LETTER TO THE EDITOR

Novel PROTAC to target FKBP12: the potential to enhance bone morphogenetic protein activity and apoptosis in multiple myeloma

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https://doi.org/10.3324/haematol.2024.287047

Received: November 25, 2024.
Accepted: April 9, 2025.
Early view: April 17, 2025.

Supplementary Figures

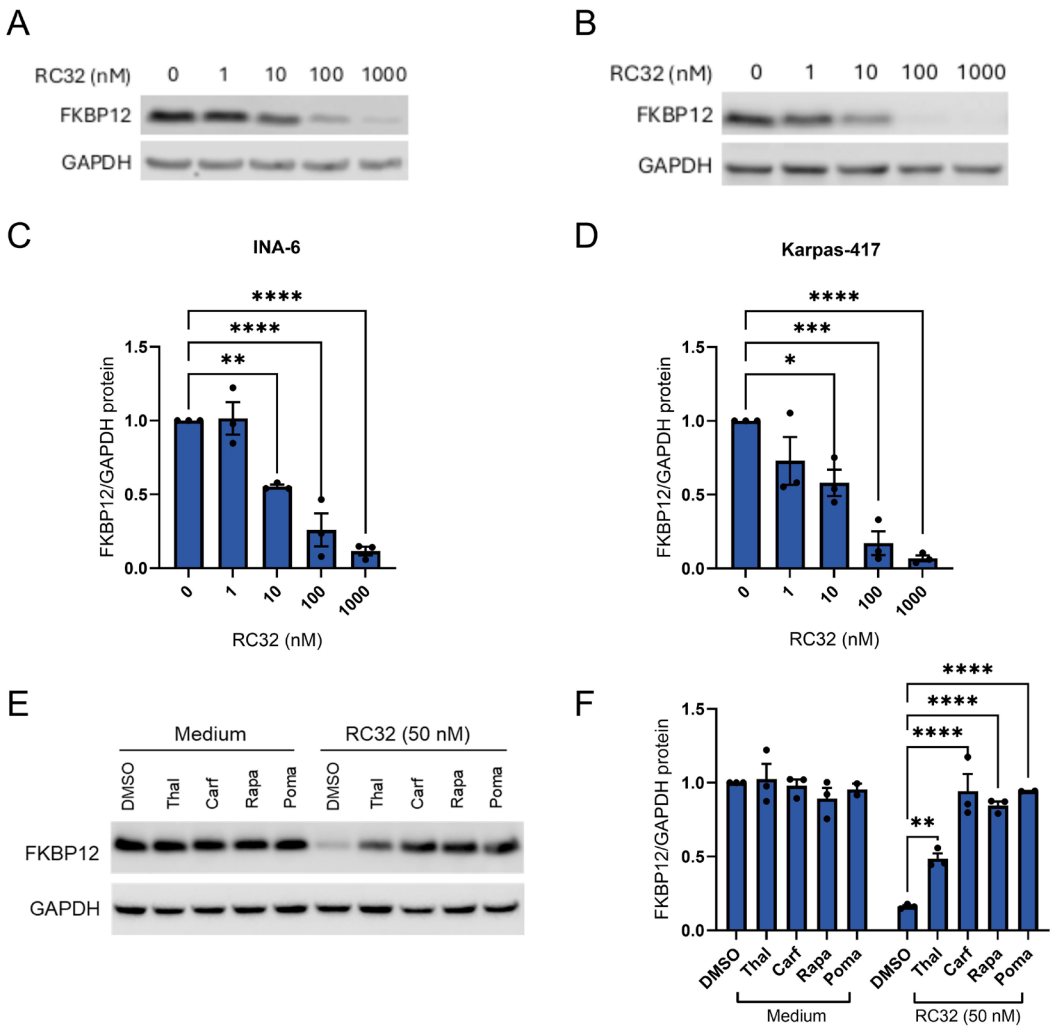

Figure S1. FKBP12 PROTACs dose-dependently degraded FKBP12 protein. FKBP12 protein expression was measured using western blot in INA6 (A) and Karpas-417 (B) after treatment with increasing doses of RC32 for 4 h. GAPDH was used as loading control and the FKBP12 signals relative to GAPDH were measured and plotted (C, D). The graphs show the mean with SEM of n=3 independent experiments. (E) INA-6 cells were pretreated with thalidomide (Thal, 100 µM), carfilzomib (Carf, 1 µM), rapamycin (Rapa, 0.5 µM), or pomalidomide (Poma, 100 µM) for 0.5 h, before incubating with or without RC32 (50 nM) for 4 h. Western blotting was done with FKBP12 (RRID: AB_2102847, #SC-133067, Santa Cruz, TX, USA) and GAPDH (RRID: AB_2107448, #Ab8245, Abcam, Cambridge, UK) primary antibodies. The result shown is one of n=3 independent experiments, except Poma (n=2), and in (F) the relative signal intensities of all experiments are shown as the mean and SEM. One-way ANOVA (C, D) or mixed-effects analysis (F,) with Dunnett's multiple comparisons test was used to test significance, *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, and ****; $p < 0.0001$.

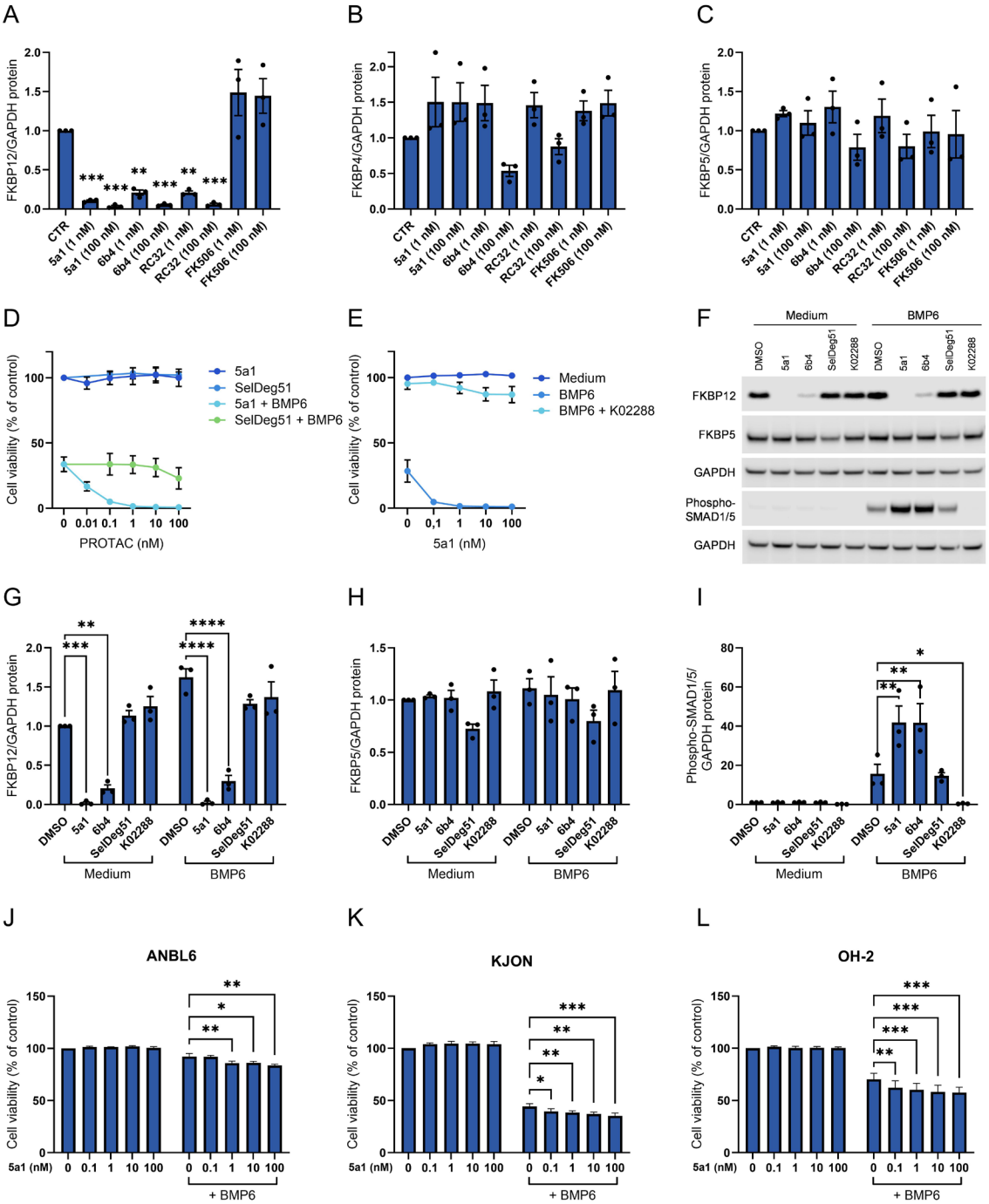

Figure S2. Effects of FKBP12-targeting PROTACs compared with FK506 and SelDeg51. The relative signal intensities of western blot in Fig. 2A; (A) FKBP12, (B) FKBP4, and (C) FKBP5, normalized to GAPDH and control. D. INA-6 cells were treated with increasing doses of 5a1 or SelDeg51, with and without BMP6 (7,5 ng/mL) for 72 h before measuring cell viability with CellTiter-Glo. E. INA-6 cells were treated with increasing doses of 5a1 or SelDeg51, with and without BMP6 (7,5 ng/mL) or K02288 (100 nM) for 72 h before measuring cell viability with CellTiter-Glo. F. INA-6 cells were treated with 5a1, 6b4, SelDeg51, or K02288, all at 100 nM, for 4 h, before incubating with or without BMP6 (7.5 ng/mL) for 1

h. Then, western blotting was done using primary antibodies targeting FKBP12 (RRID: AB_2102847, #SC-133067, Santa Cruz, TX, USA), FKBP5 (RRID:AB_2797846, #12210), phospho-SMAD1/5 (RRID: AB_491015, #9516) (Cell Signaling Technology, BioNordika AS, Oslo, Norway), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (RRID: AB_2107448, #Ab8245, Abcam, Cambridge, UK). One representative out of n=3 independent experiments is shown. The signal intensities relative to GAPDH of all n=3 experiments are shown for (G) FKBP12, (H) FKBP5, and (I) phospho-SMAD1/5. The three human myeloma cell lines ANBL6 (J), KJON (K), and OH-2 (L) were treated with increasing doses of 5a1 with and without BMP6 (100 ng/mL) for 72 h before cell viability was measured using CellTiter-Glo luciferase assay. All graphs show the average and SEM for n=3 independent experiments. One-way (A-C) or two-way (G-L) ANOVA and Dunnett's multiple comparisons test was used to test significance, *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, and ****; $p < 0.0001$.

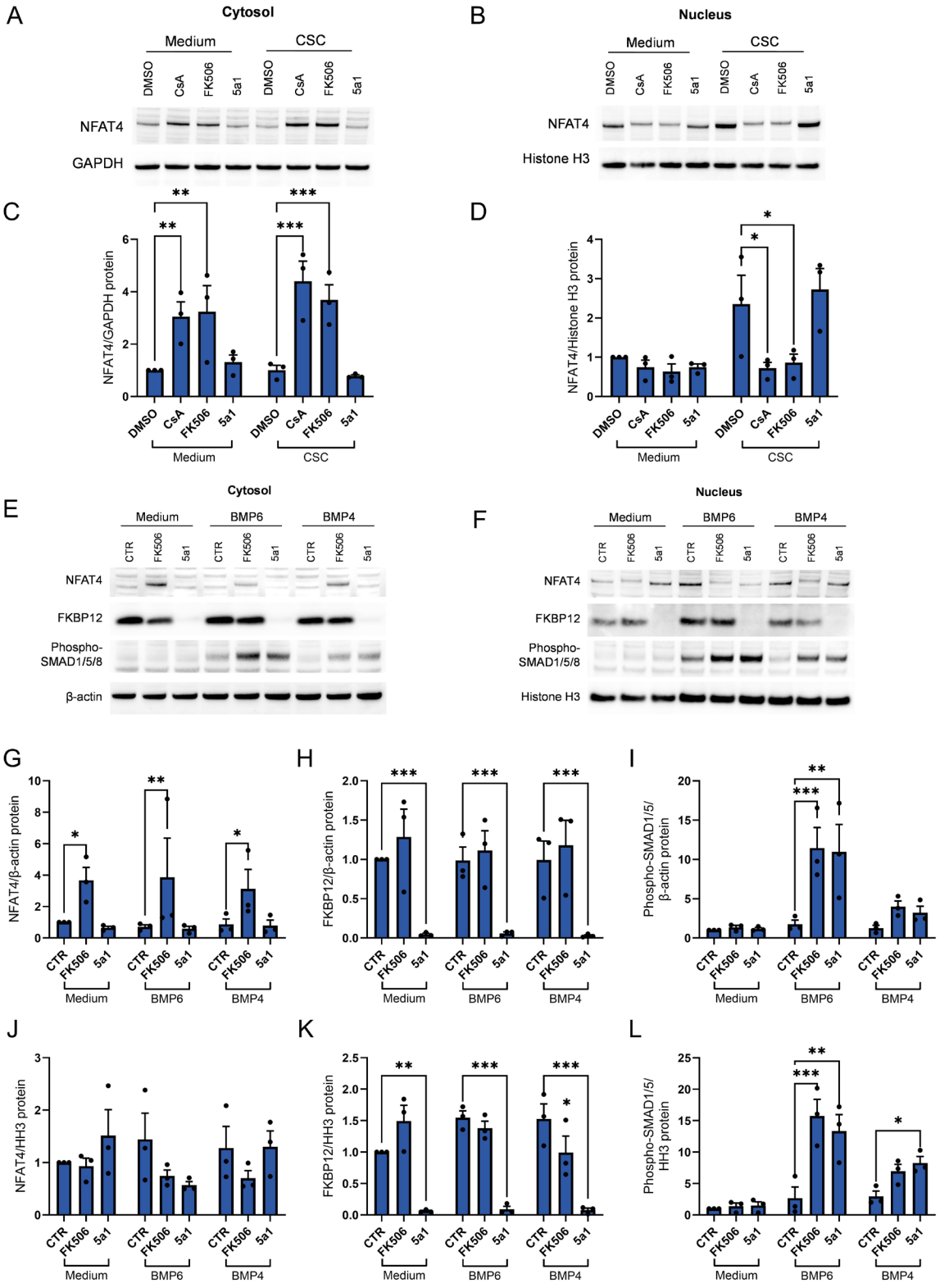

Figure S3. FKBP12-targeting PROTACs did not affect calcineurin activity. The Jurkat T-cell line was treated for 1 h with or without Cell Stimulation Cocktail (CSC) (eBioscience, #00-4975) that contains phorbol 12-myristate 13-acetate and ionomycin, combined with the calcineurin inhibitor cyclosporin A (CsA, 1 µM), FK506 (100 nM) or 5a1 (1 µM). Then, western blotting was performed on cytoplasmic

(A) and nuclear (B) fractions using primary antibodies targeting NFAT4 (RRID: AB_2152771, #4998, Cell Signaling Technology), GAPDH (RRID: AB_2107448, #Ab8245, Abcam) and Histone H3 (RRID: AB_331563, #9715, Cell Signaling Technology). C-D shows densitometric analysis of n=3 independent experiments as in A and B with the average and SEM. Then, INA-6 cells were treated with FK506 (100 nM) or 5a1 (100 nM) with and without BMP6 (7.5 ng/mL) or BMP4 (20 ng/mL) for 18 h. Western blotting was performed on cytoplasmic (E) and nuclear (F) fractions. The primary antibodies used were FKBP12 (RRID: AB_2102847, #SC-133067, Santa Cruz), phospho-SMAD1/5 (RRID: AB_491015, #9516), NFAT4 (RRID: AB_2152771, #4998), β-actin (RRID: AB_2223172, #4970) and Histone H3 (RRID: AB_331563, #9715), all Cell Signaling Technology. One representative out of n=3 independent experiments is shown. G-I (cytosol) and J-L (nucleus) show densitometric analysis of n=3 independent experiments as in E and F with the average and SEM. Two-way ANOVA and Dunnett's multiple comparisons test were used to test significance, *; $p < 0.05$, **; $p < 0.01$, and ***; $p < 0.001$.