATL cells, whereas little effect was observed on normal peripheral blood mononuclear cells. NIK-333 treatment decreases the levels of cyclin D1, cyclin D2, cIAP2, and XIAP proteins. Further analysis showed that NIK-333 inactivated nuclear factor-κB in HTLV-I-infected T-cell lines. In animal studies, treatment with NIK-333 (100 mg/kg given orally every other day) produced partial inhibition of growth of tumors of a HTLV-I-infected T-cell line transplanted s.c. in severe combined immunodeficient mice. Our results indicate that NIK-333 is a potentially useful therapeutic agent for patients with ATL. [Mol Cancer Ther 2006;5(3):704–12]

Introduction

Human T-cell leukemia virus type I (HTLV-I) is an etiologic agent for adult T-cell leukemia (ATL), a unique malignancy of mature CD4+ T cells, and other chronic inflammatory diseases, such as tropical spastic paraparesis/HTLV-I-associated myelopathy and HTLV-I-associated uveitis (1–6). ATL is subclassified into four subtypes: acute, lymphoma, chronic, and smoldering. In the relatively indolent smoldering and chronic types, the median survival time is ≥2 years. However, at present, there is no accepted curative therapy for ATL and the condition often progresses to death with a median survival time of 13 months in aggressive ATL (7). The prognosis of aggressive ATL remains poor, and death is usually due to severe infection or hypercalcemia, often associated with resistance to intensive, combined chemotherapy. Therefore, the establishment of new therapeutic strategies for ATL is very important.

ATL develops after a very long latency period and is preceded by oligoclonal expansion of HTLV-I-infected activated T cells (8). Such clonal expansion results from the expression of the viral transcriptional transactivator protein Tax, which activates various cellular genes and creates an autocrine loop involving interleukin (IL)-2 and its cognate receptor (9). Tax alters many transcriptional pathways; it activates cyclic AMP–responsive element–binding protein/activating transcription factor, activator protein-1 (AP-1), and nuclear factor-κB (NF-κB), represses p53, and interferes with several cell cycle regulators, including cyclins and cyclin-dependent kinase inhibitors (10). Thus, Tax is considered to play a crucial role in several pathways on the transformation of T cells by HTLV-I. However, treatment of transformed fibroblast cell lines derived from Tax transgenic mice with Tax antisense oligonucleotides caused a 90% reduction in Tax expression but had no effect on the cell growth rate or their ability to form tumors in vivo (11), suggesting that Tax expression is not required to maintain the transformed phenotype. Although fresh ATL cells display exactly the same biochemical phenotype as Tax-expressing cells, viral proteins, including Tax, are undetectable in circulating ATL cells by several mechanisms (12). Therefore, Tax may not be a good therapeutic target for ATL.

The role of various transcription factors in tumorigenesis has been described (13). NF-κB and AP-1 have recently
been implicated in cell survival and proliferation pathways. The NF-κB pathway is activated in ATL cells that do not express Tax, although the mechanism of activation remains unknown (14). One of the potential mechanisms by which ATL cells could develop resistance to apoptosis is through the activation of NF-κB (15). From this point of view, NF-κB has become an attractive target for therapeutic intervention. Indeed, inhibition of the NF-κB pathway by Bay 11-7082, an irreversible inhibitor of IκB phosphorylation (16, 17), by dehydroxymethylpoxyquinomicin, an inhibitor of nuclear translocation of p65, a component of NF-κB (18–20), and by bortezomib, a proteasome inhibitor (21), induced apoptosis of HTLV-I-infected T-cell lines and ATL cells, suggesting that inhibitors of NF-κB are effective against ATL cells in vivo.

Retinoids are regulators of cellular proliferation and differentiation (22). Natural retinoids, such as all-trans retinoic acid (ATRA), are currently used as chemopreventive and therapeutic agents in human cancers, particularly acute promyelocytic leukemia (23–25). The clinical usefulness of ATRA in indolent ATL was also reported (26). Unfortunately, the use of natural retinoids is limited due to their side effects and acquired in vitro and in vivo resistance after prolonged exposure (27). Therefore, synthetic retinoid analogues that couple increased specificity and reduced toxicity have been developed. The novel synthetic retinoid, NIK-333, has been shown to prevent the recurrence of hepatoma after surgical resection of primary tumors (28, 29). In these clinical studies, NIK-333 did not cause the typical toxic effects seen with conventional retinoids (28, 29). This unique agent was also reported to reduce the incidence of carcinogen-induced skin tumors and spontaneous hepatomas in mice (30). NIK-333 was also shown to induce apoptosis of human hepatoma cell lines (31, 32).

To our knowledge, no study has tested the antileukemic effects of NIK-333. The main theme of the present study was to test whether NIK-333 is a pharmacologically safe and effective inhibitor of cell growth of HTLV-I-infected T cells. The results showed that NIK-333 is a potent and selective inhibitor of cell growth of HTLV-I-infected T-cell lines and primary ATL cells. This effect on cell growth was mediated through NF-κB inhibition and associated with down-regulation of expression of gene products related to NF-κB, which induced G1 cell cycle arrest and apoptosis of HTLV-I-infected T-cell lines. Moreover, the results also showed the inhibitory effects of NIK-333 on the growth of tumors of HTLV-I-infected T-cell line transplanted s.c. in severe combined immunodeficient (SCID) mice in vivo. These studies provide the rationale for use of NIK-333 as a novel therapeutic agent in ATL.

**Materials and Methods**

**Chemicals**

The acyclic retinoid NIK-333 [(2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid, C20H30O2, molecular weight 302.46] was synthesized by Nikken Chemicals Co. (Saitama, Japan).

**Cell Lines**

The HTLV-I-uninfected human T-cell leukemia cell line (Jurkat) and HTLV-I-infected T-cell lines [MT-2 (33), MT-4 (34), C5/MJ (35), SLB-1 (36), HUT-102 (1), MT-1 (37), TL-OmL (38), and ED-40515(−) (39); C5/MJ, HUT-102, and MT-1 were generous gifts from the Fujisaki Cell Center Hayashibara Biomedical Laboratories (Okayama, Japan) and ED-40515(−) was a gift from Dr. M. Maeda, Kyoto University, Kyoto, Japan] were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS) and antibiotics. MT-2, MT-4, C5/MJ, and SLB-1 are HTLV-I-transformed T-cell lines established by an in vitro coculture protocol. MT-1, TL-OmL, and ED-40515(−) are T-cell lines of leukemic cell origin established from ATL patients. HUT-102 was established from a patient with ATL, but its clonal origin is unclear.

**Patient Samples**

The diagnosis of ATL was based on clinical features, hematologic findings, and the presence of anti-HTLV-I antibodies in the sera. Monoclonal HTLV-I provirus integration into the DNA of leukemic cells was confirmed by Southern blot hybridization in all patients (data not shown). Peripheral blood mononuclear cells (PBMC) from healthy volunteers and patients with ATL were analyzed. Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and washed with PBS. PBMC from healthy volunteers were stimulated with phytohemagglutinin (PHA; 10 μg/mL) for 48 hours. All samples were obtained after informed consent.

**Cell Viability and Apoptosis Assays**

The effects of NIK-333 and ATRA on cell growth were examined by use of the cell proliferation reagent, water-soluble tetrazolium-8 (Wako Chemicals, Osaka, Japan). Briefly, cell lines (1 × 10⁶/mL) or PBMC (1 × 10⁶/mL) were incubated in a 96-well microculture plate in the absence or presence of various concentrations of NIK-333 and ATRA. After 48 hours of culture, water-soluble tetrazolium-8 (5 μL) was added for the last 4 hours of incubation and the absorbance at 450 nm was measured using an automated microplate reader. Measurement of mitochondrial dehydrogenase cleavage of water-soluble tetrazolium-8 to formazan dye provides an indication of the level of cell proliferation. IC₅₀ values were extrapolated from trend line data. For detection of apoptosis, the Annexin V–binding capacity of the treated cells was examined by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA) using Annexin V-Fluos (Roche Diagnostics, Mannheim, Germany).

**Cell Cycle Analysis**

Cell cycle analysis was done with the CycleTEST PLUS DNA reagent kit (Becton Dickinson). In brief, 1 × 10⁶ cells were washed with a buffer solution containing sodium citrate, sucrose, and DMSO suspended in a solution containing RNase A and stained with 125 μg/mL propidium iodide for 10 minutes. After passing the cells through a nylon mesh, cell suspensions were analyzed on a FACSCalibur using CellQuest. The population of cells in each cell cycle phase was determined with ModFit software.
Western Blot Analysis

Cells were lysed in a buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 6% 2-mercaptoethanol, and 0.01% bromophenol blue. Samples were subjected to electrophoresis on SDS-polyacrylamide gels followed by transfer to a polyvinylidene difluoride membrane and probing with the specific antibodies. Rabbit polyclonal antibodies to cyclin D2, cIAP2, survivin, IκBα, and JunD were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody to Bcl-xL was purchased from BD Transduction Laboratories (San Jose, CA). Mouse monoclonal antibodies to XIAP and Cdk6, c-myc, and actin were purchased from NeoMarkers (Fremont, CA). Mouse monoclonal antibodies to Bcl-2, Bax, p53, p50, c-Rel, and p52 and various AP-1 family proteins, including c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, and JunD (Santa Cruz Biotechnology), to elicit a supershift DNA mobility shift assay. These antibodies were incubated with nuclear extracts for 15 minutes before incubation with probes. The probes or competitors used were prepared by annealing the sense and antisense synthetic oligonucleotides: a typical NF-κB element from the IL-2 receptor (IL-2R) α-chain gene (5′-gatcGGCGCAATGCTCCCGTCTCTC-3′), an AP-1 element of the IL-8 gene (5′-gatcTGATGACTCAGTTT-3′), and an Oct-1 element (5′-gatcTGCTGAATGCAAAATCTAGAAAC-3′). Underlined sequences represent the NF-κB, AP-1, or Oct-1 binding site. To identify NF-κB and AP-1 proteins in the DNA-protein complex revealed by electrophoretic mobility shift assay, we used antibodies specific for various NF-κB family proteins, including p65, p50, c-Rel, and p52 and various AP-1 family proteins, including c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, and JunD (Santa Cruz Biotechnology), to elicit a supershift DNA mobility shift assay.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay

Cells were placed in culture and examined for inhibition of NF-κB, AP-1, and Oct-1 after exposure to NIK-333 (25 or 50 μmol/L) for 48 hours. Nuclear proteins were extracted, and NF-κB, AP-1, and Oct-1 binding activities to NF-κB, AP-1, or Oct-1 element were examined by electrophoretic mobility shift assay as described previously (14, 41). To examine the specificity of the NF-κB or AP-1 element probe, unlabeled competitor oligonucleotides were preincubated with nuclear extracts for 15 minutes before incubation with probes. The probes or competitors used were prepared by annealing the sense and antisense synthetic oligonucleotides: a typical NF-κB element from the IL-2 receptor (IL-2R) α-chain gene (5′-gatcGGCGCAATGCTCCCGTCTCTC-3′), an AP-1 element of the IL-8 gene (5′-gatcTGATGACTCAGTTT-3′), and an Oct-1 element (5′-gatcTGCTGAATGCAAAATCTAGAAAC-3′). Underlined sequences represent the NF-κB, AP-1, or Oct-1 binding site. To identify NF-κB and AP-1 proteins in the DNA-protein complex revealed by electrophoretic mobility shift assay, we used antibodies specific for various NF-κB family proteins, including p65, p50, c-Rel, and p52 and various AP-1 family proteins, including c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, and JunD (Santa Cruz Biotechnology), to elicit a supershift DNA mobility shift assay.

Transfection and Reporter Assay

Reporter plasmid κB-LUC (a generous gift from Dr. J. Fujisawa, Kansai Medical University, Osaka, Japan) is a luciferase expression plasmid controlled by five tandem repeats of the NF-κB-binding sequences of the IL-2R α-chain gene. Expression plasmid for HTLV-I Tax (a generous gift from Dr. K. Matsumoto, Osaka Red Cross Blood Center, Osaka, Japan) has been described previously (42). Transient transfections were done in Jurkat cells by electroporation using 5 × 10⁶ cells and 2 μg κB-LUC with 0.1 μg Tax expression plasmid or empty vector. In all cases, the reference plasmid pRL-TK, which contains the Renilla luciferase gene under the control of the herpes simplex virus thymidine kinase promoter, was cotransfected to correct for transfection efficiency. After 16 hours, the cells were treated with NIK-333 (25 or 50 μmol/L) for 24 hours and then collected by centrifugation, washed with PBS, and lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase assays were done by using the Dual-Luciferase Reporter System (Promega), in which the relative luciferase activity was calculated by normalizing transfection efficiency according to the Renilla luciferase activities.

In vivo Administration of NIK-333 to SCID Mice

Five-week-old female C.B-17/scid/scid mice obtained from Ryukyu Biotec Co. (Urasoe, Japan) were maintained in containment level 2 cabinets and provided with autoclaved food and water ad libitum. Mice were engrafted with 5 × 10⁶ HUT-102 cells by s.c. injection in the postauricular region and then randomly placed into two cohorts of five mice each that received vehicle and NIK-333, respectively. Treatment was initiated on the day of cell injection. NIK-333 was dissolved in soybean oil at a concentration of 10 mg/mL, and NIK-333 (100 mg/kg body weight) was given by oral gavage every other day. Control mice received the same volume of the vehicle (soybean oil) only. Tumor size was monitored once weekly. All mice were sacrificed on day 21, and the tumors were dissected out and their weight was physically measured. This experiment was done according to the guidelines for the Animal Experimentation of the University of the Ryukyus and approved by the Animal Care and Use Committee of the University of the Ryukyus.

Statistical Analysis

Data are mean ± SD. For data analysis, the unpaired Student’s t test was used. Volume and weight of tumors from NIK-333-treated mice were compared with those of the vehicle-treated controls by the Mann-Whitney U test. P < 0.05 was considered statistically significant.

Results

NIK-333 Inhibits Growth of HTLV-I-Infected T-Cell Lines and Primary ATL Cells

We first examined the effects of NIK-333 and ATRA on the growth of HTLV-I-infected T-cell lines and PBMC from normal healthy controls. Tax protein was detected by immunoblot analysis in the five HTLV-I-infected T-cell lines (MT-2, MT-4, C5/MJ, SLB-1, and HUT-102) but not in the three ATL-derived T-cell lines [MT-1, TL-OmI, and ED-40515(−)]; refs. 41, 43]. Culture of cells with various concentrations (0–50 μmol/L) of NIK-333 or ATRA for 48 hours resulted in the suppression of cell growth in a dose-dependent manner in all eight lines tested as assessed by the water-soluble tetrazolium-8 assay (Fig. 1A). The concentrations of NIK-333 and ATRA required to inhibit growth of HTLV-I-infected T-cell lines by 50% (IC₅₀) ranged from 9.2 to 24.2 and 7.9 to 22.7 μmol/L, respectively (Table 1). Although the sensitivity to NIK-333 and ATRA
varied among the cell lines studied. Tax did not influence the susceptibility to both compounds among the HTLV-I-infected T-cell lines. HTLV-I-uninfected acute lymphoblastic T-cell leukemia, Jurkat, was less susceptible to NIK-333 than HTLV-I-infected T-cell lines (IC50: 35.4 μmol/L). Importantly, normal PBMC were resistant to NIK-333 but sensitive to ATRA up to 50 μmol/L. In contrast, PHA-stimulated PBMC proliferation was inhibited by NIK-333 in a dose-dependent manner (Fig. 1B). We also examined the effects of NIK-333 on freshly isolated ATL cells from eight acute type patients. Tax protein was not detected by immunoblot analysis in all patients (data not shown). ATL cells treated with NIK-333 showed reduced cell survival compared with normal healthy controls (Fig. 1B). IC50s ranged from 18.5 to 45.3 μmol/L.

Retinoic Acid Receptors, Retinoid X Receptors, and Peroxisome Proliferator-Activated Receptor-γ Expression Levels

The activity of various retinoids is thought to be mediated by interactions with two subfamilies of nuclear retinoic acid receptors (RAR), RARs and retinoid X receptors (RXR; ref. 44). These receptors are also thought to bind a variety of synthetic retinoids (45). We then examined by reverse transcription-PCR assays the mRNA expression levels of RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ, and peroxisome proliferator-activated receptor-γ, which heterodimerizes with RXRα (44). Screening for these retinoid receptors in HTLV-I-infected T-cell lines and primary ATL cells showed that RXRα and RXRβ were abundantly expressed in the all cell lines, whereas RARα, RARγ, RXRα, and peroxisome proliferator-activated receptor-γ were detected in all ATL cells. The degree and pattern of RARs did not explain the sensitivity to NIK-333 or ATRA in HTLV-I-infected T-cell lines and primary ATL cells (data not shown).

**NIK-333 Induces Accumulation of Cells in the G1 Phase of the Cell Cycle**

We next investigated the effect of NIK-333 on the cell cycle progression in normal PBMC and cell lines. The cells were incubated with NIK-333 for 24 hours and analyzed for cell cycle distribution by flow cytometry (Fig. 2A). NIK-333 could not affect cell cycle distribution of normal PBMC. However, when PBMC were treated with 25 μmol/L NIK-333 in the presence of PHA, cell cycle progression was affected. The percentage of cells in G1 was increased in PHA-stimulated PBMC (72.3–93.2%). Whereas 21.8% of PHA-stimulated PBMC were at S phase, only 2.6% of PHA-stimulated PBMC were at S phase after treatment with NIK-333. Cultivation with NIK-333 for 24 hours increased the proportion of cells in the G1 phase, with a reduction of cells in the S phase in all cell lines, except TL-Oml. These results clearly show that NIK-333 induces accumulation of cells in the G1 phase.

**NIK-333 Induces Apoptosis of HTLV-I-Infected T-Cell Lines**

To examine whether induction of apoptosis accounts for the cell growth inhibition observed in HTLV-I-infected T-cell lines, cells treated with NIK-333 were examined by the Annexin V method. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic found in cells entering apoptosis. NIK-333

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**Table 1. IC50s for inhibition of cell growth of NIK-333 and ATRA**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HTLV-I status</th>
<th>Tax status</th>
<th>IC50 for inhibition of cell growth (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NIK-333</td>
</tr>
<tr>
<td>MT-2</td>
<td>+</td>
<td>+</td>
<td>19.2</td>
</tr>
<tr>
<td>MT-4</td>
<td>+</td>
<td>+</td>
<td>18.0</td>
</tr>
<tr>
<td>C5/MJ</td>
<td>+</td>
<td>+</td>
<td>16.7</td>
</tr>
<tr>
<td>SLB-1</td>
<td>+</td>
<td>+</td>
<td>20.2</td>
</tr>
<tr>
<td>HUT-102</td>
<td>+</td>
<td>+</td>
<td>20.2</td>
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<tr>
<td>MT-1</td>
<td>-</td>
<td>-</td>
<td>9.2</td>
</tr>
<tr>
<td>TL-Oml</td>
<td>+</td>
<td>-</td>
<td>24.2</td>
</tr>
<tr>
<td>ED-40515(+)</td>
<td>-</td>
<td>-</td>
<td>19.7</td>
</tr>
<tr>
<td>Jurkat</td>
<td>-</td>
<td>-</td>
<td>35.4</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td>Normal PBMC 2</td>
<td>-</td>
<td>-</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td>PHA-blast</td>
<td>-</td>
<td>-</td>
<td>19.3</td>
</tr>
</tbody>
</table>

**NOTE:** Tax expression can be detected by Western blotting. Abbreviation: ND, not done.
NIK-333-Treated HTLV-I-Infected T-Cell Lines and Primary ATL Cells Express Intracellular Regulators of Cell Cycle and Apoptosis

To clarify the molecular mechanisms of NIK-333-induced inhibition of cell growth and apoptosis of HTLV-I-infected T-cell lines, we examined the expression of viral Tax and several intracellular regulators of cell cycle and apoptosis, including cyclin D1, cyclin D2, Cdk6, c-myc, p53, Bcl-2, Bcl-xL, Bax, cIAP2, XIAP, and survivin by Western blot analysis. As shown in Fig. 3, at 50 μmol/L, NIK-333 did not alter Bcl-2, Bcl-xL, Bax, survivin, Cdk6, p53, and c-myc levels. In contrast, NIK-333 at the same concentration significantly decreased the expression of cIAP2, XIAP, cyclin D1, and cyclin D2 in MT-2 cells. Comparable loading of protein was confirmed with a specific antibody for the housekeeping gene product actin (Fig. 3). We confirmed down-regulation of cyclin D2, cIAP2, and XIAP by NIK-333 in HUT-102 cells, although HUT-102 did not express cyclin D1. Because cIAP2, XIAP, cyclin D1, and cyclin D2 are Tax-responsive genes (46–49), we also examined the level of Tax expression. NIK-333 did not change the protein level of Tax in MT-2 (Fig. 3). We explored the effect of NIK-333 on expression of these proteins in freshly isolated ATL cells without detectable Tax expression. NIK-333 decreased XIAP but not Bcl-xL expression. Cyclin D1, cyclin D2, and cIAP2 were not detectable in this case. These results indicate that the altered expression levels of cIAP2, XIAP, cyclin D1, and cyclin D2 proteins did not result from Tax down-regulation.

NIK-333 Modulates Activated NF-κB

Several reports have suggested that NF-κB can act as a survival factor and is required for the proliferation of a variety of tumor cell types (50). Because NF-κB is constitutively active in Tax-expressing and HTLV-I-infected T-cell lines as well as primary ATL cells (14), and Tax stimulates the expression of XIAP, cyclin D1, and cyclin D2 through the NF-κB pathway (47–49), we examined whether NIK-333 inhibits the NF-κB pathway. To study the DNA-binding activity of NF-κB, we did electrophoretic mobility shift assay with radiolabeled double-stranded NF-κB oligonucleotides and nuclear extracts from untreated or NIK-333-treated HTLV-I-infected T-cell lines. NF-κB oligonucleotide probe with nuclear extracts from untreated HTLV-I-infected T-cell lines generated DNA-protein gel shift complexes (Fig. 4A). These complexes were due to specific bindings of nuclear proteins to the NF-κB sequences because these binding activities were reduced by the addition of cold probe but not by an unrelated sequence (Fig. 4B, left, lanes 2 and 3). We also showed that NF-κB complexes contain p50, p65, and c-Rel (Fig. 4B, left, lanes 4–6). As shown in Fig. 4A, nuclear extracts prepared from HTLV-I-infected T-cell lines treated with NIK-333 for 48 hours exhibited a decrease in the intensity of the NF-κB-containing gel shift complexes, suggesting that NIK-333 down-regulates the DNA-binding activities of NF-κB. Inhibition appeared specific to NF-κB and not due to cell cycle arrest.
death, because no significant change in binding activity of AP-1 and Oct-1 was observed after treatment of cells with NIK-333 (Fig. 4A). In addition, NF-κB activation in PHA-stimulated PBMC was inhibited by NIK-333. We also examined the effect of NIK-333 on the expression of JunD by immunoblotting, because AP-1 complexes contain JunD in HTLV-I-infected T-cell lines (Fig. 4B, right, lane 10; ref. 41). JunD was expressed in a HTLV-I-infected T-cell line (MT-2) and treatment of cells with NIK-333 did not alter JunD level (Fig. 3).

In addition to NF-κB DNA-binding activity, we investigated the effect of NIK-333 on transcriptional activity of NF-κB. We examined the effect of NIK-333 on Tax-induced NF-κB transcriptional activity. Tax expression plasmid, together with luciferase reporter plasmid regulated by NF-κB elements (κB-LUC) were transfected into Jurkat cells. Then, the cells were treated with NIK-333 for 24 hours. The NF-κB transcriptional activation by Tax was suppressed by NIK-333 (Fig. 4C).

Degradation of IκBα and subsequent release of NF-κB requires prior phosphorylation at Ser32 and Ser36 residues (51). To investigate whether the inhibitory effect of NIK-333 is mediated through alteration of Ser32 and Ser36 residues, MT-2 cells were treated with NIK-333, and their protein extracts were checked for phosphorylated IκBα expression. Untreated MT-2 cells constitutively expressed Ser32/36-phosphorylated IκBα, whereas NIK-333 treatment decreased the phosphorylated IκBα (Fig. 3). NIK-333 treatment increased total IκBα protein (Fig. 3), suggesting that inhibition of phosphorylation of IκBα leads to stabilization of IκBα by blocking degradation of IκBα protein.

Antitumor Effects of NIK-333 on s.c. HUT-102 Tumors

Finally, we examined the effects of NIK-333 against ATL in vivo. SCID mice (n = 10) were inoculated with HUT-102 and then divided into two groups: untreated mice (n = 5) and NIK-333-treated mice (n = 5). Treatment commenced on the day of the inoculation. At day 21 post-treatment, the mean tumor volume (Fig. 5A) and weight (Fig. 5B) were significantly lower than those of vehicle-treated mice (P < 0.05, Mann-Whitney U test; Fig. 5). There was no significant difference in body weight gain found during the period from days 0 to 21 among the vehicle group and the group treated with NIK-333. During this period, the control mice showed signs of severe disease, including piloerection. In contrast, mice treated with NIK-333 appeared generally healthy. These results suggest that NIK-333 also has in vivo anti-ATL effect.

![Image](https://example.com/image1.png)

Figure 4. NIK-333 suppresses nuclear NF-κB activity in HTLV-I-infected T-cell lines and PHA-stimulated PBMC. A, effect of 48-h treatment with NIK-333 (25 or 50 μmol/L) in HTLV-I-infected T-cell lines and PHA-stimulated PBMC on the activation of transcription factors NF-κB, AP-1, and Oct-1 assessed by electrophoretic mobility shift assay using oligonucleotide probes for NF-κB, AP-1, and Oct-1, respectively. B, electrophoretic mobility shift assay using untreated MT-2 nuclear extracts and radiolabeled NF-κB and AP-1 probes generated DNA-protein complexes (arrows), which were eliminated by 100-fold molar excess of self-competitors but not by the same molar excess of the respective oligonucleotides. Supershift assays using the radiolabeled NF-κB and AP-1 probes, untreated nuclear extracts, and the indicated polyclonal antibodies to NF-κB and AP-1 components showed that the NF-κB and AP-1 bands consisted of p50, p65, and c-Rel subunits and JunD subunit, respectively. C, NIK-333 inhibits Tax-induced NF-κB transcriptional activity. κB-LUC was transfected into Jurkat cells with Tax expression plasmid or empty vector. pRL-TK was also cotransfected as an internal control plasmid. Then, 16 h after transfection, cells were treated with NIK-333 (25 or 50 μmol/L) for 24 h. The cells were harvested for extract preparation and measurement of luciferase activity. *, P < 0.05; **, P < 0.0001, compared with untreated Tax-transfected cells (Student’s t test). Luciferase activity is expressed relative to the basal level measured in cells transfected with the reporter plasmid and Tax without further treatment, which was defined as 100. Columns, mean of three separate transfections; bars, SD.
The HTLV-I encodes an oncprotein Tax from its \( pX \) gene, which plays a central role in leukemogenesis of ATL (10). We examined the level of Tax expression by Western blot analysis in HTLV-I-infected T-cell lines, but Tax was not a molecular target of NIK-333 treatment. The growth-inhibitory effect of NIK-333 on HTLV-I-infected T cells was mainly due to the induction of cell cycle arrest and apoptosis because a significant population of cells remained in the G1 phase of the cell cycle and underwent apoptosis after treatment with NIK-333.

Detailed mapping of intracellular molecules and signaling pathways might provide more efficient, less toxic treatment opportunities in which cellular components, critical for survival of the tumor, can be selectively targeted. Because of the central role of NF-\( \kappa B \) in cell survival and proliferation, we explored the role of this transcription factor as a target for the treatment of ATL by using NIK-333. Our results indicate that NIK-333 down-regulated NF-\( \kappa B \) activity and suppressed constitutive I\( \kappa B \)-Re Mo n phosphorylation. We found that suppression of NF-\( \kappa B \) by NIK-333 correlated with the down-regulation of the expression of several gene products regulated by NF-\( \kappa B \). The expression of cIAP2, XIAP, cyclin D1, and cyclin D2, the synthesis of which is regulated by NF-\( \kappa B \) (47–49, 52), was suppressed by NIK-333. This led to the suppression of proliferation, accumulation of cells in the G1-S-phase boundary of the cell cycle, and induction of apoptosis. These results provide the framework for targeting NF-\( \kappa B \) in ATL therapy.

The potent and selective apoptotic effect of NIK-333 against HTLV-I-infected T-cell lines and ATL cells in vitro prompted us to evaluate its in vivo anti-ATL effect in SCID mice bearing a HTLV-I-infected T-cell line (HUT-102). Importantly, NIK-333 inhibited the growth of HUT-102 cells in our model.

Several novel anti-ATL agents, such as arsenic trioxide (53, 54), a proteasome inhibitor (21), ATRA (55), can inhibit NF-\( \kappa B \) activity. Thus, NF-\( \kappa B \) inhibition may be at least a component of their antitumor activity. Nonspecific drug toxicity is one of the major problems in drug development. Numerous studies have shown that NIK-333 is pharmacologically safe (28, 29). In conclusion, we have clearly shown the efficiency of NIK-333 in inducing cell death through inactivation of NF-\( \kappa B \) pathway in HTLV-I-infected T cells. Our studies provide the rationale for clinical trials of NIK-333 in patients with ATL.

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Effects of Acyclic Retinoid in Adult T-Cell Leukemia


NIK-333 inhibits growth of human T-cell leukemia virus type I-infected T-cell lines and adult T-cell leukemia cells in association with blockade of nuclear factor-κB signal pathway

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