Supporting Information

Irreversible Nek2 kinase inhibitors with cellular activity

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Mutagenesis and protein expression

Bacterial expression plasmids (pET22b, Invitrogen) containing N-terminally His6-tagged Nek2 kinase domain (aa 1-271) and full length Nek2, were previously reported.[1] These Nek2 constructs contain a T175A mutation required to render the kinase non-toxic to E. Coli. Expression plasmids for C22V Nek2 kinase domain, and full length C22V Nek2 were prepared by performing quickchange mutagenesis on the parent pET22b vectors using the following primers:
5'-ggctcctacggccgcgtccagaagatccggag-3' and 5'-ctccggatcttctggacgcggccgtaggagcc-3' (Operon). Expression and purification of all Nek2 protein constructs was carried out according to a previously reported procedure.[1]

Generation of stable cell lines

PCR was used to install Gateway recombination sequences and a triple C-terminal HA-tag onto the 3'-end of WT or C22V Nek2 using the following three oligonucleotide primers in one reaction (PCR with one forward and two overlapping reverse primers).
Nek2 forward primer
5'-ggggacaagtttgtacaaaaaagcaggctgcggccgcaccatgccttcccgggctgagg
Nek2 reverse primer (used at 1 mol% of the other primers)
5'-cgcatagtcagggacgtcataaggatatccagcgtaatctggaacatcataaggatatccgcgcatgcccaggatctgtc
Reverse primer(2)
5'-ggggaccactttgtacaagaaagctgggtggggatcctaagcgtaatctggaacatcgtatgggtagcccgcatagtca gggacgtcat

BP Clonase-mediated recombination was used to insert the PCR products into the Gateway donor vector pDONR221 (Invitrogen). LR Clonase-mediated recombination was then used to shuttle the Nek2 constructs from pDONR221 into a version of the tet-inducible expression vector pCDNA5/FRT/TO (Invitrogen) that had previously been modified to contain the appropriate Gateway recombination sequences. Tetracycline-inducible Hek293 cell lines expressing C-terminal triple HA-tagged WT or C22V Nek2 were made using the Invitrogen Flp-in 293 T-rex system. This was achieved by co-transfecting Flp-in 293 T-rex cells (Invitrogen) with pOG44 (Invitrogen) and the Nek2 containing expression plasmid pCDNA5/FRT/TO. After transfection, hygromycin was used to select for stable cell lines.

Preparation of Poly-L-lysine coated coverslips

Poly-L-lysine coated coverslips were prepared by treating coverslips (Fisherbrand) with 1 M HCl at 40 °C for 4 h, washing three times with water, rocking in 0.1% poly-L-lysine solution (Sigma) at room temperature for 1 h, then washing three times with water and once with ethanol. Next, coverslips were air dried and stored at 4 °C. Coated coverslips were sterilized under a germicidal lamp and washed with tissue culture media prior to use.

Immunofluorescence microscopy

After removal of growth media, cells were formaldehyde-fixed for 10 min at room temperature with fixing buffer (80 mM PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X100, 4% formaldehyde) and blocked for 30 min with blocking buffer (20 mM TRIS pH 7.4, 150 mM NaCl, 0.1% Triton X100, 2% BSA, 0.1% sodium azide).

Cells were then stained with 1:200 anti-phospho-Ser10 histone-H3 antibody (Millipore) in blocking buffer for 1h, then washed with TBS-TX100 (20 mM TRIS pH 7.4, 150 mM NaCl, 0.1% Triton X100). Next, cells were stained with a mixture of 1:500 Alexa Fluor-A594 conjugated anti-rabbit antibody (Molecular Probes) and 1:500 anti-α-tubulin FITC-conjugated antibody (Sigma) in blocking buffer for 1 h, then washed with TBS-TX100. Next, cells were stained with 500 ng/μL of DAPI (Sigma) in TBS-TX100 for 10 min. Following DAPI staining, cells were washed with TBS-TX100 and mounted in Vectashield mounting media (Vector Labs). Images were captured using a Carl Zeiss Axiovert 200 inverted fluorescence microscope equipped with a C4742-98 CCD camera (Hamamatsu). Images were acquired and processed using Slide Book (Intelligent Imaging Innovations) and Photoshop software (Adobe).

Western blot analysis

Beads from immunoprecipitations were suspended in 30 μL of kinase buffer (20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1x PhosSTOP), then treated with 8 μL of sample buffer (300 mM TRIS pH 6.8, 10% SDS, 50% glycerol, 0.01% bromophenol blue, 0.5 M DTT) followed by NuPAGE Antioxidant (Invitrogen), and heated to 65 °C for 5 min. Samples were resolved on NuPAGE Novex Bis-Tris 4-12% gradient gels (Invitrogen) and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% milk in TBST (50 mM Tris pH 7.6, 150 mM NaCl, 0.05% tween 20) for 1 h and washed three times with TBST.

For Western blot detection of endogenous Nek2, blocked membranes were treated with 1:500 anti-Nek2 antibody (610593, BD Biosciences) in AB-Dil (50 mM Tris pH 7.6, 150 mM NaCl, 0.05% tween 20, 0.1% NaN_3, 2% BSA) for 1 h and washed three times

with TBST. Membranes were then treated with 1:1000 anti-mouse-HRP conjugated antibody (Santa Cruz Biotech) in 5% milk-TBST for 30 min, washed three times with TBST, and detected using Supersignal West Femto Substrate (Thermo Scientific).

For Western blot detection of Nek2-3HA, blocked membranes were treated with 1:2000 anti-HA antibody (SAB4300603, Sigma) in AB-Dil for 1 h and washed three times with TBST. Membranes were then treated with 1:1000 anti-rabbit-HRP antibody (Santa Cruz Biotech) in 5% milk-TBST for 30 min, washed three times with TBST, and detected using Supersignal West Pico Substrate (Thermo Scientific).

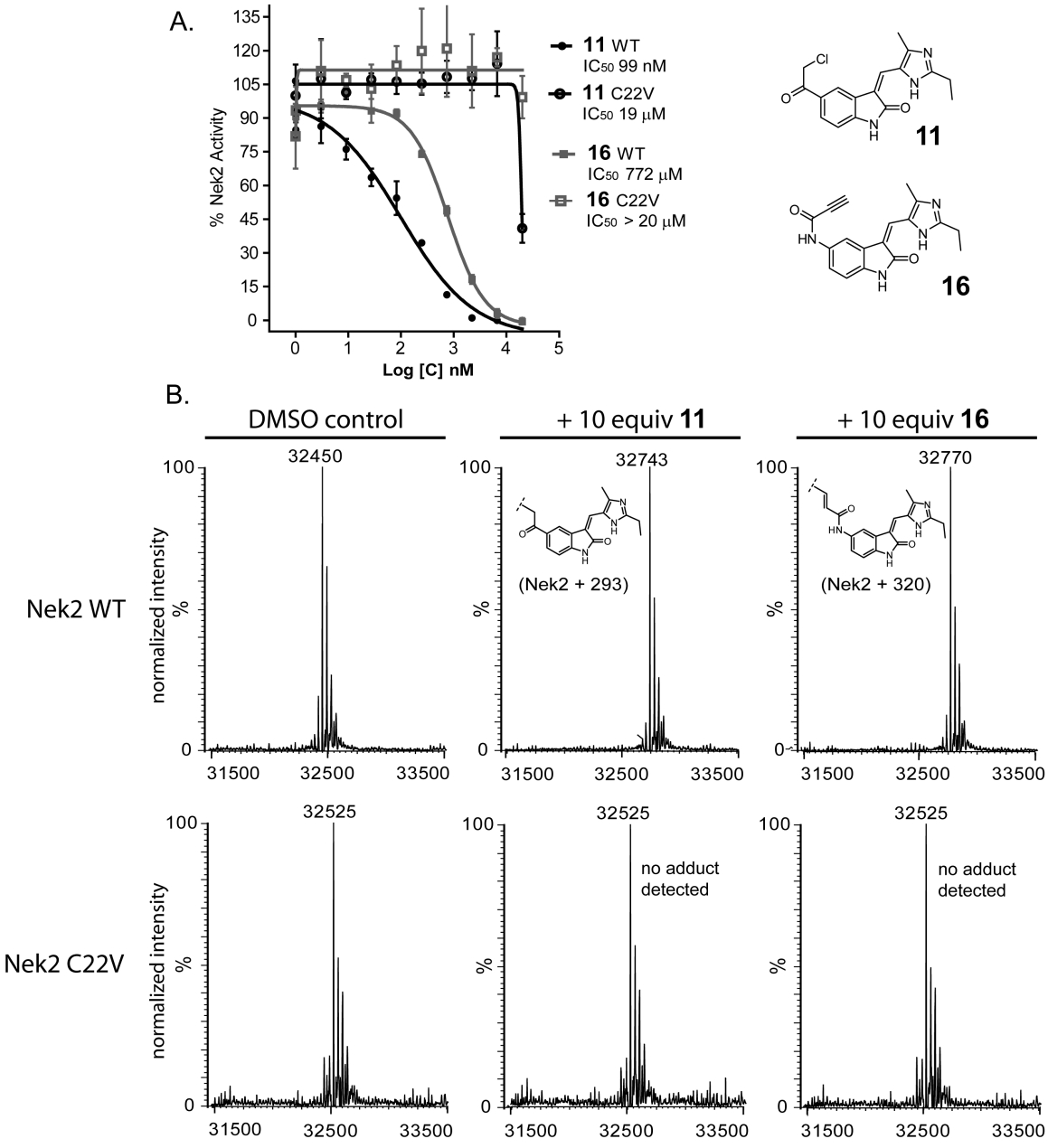

Figure 1S. (A) Dose-response curves from in vitro kinase assays with **11** and **16** vs. WT and C22V Nek2. (B) Electrospray mass spectrometry reveals stoichiometric alkylation of the Nek2 kinase domain (aa 1-271) by compounds **11** and **16** (10 equiv).

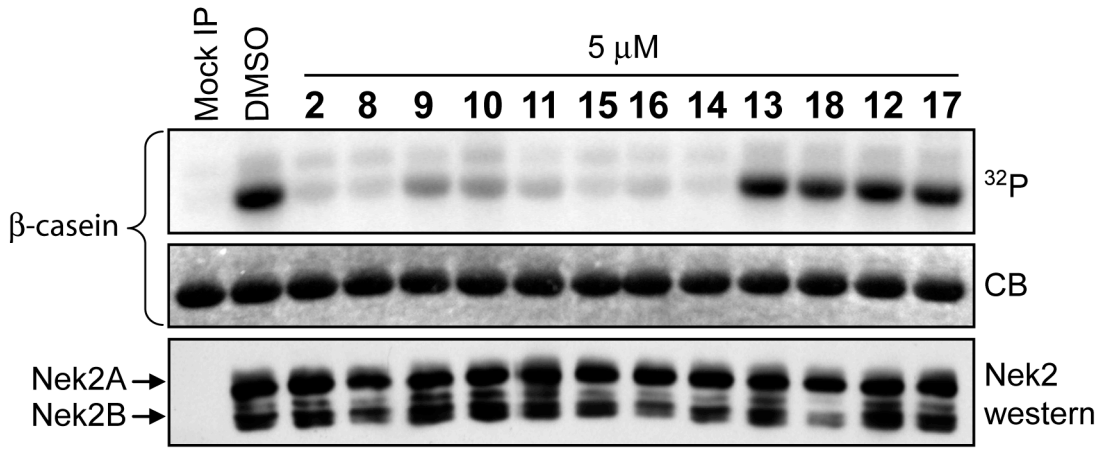

Figure 2S. IP kinase assays of endogenous Nek2 derived from A549 cells that had been pre-treated with 5 μM of the indicated compounds for 45 min.

Supporting Reference

(1) Rellos, P.; Ivins, F. J.; Baxter, J. E.; Pike, A.; Nott, T. J.; Parkinson, D-M.; Das, S.; Howell, S.; Fedorov, O.; Shen, Q. Y.; Fry, A. M.; Knapp, S.; Smerdon, S. J. Structure and regulation of the human Nek2 centrosomal kinase. *J. Biol. Chem.* **2006**, *282*, 6833–6842.