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A homogeneous time-resolved fluorescence-based high-throughput screening system for discovery of inhibitors of IKKβ–NEMO interaction

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ABSTRACT

The nuclear transcription factor NF-κB is crucial to the expression of numerous cytokines, enzymes, and cell adhesion molecules, all of which can drive inflammatory and autoimmune disorders such as rheumatoid arthritis. The IKK complex plays the most important role in the signal cascade leading to NF-κB activation. Recently, inhibition of the interaction between NEMO (NF-κB essential modulator) and the catalytic subunits of IKK, especially IKKβ, has received particular attention as a possible new therapeutic approach to treatment of inflammatory disorders, and several reports have shown the efficacy of cell permeable NEMO binding domain (NBD)-containing peptides in blocking the IKK/NF-κB pathway. In this article, we describe in detail the development and validation of two novel binding assays, a homogeneous time-resolved fluorescence (HTRF)-based assay and an enzyme-linked immunosorbent assay (ELISA)-based assay, suitable for the discovery of small molecules that inhibit IKKβ–NEMO interaction. Using the HTRF-based assay, we screened approximately 15,000 compounds from our chemical library and eliminated false positive hits by the ELISA-based assay and IKK complex kinase assay. As a result, seven positive hit compounds that inhibit IKK complex activity through inhibition of IKKβ–NEMO interaction were identified. These hit compounds may have a good potential in the treatment of inflammatory and autoimmune disorders such as rheumatoid arthritis.

NF-κB is an ubiquitous transcription factor involved in the regulation of cell signaling response and is crucial to the expression of numerous cytokines, enzymes, and cell adhesion molecules, all of which can drive inflammatory and autoimmune disorders such as rheumatoid arthritis. As such, numerous approaches are ongoing within pharmaceutical discovery laboratories to identify potential therapeutic agents that can modulate NF-κB activation. In most cell types, NF-κB is retained in the cytoplasm by IκB, an inhibitory protein of NF-κB. Activation of NF-κB typically involves phosphorylation of IκB by the IκB kinase (IKK)[1] complex, which results in IκB degradation. This IκB degradation allows NF-κB to translocate freely to the nucleus. Thus, cytokine-mediated activation of IKK complex is a key step in the activation of the NF-κB pathway. IKK signalosome is a 600- to 900-kDa complex that encompasses IκB kinases, IKKα and IKKβ, in addition to the tightly associated scaffold protein NEMO (NF-κB essential modulator, also termed IKKγ). This scaffolding protein NEMO also binds a number of other molecules that are involved in the regulation of IKK activity [1–7]. Although NEMO does not contain a kinase domain, its elimination in cells inhibits the formation of IKK complex, thereby inhibiting cytokine-induced activation of NF-κB pathway [8,9]. Therefore, disturbance of the interaction between NEMO and the catalytic subunits of IKK, especially IKKβ, has recently received particular attention as a possible new therapeutic approach to treatment of inflammatory disorders.

Studies with truncation mutants of IKKβ and NEMO have shown that the carboxyl terminus of IKKβ interacts with the amino terminus of NEMO [10,11]. Using deletion and point mutants of IKKβ, May and coworkers further narrowed the interacting region to amino acids 737–742, LDWSWL (called the NEMO binding domain [NBD]), located in the carboxyl terminus of IKKβ [11]. Within this NBD, the aspartate and two tryptophan residues were shown to be critical to IKKβ ability to interact with NEMO [11,12]. In addition, single point mutations of these residues to alanine prohibit IKKβ from interacting with NEMO. Therefore, the identification of this relatively small IKKβ–NEMO interacting region suggests that peptides or small molecules might inhibit this interaction and

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[1] *Abbreviations used:* IKK, IκB kinase; NEMO, NF-κB essential modulator; NBD, NEMO binding domain; HTRF, homogeneous time-resolved fluorescence; ELISA, enzyme-linked immunosorbent assay; HTS, high-throughput screening; anti-FLAG-K, Eu^{3+} cryptate-conjugated anti-FLAG antibody; anti-6His-XL665, XL665-conjugated anti-6×His antibody; cDNA, complementary DNA; RT-PCR, reverse transcription polymerase chain reaction; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT, room temperature; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; HRP, horseradish peroxidase; TR–FRET, time-resolved fluorescence resonance energy transfer.

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prevent formation of the IKK complex. Such inhibition could have implications for the treatment of inflammatory diseases that are regulated by the IKK/NF-κB pathway. Several reports have shown the efficacy of cell permeable NBD-containing peptides in blocking the IKK/NF-κB pathway [11,13]. In all of those reports, the 6-residue LDWSWL (NBD) was extended to encompass additional IKKβ residues at both the N and C termini, resulting in an 11-residue peptide, TALDWSWLQTE. This NBD-containing peptide was synthesized in tandem with a cell permeable sequence that assists in its delivery into cells. Treatment with this cell permeable NBD-containing peptide inhibited formation of the IKK complex in various cell types, effectively reduced inflammation, and ameliorated the condition in several animal models [14–20]. These findings support the potential role of this peptide as a novel therapeutic approach in inflammatory diseases.

In this study, we describe in detail the development and validation of two binding assays, a homogeneous time-resolved fluorescence (HTRF)-based assay and an enzyme-linked immunosorbent assay (ELISA)-based assay, suitable for the discovery of small molecular compounds that inhibit the interaction between IKKβ and NEMO. The HTRF-based binding assay, which is characterized by an excellent Z' factor, is simple, sensitive, robust, and suitable for a high-throughput screening (HTS) format. In fact, using this assay, we screened approximately 15,000 compounds from our chemical library and eliminated false positive hits by the ELISA-based assay and IKK complex kinase assay. As a result, we found seven positive hit compounds that inhibit IKK complex activity through inhibition of interaction between IKKβ and NEMO. These hit compounds may have a good potential in the treatment of inflammatory and autoimmune disorders such as rheumatoid arthritis.

Materials and methods

Antibodies, chemicals, and peptides

Eu^{3+} cryptate-conjugated anti-FLAG antibody (anti-FLAG-K) and XL665-conjugated anti-6×His antibody (anti-6His-XL665) were purchased from CIS Biointernational. Anti-IKKα was purchased from Cell Signaling Technology (Cat. No. 2682). Anti-IKKβ was purchased from Abcam (Cat. No. ab12139). Anti-IKKγ (NEMO) was purchased from BD Pharmingen (Cat. No. 559675). Manumycin A was purchased from Sigma (Cat. No. M6418). BMS-345541 and TPCA were purchased from Calbiochem (Cat. Nos. 401480 and 401481, respectively). Cell permeable antennapedia-NBD (wild-type and mutant) fusion peptides were purchased from Sigma Genosys. These peptide sequences were as follows: NBD (wild-type), DRQIKIWFQNRRMKWKKTALDWSWLQTE; NBD (mutant), DRQIKIWFQNRRMKWKKTALDASALQTE.

Expression constructs

Complementary DNA (cDNA) of human IKKβ was amplified by reverse transcription polymerase chain reaction (RT-PCR) from human brain total RNA and subcloned into pBluescript vector (Stratagene). pcDNA3.1/hIKKβ/3′FLAG was made by insertion of hIKKβ full-length PCR fragment into pcDNA3.1/3′FLAG vector. pET/hIKKβ/3′FLAG was made by insertion of hIKKβ-3′FLAG PCR product into the pET vector (Novagen). cDNAs of human NEMO and IκBα were amplified by RT-PCR from human leukemic monocyte lymphoma cell line U937 total RNA and subcloned into the pBluescript vector. pcDNA3.1/hNEMO was made by insertion of the full-length hNEMO PCR product into the pcDNA 3.1 vector. pET/hNEMO (1–265)/3′6×His was made by insertion of hNEMO (aa 1–265) PCR product into the pET/3′6×His vector. pGEX-2T/hNEMO, pGEX-2T/hNEMO (1–265), pGEX-2T/hNEMO (135–265), and pGEX-2T/hNEMO (135–231) were made by insertion of hNEMO corresponding-length PCR products into the pGEX-2T vector (GE Healthcare). For expression of various lengths of glutathione *S*-transferase (GST)-tagged hNEMO in mammalian cells, pcDNA3.1/GST-hNEMO vectors were made by insertion of GST-tagged hNEMO fragments from pGEX-2T/hNEMO vectors. pET/5′6×His/hIκBα (1–54) was made by insertion of hIκBα (aa 1–54) PCR product into the pET vector. All sequences of PCR amplified cDNAs were actually confirmed by sequencing.

Protein expression and purification

pET/hNEMO (1–265)/3′6×His and pET/5′6×His/hIκBα both were transformed into *Escherichia coli* BL21(DE3)pLysS, grown at 37 °C to OD_{600} 0.5 to 0.6, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and grown for 3 h at 30 °C. The collected cells were lysed and sonicated at 4 °C in lysis buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 5 mM dithiothreitol [DTT], 0.1% Tween 80, 2.5 mg/ml lysozyme, and protease inhibitor cocktail [Roche]). After centrifugation, His-hNEMO (1–265) and His-hIκBα (1–54) in the cleared lysates both were purified using Ni–nitrilotriacetic acid (NTA) column. pET/hIKKβ/3′FLAG was transformed into BL21(DE3)pLysS, grown at 37 °C to OD_{600} 0.6, induced with 0.5 mM IPTG, and grown for 2 h at 25 °C. The collected cells were lysed and sonicated at 4 °C in lysis buffer. After centrifugation, the insoluble inclusion body pellet was lysed in lysis buffer containing 6 M guanidine hydrochloride and then dialyzed six times against ×100 volume of lysis buffer containing either 3, 2, 1, 0.5, 0.25, or 0 M guanidine hydrochloride. Refolded hIKKβ-FLAG in the dialyzed lysate was purified using anti-FLAG M2 affinity gel (Sigma, Cat. No. A2220) and FLAG peptide (Sigma, Cat. No. F3290) according to the manufacturer's protocol.

Cell culture and plasmid transfection

CHO-K1 cells were grown in Ham's F12 medium containing 10% fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 5% CO_2. HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and incubated at 37 °C in a humidified atmosphere of 5% CO_2. Transfection of the expression plasmids of hIKKβ and hNEMO was performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. After transfection, the cells were cultured for 24–48 h and assayed in various experiments.

Immunoprecipitation-based binding assay

In cell-based immunoprecipitation assay, HeLa cells were cotransfected with the expression plasmids of hIKKβ-FLAG and hNEMO (1–265)-6×His. These cells were collected and lysed at 4 °C in lysis buffer (phosphate-buffered saline [PBS](−) containing 0.5% Tween 80 and protease inhibitor cocktail). After centrifugation of the cell lysate, glutathione Sepharose 4B was added to the cleared lysate and incubated at 4 °C for 2 h. After centrifugation and wash of the resins three times, the resulting immunoprecipitates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analysis with the anti-GST antibody and the anti-FLAG antibody. In purified protein-based immunoprecipitation assay, 45 μl of 10 nM hNEMO (1–265)-6×His, 45 μl of 10 nM hIKKβ-FLAG, and 10 μl of NBD peptide were mixed in PBS (pH 7.4). After 10 min of incubation at room temperature (RT), the anti-6 His antibody and protein G Sepharose were added to the reaction mixture and the mixture was incubated at RT for 2 h. After centrifugation and wash of the resins three times, the resulting immunoprecipitates were subjected to SDS–PAGE and immunoblot analysis with the anti-6×His antibody and the anti-FLAG antibody.

The obtained image was analyzed and measured with Scion Image (Scion) to calculate percentage inhibition.

HTRF-based binding assay and HTS format

For HTRF assay in black 384-well low-volume microplates (Corning Coaster, Cat. No. 3676), 4 μl of 10 nM hIKKβ-FLAG, 4 μl of 10 nM hNEMO (1–265)-6×His, and 2 μl of NBD peptide or test compound were mixed in the binding buffer (50 mM Tris–HCl [pH 7.4], 10 mM phosphate, 10 mM citrate, 150 mM NaCl, 0.1% Tween 80, 0.5% Tween 20, 0.5% bovine serum albumin [BSA], and 1% dimethyl sulfoxide [DMSO]) and incubated at RT for 1 h. After incubation, 0.5 ng/5 μl of anti-FLAG-K and 10 ng/5 μl of anti-6His-XL665 were added into each well. After 2 h of incubation at RT, HTRF signals were measured using a RUBYstar plate reader (BMG) at 620 and 665 nm emission. Results represented the addition of 20 readings/well with an integration delay of 50 μs and an integration time of 400 μs. HTRF signal was calculated as a two-wavelength signal ratio: [intensity (665 nm)/intensity (620 nm)] × 10^4. Percentage inhibition was calculated using following equation: % inhibition = [(signal of compound) − (signal of min)]/[(signal of max) − (signal of min)] × 100, where (signal of max) and (signal of min) are the signal ratios for bound hIKKβ-FLAG/hNEMO (1–265)-6×His and free hIKKβ-FLAG alone, respectively. The signal/background (S/B) ratio and the Z' factor were calculated using the following equations: S/B = (signal of max)/(signal of min) and Z' = 1–3 × [(SD of max) + (SD of min)]/[(signal of max) − (signal of min)], where SD is standard deviation. In HTS, assay data were analyzed using Assay Explorer (Symyx). Because the standard deviations of (signal of max) in each plate were less than 9% and the 3 × SD was less than 27%, the activity cutoff was set as a percentage inhibition greater than 30%.

ELISA-based binding assay

For ELISA-based assay, 30 μl of 5 nM hIKKβ-FLAG, diluted in 50 mM citrate buffer (pH 5.0), was added into the well of a clear 96-well half-area microplate (Corning Coaster, Cat. No. 3690) and incubated at 4 °C overnight. After three washes each with 100 μl of wash buffer (PBS containing 0.05% Tween 20), 100 μl of blocking solution (5% skim milk, 3% BSA, and 0.05% Tween 20 in PBS) was added and the microplate was incubated at 37 °C for 1 h. After five washes each with 100 μl of wash buffer, 30 μl of 5 nM hNEMO (1–265)-6×His, diluted in assay buffer (100 mM Tris, 10 mM phosphate, 10 mM citrate, 150 mM NaCl, 0.1% Tween 80, 0.5% Tween 20, 10% BlockAce [DS Pharma Biomedical, Cat. No. UK-B500], and 1% BSA, pH 7.4) with NBD peptide or test compound, was added and the microplate was incubated at 37 °C for 1 h. After five washes each with 100 μl of wash buffer, 30 μl of biotinylated anti-6×His antibody appropriately diluted in antibody buffer (0.05% Tween 20, 10% BlockAce, and 1% BSA in PBS) was added and the microplate was incubated at 37 °C for 1 h. After five washes each with 100 μl of wash buffer, 30 μl of streptavidin-labeled horseradish peroxidase (HRP) appropriately diluted in antibody buffer was added and the microplate was incubated at 37 °C for 1 h. After five washes each with 100 μl of wash buffer, 80 μl of reaction solution (3.7 mM *o*-phenylenediamine and 0.034% H_2O_2 in 50 mM citrate, pH 5.0) was added and the microplate was incubated at 37 °C for 10 min. To terminate the reaction, 20 μl of 4 N H_2SO_4 was added to the mixture. ELISA signals were measured using a SpectraMax M2 plate reader (Molecular Devices) at 492 nm absorbance.

In vitro IKK complex kinase assay

CHO-K1 cells were cotransfected with the expression plasmids of hIKKβ-FLAG and full-length hNEMO. The transfected cells were collected and lysed at 4 °C in lysis buffer (50 mM Hepes [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1% Triton X-100, 2 mM NaF, 0.5 mM Na_3VO_4, and protease inhibitor cocktail). The IKK complex in the cleared lysate was purified using anti-FLAG M2 affinity gel and FLAG peptide according to the manufacturer's protocol. For in vitro kinase assay, IKK complex was diluted at the described concentration in 12.5 μl of kinase reaction buffer (25 mM Hepes [pH 7.4], 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 5% glycerol, 0.5% Triton X-100, 1 mM NaF, 0.5 mM Na_3VO_4, 50 μg/ml 6×His-IκBα (1–54), 20 μM ATP, 0.02 μCi/μl [^{32}P]γATP, and test compound). The suspension was incubated at 30 °C for 30 min, after which the reaction was stopped by adding 12.5 μl of 0.1 M EDTA. IKK complex suspension was then filtered using 1 cm^2 cut P81 phosphocellulose filter paper (Whatman), which was subsequently air-dried and washed four times each with 75 mM phosphoric acid for 20 min. The air-dried P81 paper was then transferred into a scintillation vial (Wheaton), which was filled with 5 ml of ACSII scintillation cocktail (GE Healthcare) and counted in a TopCount scintillation counter (Packard).

Results

Immunoprecipitation binding assay: Importance of NEMO N-terminal region for NEMO–IKKβ interaction, which can be inhibited by NBD (wild-type) peptide.

First, to determine the important region of NEMO for interaction with IKKβ, we expressed various lengths of GST-hNEMO with hIKKβ-FLAG in HeLa cells and performed cell-based immunoprecipitation binding assay. As shown in Fig. 1A, both hNEMO (full) and hNEMO (1–265) interacted with hIKKβ. On the other hand, the two N-terminal truncated forms, hNEMO (135–231) and hNEMO (135–265), did not interact with hIKKβ. These results, which are consistent with those of previously reports [11,21,22], indicate that the N-terminal region of NEMO is essential for IKKβ–NEMO interaction. Next, to examine the effect of NBD peptide on IKKβ–NEMO interaction, we used purified protein binding assay. As shown in Fig. 1B, hIKKβ-FLAG was immunoprecipitated with hNEMO (1–265)-6×His, and this interaction was dose-dependently inhibited by NBD (wild-type) peptide with an IC_{50} = 7.2 μM (Fig. 1C). On the other hand, NBD (mutant) peptide did not inhibit IKKβ interaction with NEMO. These results indicate that small molecule inhibitors of IKKβ–NEMO interaction can be screened using the immunoprecipitation-based assay, although this assay might not be adequate for HTS.

Development of HTRF-based IKKβ/NEMO binding assay

To develop a highly sensitive assay suitable for in vitro HTS of inhibitors of IKKβ–NEMO interaction, we developed a novel HTRF-based binding assay (Fig. 2). In principle, FLAG-tagged purified hIKKβ, 6×His-tagged purified hNEMO, anti-FLAG-K antibody, and anti-6×His-XL665 antibody were mixed and incubated at RT for 3 h. IKKβ binding to NEMO was then measured as HTRF signal using the method described in Materials and methods. In the assay, 6×His-tagged hNEMO was used instead of GST-tagged hNEMO because GST-tag molecular weight is much larger than that of 6×His tag and might consequently cause noise or false positives in HTS. As shown in Fig. 3A, an increase in the concentration of hNEMO (1–265)-6×His resulted in an increase in HTRF signal. When the concentration of hIKKβ-FLAG was fixed at 2 nM, the maximal assay window for hNEMO (1–265)-6×His titration reached approximately 22,000 with a K_d of 1.2 nM. When the assay dynamic range was assessed by calculating the S/B ratio at 2 nM each, the mean of the S/B ratio was 26.1 ± 0.5 (n = 4). These data

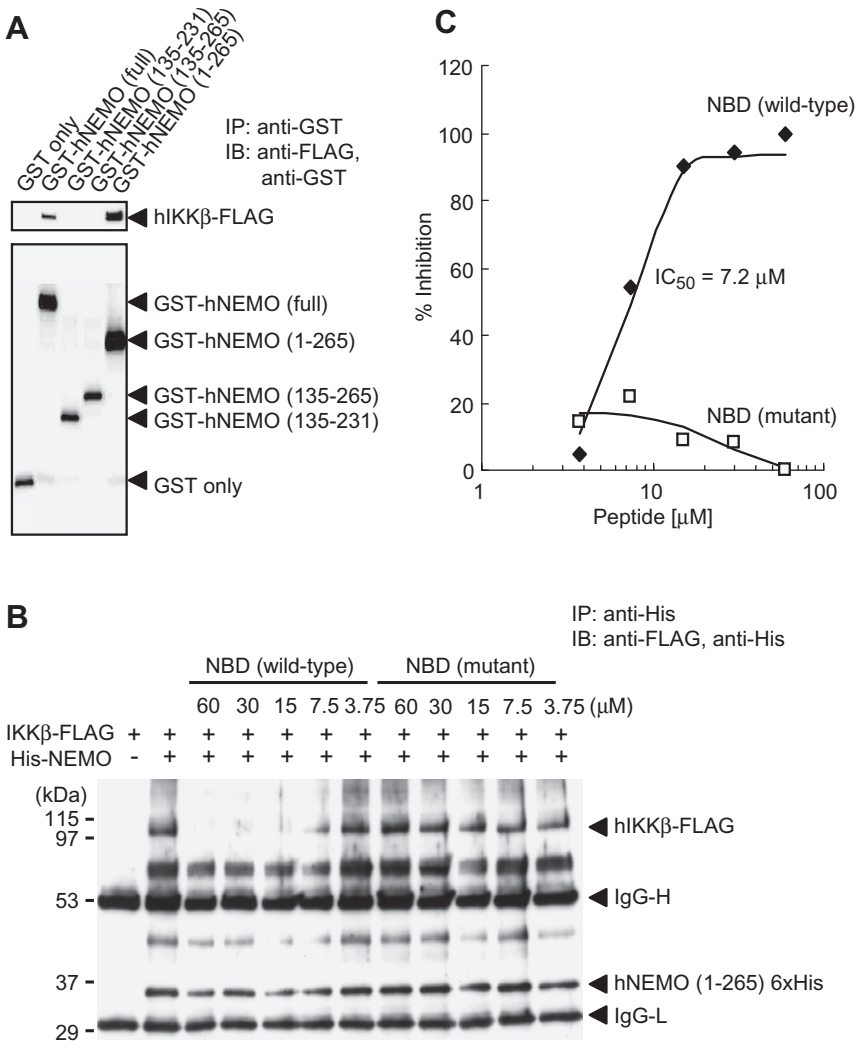


Fig. 1. Immunoprecipitation-based binding assay: IKKβ–NEMO interaction and inhibition. (A) Interaction of IKKβ with deletion mutants of NEMO. HeLa cells were cotransfected with IKKβ-FLAG and GST-hNEMO expression plasmid. Cell extracts were immunoprecipitated with glutathione Sepharose 4B. Immunoprecipitants were detected by Western blotting using anti-GST antibody and anti-FLAG antibody. (B) Direct interaction between hIKKβ-FLAG and hNEMO (1–265)-6×His with various concentrations of NBD (wild-type) or NBD (mutant) peptide was determined by Western blotting using anti-6×His antibody and anti-FLAG antibody. IgG-H, immunoglobulin G heavy chain; IgG-L, immunoglobulin G light chain. (C) The inhibitory effect of NBD peptide was calculated by quantification analysis of band density in (B). The data were fitted using nonlinear least squares regression analysis.

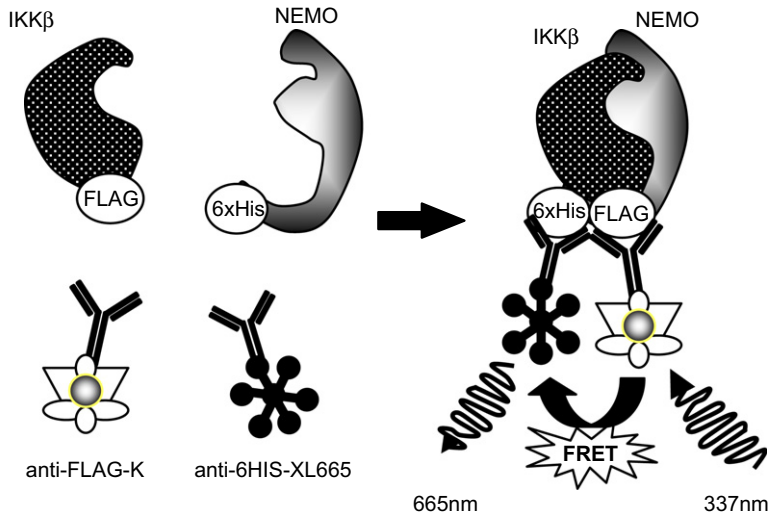


Fig. 2. Schematic representation of HTRF-based binding assay for monitoring inhibition of IKKβ–NEMO interaction.

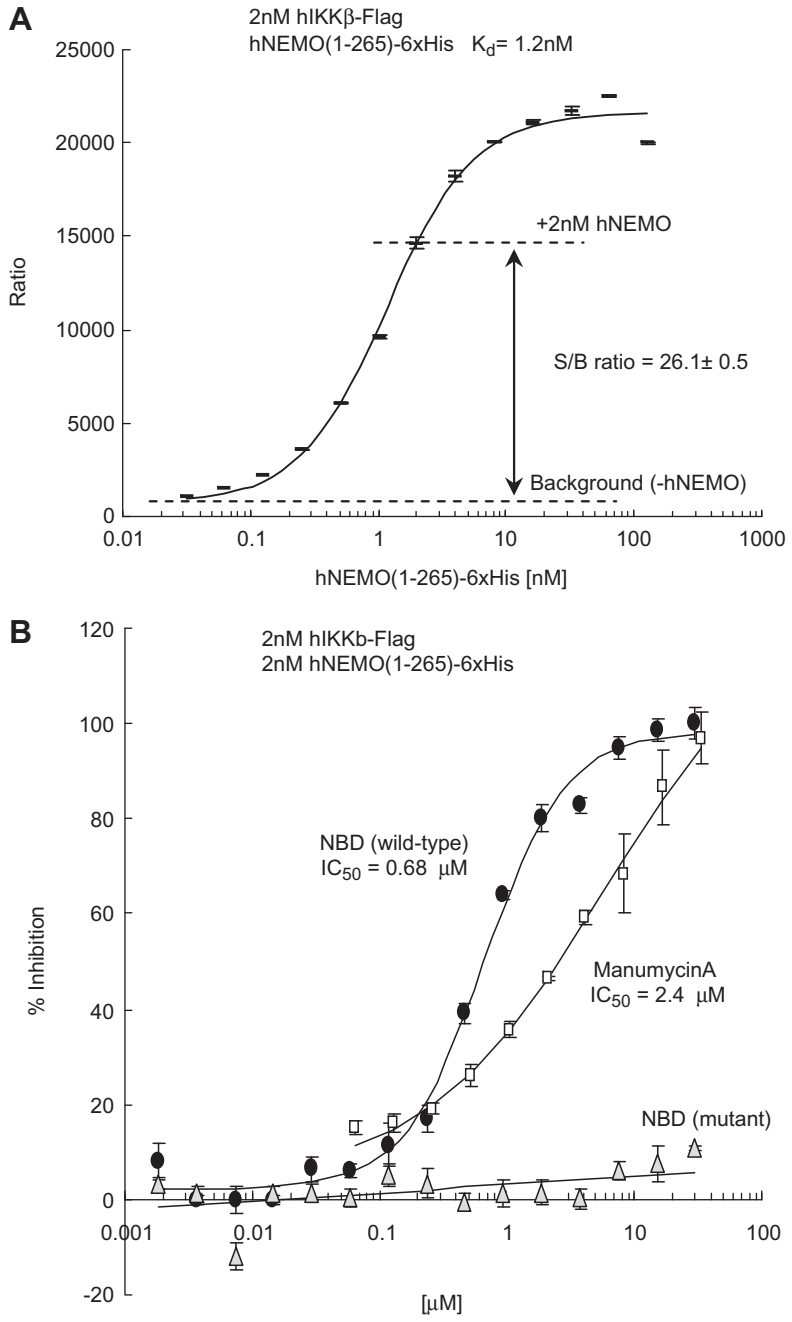


Fig. 3. (A) Concentration dependence examination in HTRF-based binding assay. hNEMO (1–265)-6×His titrated with 2 nM solution of hIKKβ-FLAG, anti-FLAG-K, and anti-6His-XL665. The data are means (± standard deviations, $n = 4$) and were fitted using nonlinear least squares regression analysis. (B) Inhibitory effect of NBD peptide and manumycin A on IKKβ–NEMO interaction in HTRF-based binding assay. NBD peptide or manumycin A was incubated with 2 nM hIKKβ-FLAG, 2 nM hNEMO (1–265)-6×His, anti-FLAG-K, and anti-6His-XL665. The data are means (± standard deviations, $n = 4$) and were fitted using nonlinear least squares regression analysis.

indicate that this novel assay is robust and suitable for monitoring in vitro interaction between IKKβ and NEMO.

Inhibition of IKKβ–NEMO interaction by NBD peptide and manumycin A

In the HTRF-based binding assay, NBD (wild-type) peptide inhibited IKKβ–NEMO interaction with an IC_{50} = 0.68 μM, and NBD (mutant) peptide did not inhibit this interaction (Fig. 3B). Furthermore, the antibiotic manumycin A, which has been reported to inhibit IKK complex kinase activity by dissociating IKKβ binding to NEMO [23], also inhibited IKKβ–NEMO interaction with an IC_{50} = 2.4 μM (Fig. 3B). However, both BMS-345541 and TPCA, which inhibit IKKβ kinase activity, did not inhibit IKKβ–NEMO interaction (data not shown). These results indicate that NBD (wild-type) peptide inhibits IKKβ–NEMO interaction in vitro and that the HTRF-based binding assay is useful for screening inhibitors of this interaction.

Development and optimization of ELISA-based IKKβ/NEMO binding assay

As a confirmatory assay for hit compounds obtained by the HTRF-based assay, we developed a novel ELISA-based assay to detect in vitro interaction between IKKβ and NEMO (Fig. 4A). Because this ELISA-based assay is a simple 96-well plate format assay, the

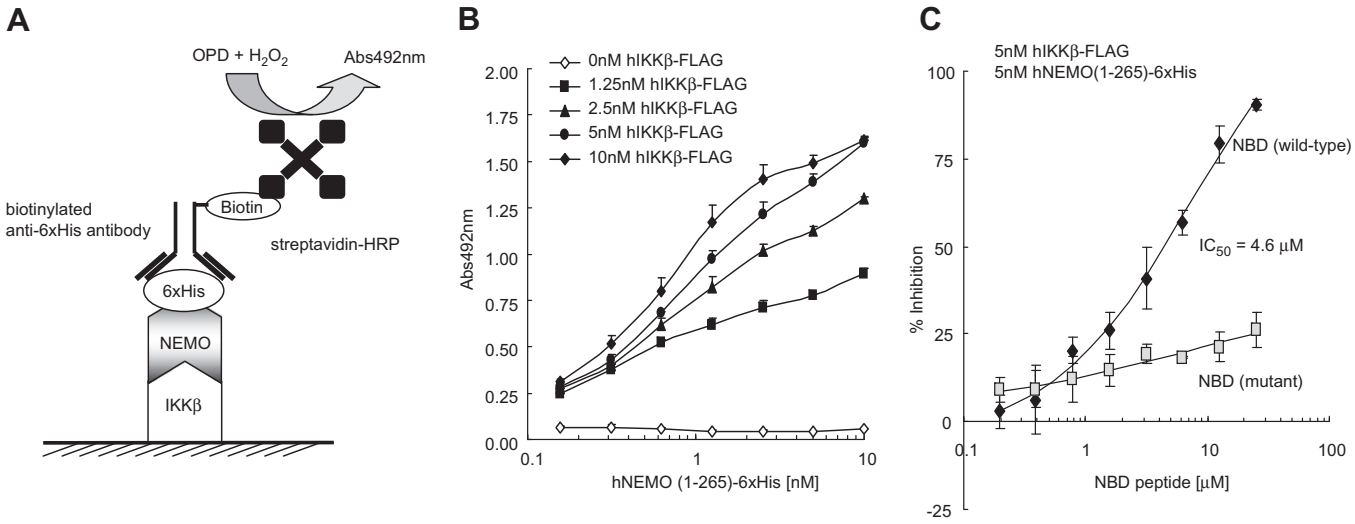


Fig. 4. ELISA-based binding assay for detection of IKKβ–NEMO interaction. (A) Schematic diagram. OPD, *o*-phenylenediamine. (B) ELISA signals across hNEMO (1–265)-6×His titration with various concentrations of hIKKβ-FLAG. (C) Inhibitory effect of NBD peptide on interaction between 5 nM hIKKβ-FLAG and 5 nM hNEMO (1–265)-6×His. The data are means (± standard deviations, $n = 4$) and were fitted using nonlinear least squares regression analysis.

throughput is higher than that of the immunoprecipitation-based assay. As shown in Fig. 4B, an increase in the concentration of either hIKKβ-FLAG or hNEMO (1–265)-6×His resulted in an increase in ELISA signal (492 nm absorbance), which stayed linear for a concentration range up to 5 nM for both proteins. To obtain a sufficient assay window, the concentration of hIKKβ-FLAG, as well as that of hNEMO (1–265)-6×His, was fixed at 5 nM. In this ELISA-based assay, NBD (wild-type) peptide inhibited IKKβ–NEMO interaction with an $IC_{50} = 4.6$ μM, although NBD (mutant) peptide showed only weak inhibition of this interaction (Fig. 4C).

Inhibition of IKK complex kinase activity by NBD peptide

To examine whether NBD peptide inhibits IKK complex kinase activity, we developed an in vitro IKK activity assay. The IKK complex was purified from CHO-K1 cells transiently coexpressed with IKKβ-FLAG and full-length NEMO. As shown in Fig. 5A, IKKβ-FLAG and NEMO were detected in this purified complex. But endogenous IKKα was not detected in this purified complex (Fig. 5A). To optimize the assay format, dose dependency of purified IKK complex was analyzed. As shown in Fig. 5B, an increase in the concentration of purified IKK complex resulted in an increased signal that stayed linear for a concentration range up to 50 μg/ml. Furthermore, this signal increase was time dependent for up to 45 min (Fig. 5C). Therefore, the assay condition was fixed as follows: the concentration of purified IKK complex was 40 μg/ml, and the reaction time was 30 min. In this assay format, NBD (wild-type) peptide inhibited IKK complex kinase activity with an $IC_{50} = 28.5$ μM, although NBD (mutant) peptide showed only weak inhibition of this activity (Fig. 5D). These results are consistent with those of the HTRF-, immunoprecipitation-, and ELISA-based binding assays. Because NBD (wild-type) peptide is known to inhibit IKK complex activity through dissociation of IKKβ–NEMO interaction, it is believed that the inhibitors of IKKβ–NEMO interaction inhibit IKK complex function, leading to NF-κB activation and subsequently inflammation.

Pilot HTS with approximately 15,000 compounds and hit conformation

To obtain small molecule inhibitors of IKKβ–NEMO interaction, approximately 15,000 compounds (48 plates, 384-well plate format, and final concentration of 10 μg/ml) from our chemical library were screened using the HTRF-based binding assay. The *S*/*B* value ranged from 8.4 to 27.3, and the average for the 48 plates was 21.5 with a standard deviation of 3.9 (Fig. 6A). The Z' factor ranged from 0.85 to 0.97 with an average of 0.93 (Fig. 6A). These results suggest that this pilot HTS was robust and consistent. As a result of screening and retest assay, we identified 11 compounds ("hit" rate of 0.07%) that showed more than 30% inhibition of IKKβ–NEMO interaction. These 11 hit compounds were confirmed by evaluating their inhibitory activity on IKKβ–NEMO interaction in the ELISA-based binding assay. Of the 11 compounds, eight showed dose-dependent inhibition of IKKβ–NEMO interaction. The 11 hit compounds were also evaluated using IKK complex kinase assay. Of the 11 compounds, seven showed dose-dependent inhibition of IKK complex kinase activity. These seven compounds were among the eight compounds found to inhibit IKKβ–NEMO interaction in the ELISA-based binding assay (Fig. 6B). These results suggest that compounds that inhibit IKK complex kinase activity act through inhibition of IKKβ–NEMO interaction and dissociation of IKK complex formation. As shown in Fig. 6C, the IC_{50} values of NBD (wild-type) peptide and six hit compounds of the kinase assay were higher than those of the ELISA-based assay. On the other hand, the IC_{50} values of compound G were virtually equivalent in the two assays. These results indicated that our screening cascade, which consists of an HTRF-based binding assay, an ELISA-based binding assay, and an IKK complex kinase assay, is useful in the discovery of novel inhibitors of IKK function.

Discussion

We have shown that a cascade of novel assays (i.e., HTRF-based binding assay, ELISA-based binding assay, and IKK complex kinase assay) is a useful tool in the HTS of compounds that inhibit the interaction between IKKβ and NEMO. Inhibition of this interaction, which interferes with IKK-NF-κB activation, has recently received particular attention as a possible new therapeutic approach to treatment of inflammatory and autoimmune disorders such as rheumatoid arthritis.

Small molecule inhibitors of IKK, which are generally screened by traditional in vitro kinase activity assays, generally act by competing with ATP in the ATP binding pocket that is shared among all kinases. This competition makes it difficult to isolate selective kinase inhibitors due to the potential for cross-reactivity to other kinases. Despite this drawback, a number of compounds have been

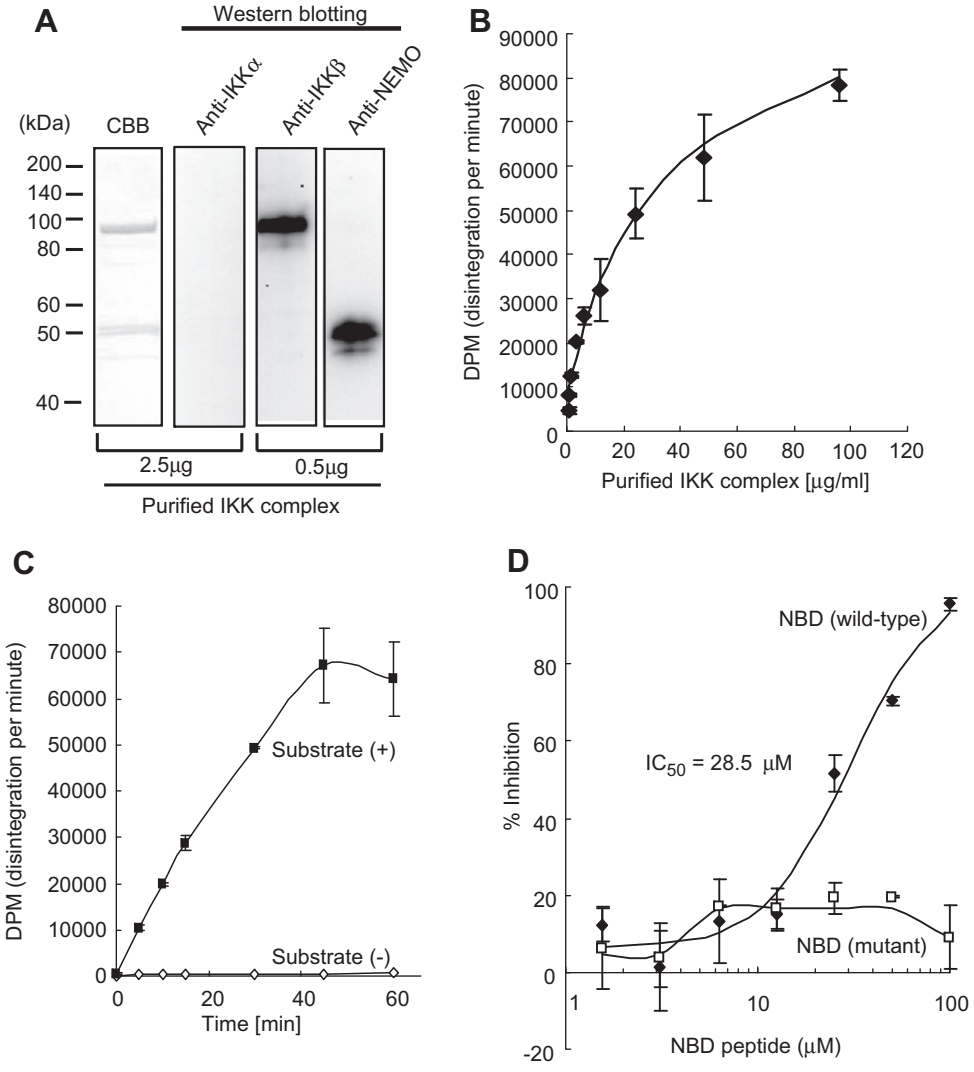
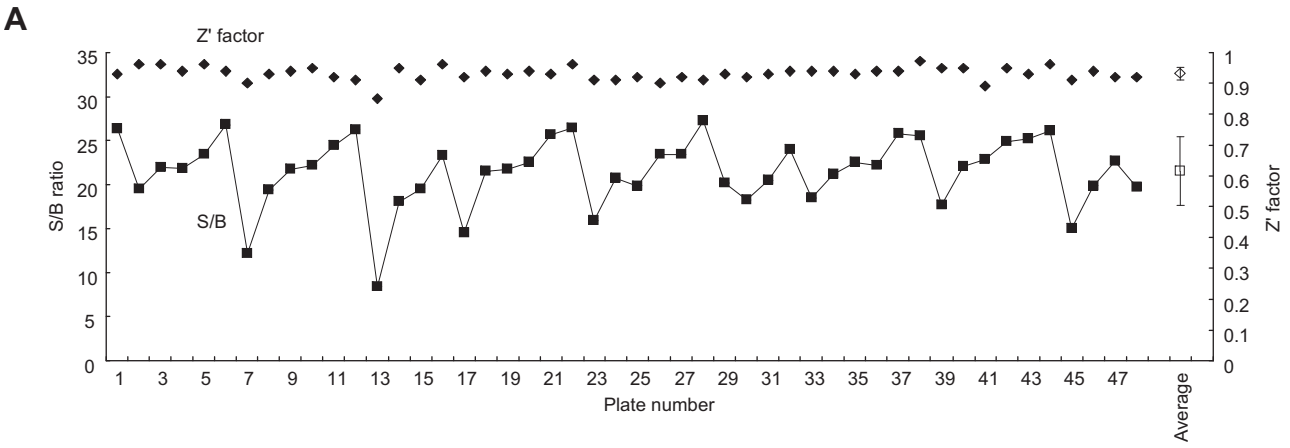


Fig. 5. In vitro kinase assay using purified IKK complex. (A) The purified IKK complex was analyzed with SDS–PAGE (Coomassie Brilliant Blue [CBB] staining) and Western blotting (anti-IKKβ, anti-IKKβ, or anti-NEMO). (B) Dose dependency of purified IKK complex. The reaction time was 30 min. The data are means (± standard deviations, $n = 3$). (C) Time course of purified IKK complex activity (40 μg/ml). (D) Inhibitory effect of NBD peptide on purified IKK complex kinase activity.

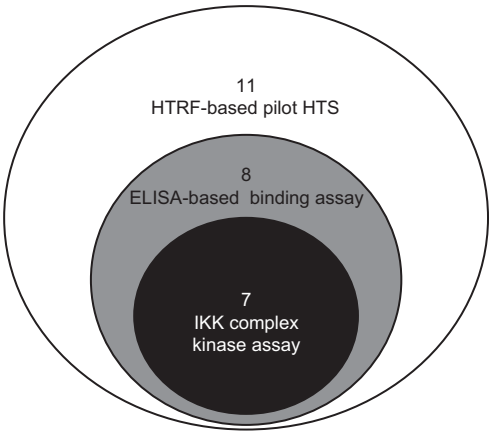
reported to specifically inhibit IKKβ [24–27]. On the other hand, inhibitors of IKK complex are thought to be more selective because IKKβ interacts with NEMO through a unique site called the NBD. Activation of IKK complex in response to inflammatory mediators depends on the presence of NEMO in the complex. For example, NEMO-deficient cells lack detectable NF-κB activity in response to TNFα, IL1β, and LPS [28]. In addition, recent studies have shown that continuous administration of NBD peptide effectively ameliorates inflammatory response in animal models without overt side effects such as liver or kidney toxicity [17,19]. In fact, NBD peptide does not influence other alternative pathways (those distinct from the canonical pathway) for NF-κB activation, thereby minimizing potential toxicity concerns. Taken together, inhibition of the interaction between IKKβ and NEMO appears to be a truly promising approach to selective suppression of NF-κB activation selectively under pathological conditions.

In this study, we validated the HTRF-based binding assay, which can be performed in a simple "mix-and-measure" format, as a robust and reliable system for the discovery of small molecule compounds that inhibit the interaction between IKKβ and NEMO. Because this assay is more high-throughput than previous assays, such as the immunoprecipitation-based assay and the ELISA-based binding assay, it is very suitable for HTS. In the HTRF-based binding assay, we used purified proteins that were tagged by FLAG or 6×His to bear time-resolved fluorescence resonance energy transfer (TR–FRET) signal effectively. Using a LANCE TR–FRET system, Rushe and coworkers recently reported a method that measures interaction between GST-NEMO and biotinylated peptides that consist of only 45 amino acids at the C-terminal region of IKKα or IKKβ [22]. However, we could develop the HTRF-based binding assay system using the full-length protein of IKKβ. Therefore, our system may have higher accuracy in screening various types of inhibitors, including those that can bind to IKKβ at different regions from the NBD site, causing structural change in IKKβ and collapse of the IKK complex. In addition, the HTRF-based binding assay might be useful in identification of other compounds that act on pharmaceutical targets based on protein–protein interaction. This principle can be applied not only to protein–protein interaction but also to cell surface binding between membrane receptors and their ligands.

As shown in Fig. 3B, both NBD (wild-type) peptide and manumycin A inhibited the interaction between IKKβ and NEMO. However, the slope of the dose–response curve of manumycin A is more moderate than that of NBD (wild-type) peptide (Fig. 3B). NBD



B



C

	HTRF (HTS assay)	ELISA	Kinase assay
	% Inhibition @20μM	IC_{50} (μM)	IC_{50} (μM)
Compound A	66.5%	6.7	24.0
Compound B	39.5%	17.1	23.1
Compound C	78.0%	0.02	<1.0
Compound D	79.2%	0.02	<1.0
Compound E	54.9%	24.8	>36.0
Compound F	69.1%	5.5	15.4
Compound G	66.1%	1.1	1.7
NBD (wild-type)	(IC_{50}=0.68 μM)	4.6	28.5

Fig. 6. Summary of pilot HTS of approximately 15,000 compounds (48 assay plates). (A) *Z′* factors and *S*/*B* ratios of assay plates. The closed diamonds are *Z′* factors of each plate, and the open diamond is the average (± standard deviation) of all plates. The closed squares are *S*/*B* ratios of each plate, and the open square is the average (± standard deviation) of all plates. (B) Summary of the hit compounds through our screening cascade. (C) Table of inhibitory potencies of the seven screening hits and NBD (wild-type) peptide in our three assay formats.

(wild-type) peptide was thought to interact with the IKKβ binding site of NEMO. Due to this competitive action, NBD (wild-type) peptide could inhibit the interaction between IKKβ and NEMO. On the other hand, it has been reported that manumycin A produces the covalent homotypic dimerization of IKKβ, that this stable IKKβ dimer does not bind to NEMO, and that manumycin A consequently disrupts IKK complex formation and inhibits IKK complex kinase activity [23]. Therefore, the inhibition style of manumycin A is thought to be different from that of NBD peptide that binds to NEMO. Because of this difference, the slope of the dose–response curve of manumycin A may be more moderate than that of NBD (wild-type) peptide.

As shown in Fig. 6C, the NBD (wild-type) peptide showed different IC_{50} values in three assays. In general, in the HTRF system, two fluorophores (i.e., Eu^{3+} cryptate and XL665) must be in close proximity to bear the sufficient FRET signal. Therefore, only direct and close interaction between IKKβ and NEMO may be detected in the HTRF system. On the other hand, in the ELISA-based binding assay system, not only the NEMO directly binding to IKKβ but also the NEMO binding indirectly to IKKβ may be detected because NEMO could form homo-oligomers and the interaction between IKKβ and NEMO oligomers was detected in this ELISA system. Furthermore, the NBD (wild-type) peptide does not inhibit the interaction between NEMO and NEMO. Therefore, the IC_{50} value of NBD peptide of the ELISA-based assay was higher than that of the HTRF-based assay.

Besides, the NBD (mutant) peptide slightly inhibited the interaction between IKKβ and NEMO in the ELISA-based assay but not in the HTRF-based assay. One reason for this inhibition in ELISA assay is that the NBD (mutant) peptide might interact with NEMO weakly and influence the interaction between NEMO and NEMO. In addition, in the IKK complex kinase assay, the NBD (wild-type) peptide was thought to inhibit the IKK complex kinase activity through the collapse of the interaction between IKKβ and NEMO. Therefore, the IC_{50} values of NBD peptide and six hit compounds of the IKK complex kinase assay were higher than those of the ELISA-based assay. On the other hand, the IC_{50} values of compound G were virtually equivalent in these two assays. This might mean that the inhibition style of compound G is different from that of NBD (wild-type) peptide and the other hit compounds.

In conclusion, we have shown that an HTRF-based binding assay, used in combination with an ELISA-based binding assay and an IKK complex kinase assay, is suitable for HTS of compounds that inhibit IKKβ–NEMO interaction. Using this assay, we identified seven hit compounds that inhibit IKK complex activity through inhibition of IKKβ–NEMO interaction. This inhibition may prove to be useful in the treatment of inflammatory and autoimmune disorders. It should be noted, however, that all of the data collected in this study were from a cell-free assay system and that the positive results obtained should be confirmed in a cell-based assay and in vivo studies. It would also be useful to perform full HTS of our chemical library and the follow-up assays so as to identify various

types of chemical scaffolds. Our goal from all of these studies would be to identify novel therapeutic agents for the treatment of inflammatory diseases.

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