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

Targeting FKBP12 with FK506 (tacrolimus) promotes the activity of bone morphogenetic proteins (BMP) to induce apoptosis in multiple myeloma. However, to avoid the immunosuppressive properties of FK506 in myeloma patients, other methods are required. Here, we show how two novel PROteolysis TArgeting Chimeras (PROTAC) efficiently potentiate BMP-induced activity and apoptosis in multiple myeloma cells.

Multiple myeloma is characterized by uncontrolled clonal expansion of plasma cells in the bone marrow, leading to osteolytic bone lesions, renal failure, anemia, and hypercalcemia.[1] Although new therapies have increased overall survival to ten years for transplant eligible patients, myeloma remains incurable, as most patients become resistant to all available therapies.[1] The growing number of patients living with multiple myeloma underscores an urgent need for new therapies.

Bone morphogenetic proteins belong to the transforming growth factor (TGF)-β family that also includes TGF-β and activins.[2] BMP are found throughout the body, including in circulation and in the bone marrow, and they have essential roles in normal tissue homeostasis and tumorigenesis. The mature dimeric BMP ligands form hexameric signaling complexes with type I and type II BMP receptors.[2] In the resulting active complexes, the type I receptors are activated by phosphorylation in the GS-domain, a conserved glycine- and serine-rich juxtamembrane domain.[3]

FKBP12, encoded by *FKBP1A*, is a cytosolic immunophilin family protein that associates with the GS-domain of TGF-β type I receptors and inhibits their activity.[4] When an active ligand-receptor complex is formed, the binding specificity of the GS-domain is altered, leading to release of FKBP12, and activation of receptor-activated SMAD1 and/or SMAD5.[3] SMAD1/5 then forms heteromeric complexes with SMAD4 that translocate to the nucleus to regulate gene expression.[3] In myeloma cells, this leads to growth arrest and apoptosis via SMAD1/5-dependent downregulation of the oncogene MYC, an important survival factor for myeloma cells.[5,6] Previously, we had found that targeting FKBP12 with FK506 potentiated BMP-induced SMAD1/5 activity and apoptosis.[7] However, the complex between FK506 and FKBP12 is known to inhibit calcineurin, a positive regulator of NFAT transcription factors, thereby suppressing T-cell activity.[8] Due to the immunosuppressive potential, FK506 is not suitable as cancer treatment. We, therefore, decided to investigate protein degradation as an alternative approach to target FKBP12, which had been found to be non-immunosuppressive in earlier studies.[9]

PROTAC are designed to degrade specific proteins and have emerged as potential therapeutics for cancer.[10] PROTAC are heterobifunctional molecules consisting of a ligand that binds the target protein, a linker, and a ligand which recruits an E3 ubiquitin ligase.[10] Here, we investigated the efficacy of two novel FKBP12-targeting PROTAC[11] on BMP-induced signaling and apoptosis in myeloma cells and compared them with the commercially available PROTAC RC32 that targets FKBP12.[12] The goal was to compare the drugs' specificity and efficacy *in vitro* as a proof-of-principle. This study adheres to the Norwegian guidelines for research ethics.

The myeloma cell line INA-6 was used to investigate the importance of FKBP12 in BMP-induced apoptosis. We first knocked out the gene encoding FKBP12 (*FKBP1A*) using CRISPR/Cas9 technology. The cells were treated with BMP4, which reduces cell viability in the presence of FK506 but not on its own or with BMP6.[7] As expected, BMP4 decreased cell viability in cells depleted of FKBP12, but not in CTR knockout (KO) cells (Figure 1A). On the other hand, BMP6 reduced cell viability in both *FKBP1A* KO and CTR KO cells, but the effect was more pronounced in the *FKBP1A* KO cells. Adding FK506 to *FKBP1A* KO cells did not further reduce cell viability, suggesting that the FK506-mediated effect is via FKBP12 and not other FKBP. We also measured caspase 3/7 activity (Figure 1B) and annexin V/PI staining (Figure 1C) to confirm that the decreased number of viable cells was mainly a result of apoptosis. Of note, SMAD1/5 activation was promoted in *FKBP1A* KO cells (Figure 1D, E). Knockout of *FKBP1A* was confirmed using Western blotting (Figure 1F, G). Combined, these results confirm the importance of FKBP12 as a gatekeeper, preventing leaky signaling from BMP receptors.

We then decided to target FKBP12 protein using PROTAC. First, we tested RC32, consisting of rapamycin for FKBP12-binding and pomalidomide to recruit the E3 ubiquitin ligase cereblon.[12] RC32 dose-dependently degraded FKBP12 after only 4 hr incubation (*Online Supplementary Figure S1A-D*). The RC32 effect was counteracted by co-treating cells with thalidomide, pomalidomide or rapamycin (drugs that compete with RC32 for binding) and with the proteasomal inhibitor carfilzomib (*Online Supplementary Figure S1E, F*). In our study, we then included two novel FKBP12-targeting PROTAC: 5a1 and 6b4.[11] 5a1 consists of a novel FKBP12 binder, a linker, and a Von Hippel Lindau ligand; 6b4 has the same linker and E3 ligase, but a different FKBP12 binder. Both molecules were recently

characterized together with an FKBP5 targeting PROTAC, SelDeg51, which is here included as a control compound.[11] A co-crystal structure illustrates the 5a1 ligand-binding to FKBP12 and its exit vector (Figure 2A).[13] All PROTAC potently down-regulated FKBP12 but 5a1 was the most efficient (Figure 2B and *Online Supplementary Figure S2A*). Also of

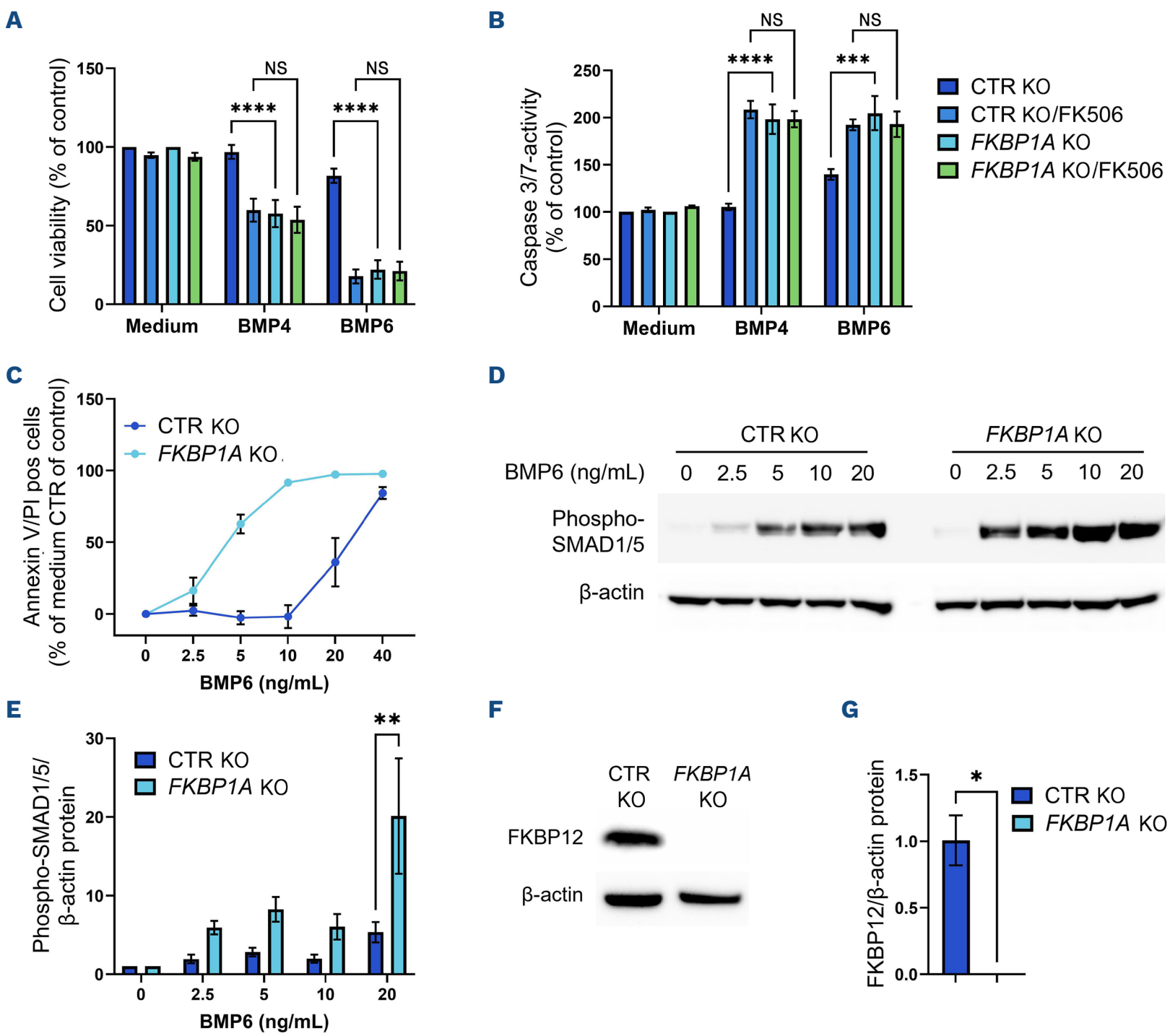


Figure 1. Depletion of FKBP12 enhanced bone morphogenetic protein-induced apoptosis in myeloma cells. (A) INA-6 CTR knock-out (KO) and *FKBP1A* KO were made using clustered regularly interspaced short palindromic repeats (CRISPR) single guide (sg) RNA targeting *FKBP1A* (GGGCGCACCTTCCCCAAGCG) and an irrelevant, targeting control (CTR), sgRNA specific for intron 2 of *UNG* (CGACCCGCGAGATGATATCA, a kind gift from Per Arne Aas, NTNU, Trondheim, Norway). The sgRNA oligo pairs were ligated into lentiCRISPR v2 (a gift from Feng Zhang; Addgene plasmid #52961; RRID:Addgene_52961). The KO cells were treated with bone morphogenetic protein (BMP)4 (100 ng/mL) or BMP6 (7.5 ng/mL) and FK506 (100 nM) for 72 hours (hr) before cell viability was measured using a CellTiter-Glo luciferase assay. (B) Cells were treated for 48 hr with BMP4 (100 ng/mL) or BMP6 (7.5 ng/mL) and FK506 (100 nM), and caspase activity was measured using Caspase-Glo 3/7 Reagent (Promega). The result is shown as relative luciferase units. (A and B) Two-way ANOVA and Tukey multiple comparisons test were used to test significance. ***P<0.001, ****P<0.0001, ns: not significant. (C) INA-6 CTR KO and *FKBP1A* KO were treated for 72 hr with BMP6. The cells were stained with annexin V and propidium iodide (PI) using Apotest Annexin A5-FITC kit (VPS Diagnostics, Hoeven, the Netherlands). The average % of cells that were positive (pos) for annexin V or PI were plotted relative to medium control (0%) with error bars representing standard error of the mean (SEM). (D) The phospho-SMAD1/5 protein levels were measured in INA-6 CTR KO and INA-6 *FKBP1A* KO treated for 1 hr with increasing doses of BMP6. The antibodies used were phospho-SMAD/5 (Ser463/465) (RRID: AB_491015, #9516) and β-actin (RRID: AB_2223172, #4970) both from Cell Signaling Technology, BioNordika AS, Oslo, Norway. (E) Graph representing signal intensities shown in (D) relative to β-actin. Two-way ANOVA and Šídák's multiple comparisons test were used to test significance. **P<0.01. (F) FKBP12 protein levels were measured in INA-6 CTR KO and INA-6 *FKBP1A* KO using antibodies targeting FKBP12 (RRID: AB_2102847, #SC-133067, Santa Cruz, TX, USA) and β-actin. (G) Graph representing signal intensities shown in (F) relative to β-actin. Significance was calculated using a paired two-sided *t* test. *P<0.05. All graphs show the mean and SEM of 3 independent experiments.

note, cells express several FKBP proteins, and some can have similar binding affinities as FKBP12 to drugs.[7] Based on such affinities, we also compared the effect of the PROTAC on protein levels of FKBP4 and FKBP5 (Figure 2B, and *Online Supplementary Figure S2B, C*). 6b4 and RC32 caused some degradation of FKBP4 and FKBP5, whereas 5a1 did not. Next, we tested the ability of the PROTAC to potentiate BMP6-induced SMAD1/5 activity using the INA-6 BRE-luc reporter cell line.[14] All the PROTAC potentiated BMP6-induced SMAD1/5 activity compared with BMP6 alone, but 5a1 and 6b4 were more potent than RC32 (Figure 2C). Using PROTAC in doses above 10 nM was not possible as this led to apoptosis of the INA-6 BRE-luc reporter cells. Control experiments including SelDeg51 and the BMP type I receptor inhibitor K02288 supported the hypothesis that 5a1 is a potent and specific degrader of FKBP12 and that the combined effect with BMP6 on cell viability is mediated via BMP6-induced SMAD1/5 activity (*Online Supplementary Figure S2D-I*).

We then performed dose-response experiments of 5a1,

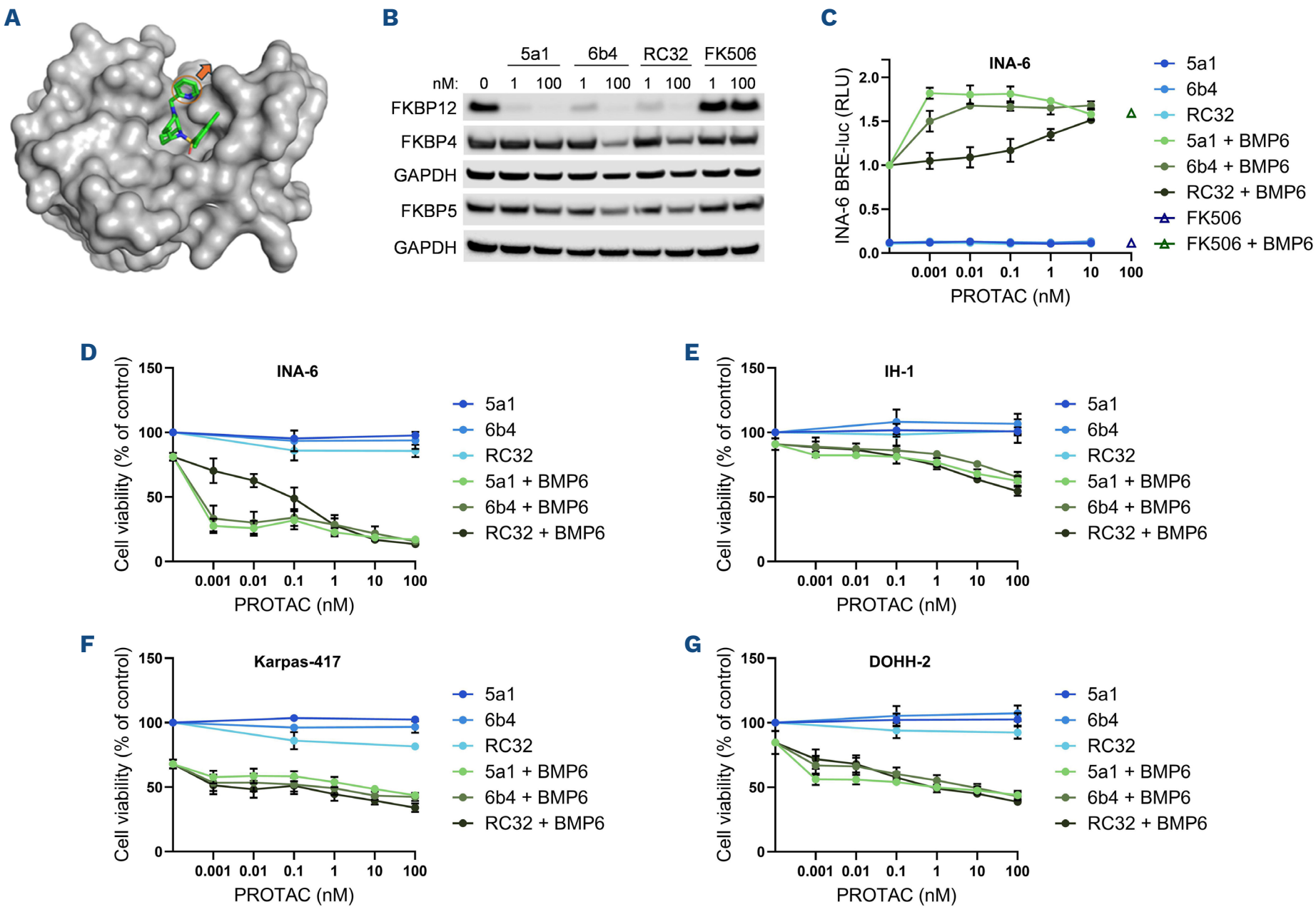
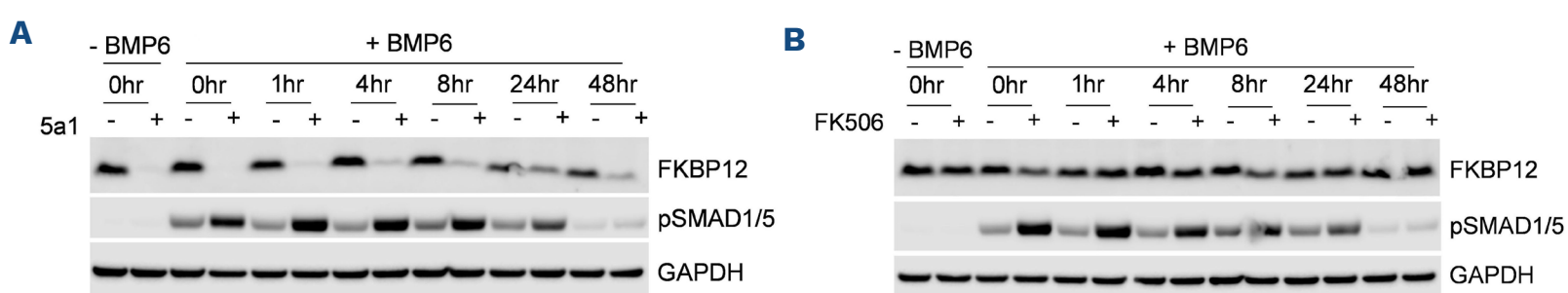


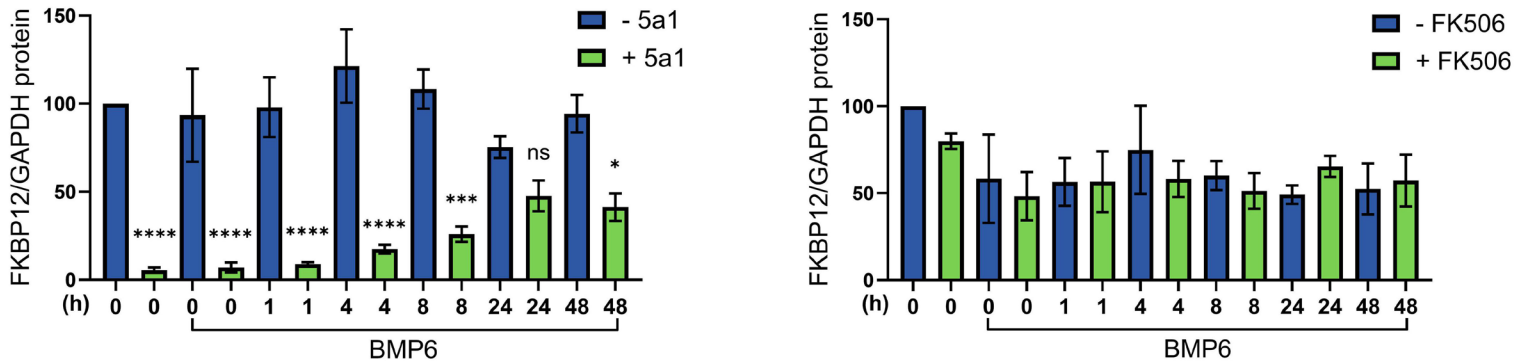
Figure 2. FKBP12-targeting PROteolysis targeting chimeras degraded FKBP12 and enhanced bone morphogenetic protein (BMP)-6-induced signaling and cell death. (A) Co-crystal structure (PDB-ID 8chl) of a prototypical [4.3.1]bicyclic sulfonamide ligand (green sticks) bound to FKBP12 (gray surface). The pyridine moiety, which is replaced by a triazole in the PROteolysis targeting chimera (PROTAC) 5a1, is circled in orange and the exit vector for the linker is indicated by the orange arrow. The structure is derived from Purder *et al.*[13] (B) The human myeloma cell line INA-6 was treated with FKBP12 PROTAC (1 or 100 nM) for 18 hours (hr) before Western blotting with primary antibodies FKBP12 (RRID: AB_2102847, #SC-133067, Santa Cruz, TX, USA), FKBP4 (RRID:AB_2797737, #11826), FKBP5 (RRID:AB_2797846, #12210) (Cell Signaling Technology, BioNordika AS, Oslo, Norway), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (RRID: AB_2107448, #Ab8245, Abcam, Cambridge, UK). The image represents one of 3 independent experiments. Densitometric analysis based on all 3 replicates can be found in *Online Supplementary Figure S2A-C*. (C) INA-6 BRE-luc cells (50,000/well) were seeded in RPMI with 0.1% bovine serum albumin and interleukin-6 (1 ng/mL) and treated with increasing doses of PROTAC alone (blue lines/circles) or with bone morphogenetic protein (BMP)6 (7.5 ng/mL, green lines/circles) for 18 hr. FK506 (100 nM) was included as an internal control both alone and with BMP6 (green or blue triangle). The luciferase activity was measured using BriteLite™ Plus Luciferase Detection Reagent (PerkinElmer Inc.) and plotted relative to BMP6 alone. Cell lines, INA-6 (D), IH-1 (E), Karpas-417 (F), and DOHH-2 (G) were treated with increasing doses of the FKBP12 targeting PROTAC 5a1, 6b4, and RC32, combined with BMP6 (7.5 ng/mL) for 72 hr before measuring cell viability with CellTiter-Glo (Promega). The graphs show the average and standard error of the mean (SEM) for 3 independent experiments.

6b4, and RC32 with a fixed dose of BMP6 in three myeloma cell lines and one diffuse large B-cell lymphoma cell line (Figure 2D-G). As expected, none of the PROTAC influenced cell viability on their own. Three additional myeloma cell lines were tested with BMP6 and 5a1 (*Online Supplementary Figure S2J-L*). The PROTAC enhanced BMP6-induced loss of cell viability in all cell lines tested. The effects were most pronounced in the INA-6 cell line; for RC32, we saw a clear effect down to the upper pM range, whereas 5a1 and 6b4 were potent even down to the lower pM range (Figure 2D). This is consistent with the results seen on signaling and support the view that using PROTAC is an efficient way of targeting FKBP12 *in vitro*.

To assess the potential calcineurin-inhibiting effects, we measured NFAT4 levels and localization in the Jurkat T-cell line. NFAT4 levels increased in the cytoplasmic fraction in cells treated with the calcineurin inhibitor cyclosporin A (CsA) and FK506, whereas 5a1 had no effect on NFAT4 localization and/or levels (*Online Supplementary Figure S3A-D*). A similar experiment was carried out using INA-6 cells, comparing 5a1 and FK506 to NFAT4 and FKBP12 levels, and to BMP-induced SMAD1/5 activity (*Online Supplementary Figure S3E-L*). With the doses and timing used here, the effect on SMAD1/5 activation was similar between 5a1 and FK506. The experiments suggest that FKBP12-targeting PROTAC are specific and have a similar potency on BMP-mediated SMAD1/5 activation as FK506, while sparing the activity of calcineurin.

Finally, we hypothesized that the PROTAC effect would be stable over time and decided to compare this with FK506.





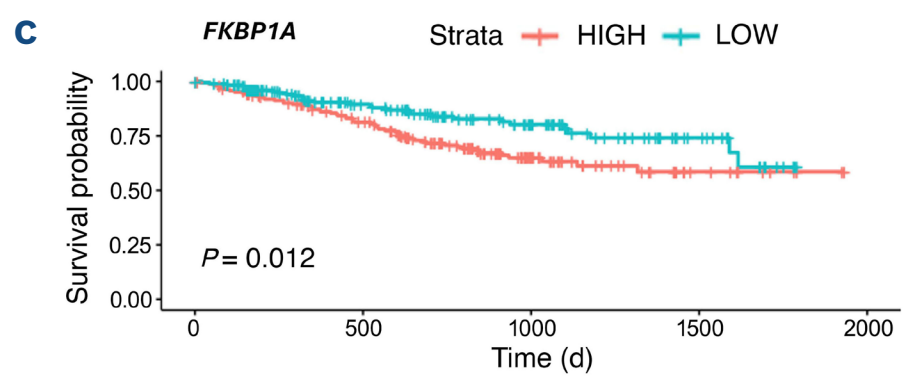


Figure 3. The effect of 5a1 on FKBP12 protein levels over time. INA-6 cells were treated for 18 hours (hr) with or without 5a1 (A) or FK506 (B). Some cells were subsequently kept as controls, and the remaining cells were washed with Hank's balanced salt solution to remove the drugs. The cells were resuspended in experimental medium and incubated for the time-points indicated in the plot. Before harvesting, the cells were treated for 1 hr with bone morphogenetic protein (BMP)6 (7.5 ng/mL) to assess the effect of 5a1 and FK506 on FKBP12 protein levels and SMAD1/5 activity. Primary antibodies used were FKBP12 (RRID: AB_2102847, #SC-133067; Santa Cruz, CA, USA), phospho-SMAD1/5 (RRID: AB_491015, #9516; Cell Signaling Technology), and GAPDH (RRID: AB_2107448, #Ab8245, Abcam). One representative out of 3 independent experiments is shown. The graphs represent densitometric analysis of 3 independent experiments with the average and standard error of the mean (SEM). Two-way ANOVA and Šídák's multiple comparisons test were used to test significance. *P<0.05, ***P<0.001, **** P<0.0001. (C) RNA-sequencing and clinical data of myeloma patients were obtained from the publicly available Multiple Myeloma Research Foundation (MMRF) CoMMpass database (https://research.themmrf.org). RNA-sequencing data of 762 patients were available. The MMRF-CoMMpass data was analyzed in R (2023.06.0+421) using TCGABiolinks and DESeq2. A Kaplan-Meyer plot was generated from the MMRF-CoMMpass dataset and shows overall survival (OS) related to FKBP1A expression (high>upper quarter percentile, low<lower quarter percentile, N=762). d: days.

We saw a marked downregulation of FKBP12 protein in 5a1 pretreated cells and this was still clear at 48 hours (hr), although the effect started to fade as fast as 1 hr after drug removal (Figure 3A). As expected, no clear effects on FKBP12 protein levels were seen with FK506 (Figure 3B). Of note, we saw the same effect on SMAD1/5-activation with FKBP12 degradation as with binding to FK506.

The use of protein degraders for cancer treatment is an expanding field. In multiple myeloma, protein degradation has been used for decades with drugs such as thalidomide and its analog lenalidomide, which bind cereblon and recruit neosubstrates like Ikaros and Aiolos for ubiquitylation and degradation.[15] In principle, PROTAC can be made to target many different combinations of ligands and ubiquitin ligases. BMP receptors are expressed in most tissues, suggesting that systemic increase in BMP activity will have side-effects. To target myeloma cells more specifically, one option is to develop FKBP12-degrading antibody PROTAC conjugates using myeloma cell surface antigens like BCMA or GPRC5D. Interestingly, we found that *FKBP1A* mRNA levels in myeloma cells can predict patients' overall survival (Figure 3C), further supporting the relevance of FKBP12 as a therapeutic target and a possible means for patient stratification in the future.

Taken together, we show two novel PROTAC that potently and specifically degraded FKBP12 in multiple myeloma cells. Loss of FKBP12 protein strongly potentiated BMP-induced SMAD activity and apoptosis in multiple myeloma cells, without inhibiting calcineurin activity. Our results suggest the potential for using FKBP12-targeting PROTAC in multiple myeloma, but further research is needed to verify efficacy and safety *in vivo*.

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Disclosures

No conflicts of interest to disclose.

Contributions

IQL and TH designed the study. IQL, MT, CAR, MAH, MW, JFNM, MAV and TH performed experiments and analyzed data. IQL and TH were responsible for study concept, preparing the figures, and writing the original draft. All authors contributed to revising and editing the draft. AS, TSS, FH and TH were responsible for study supervision and funding acquisition.

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Data-sharing statement

Original data can be shared upon request by contacting the corresponding author.

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