

## Synthesis and discovery of the first potent proteolysis targeting chimaera (PROTAC) degrader of AIMP2-DX2 as a lung cancer drug

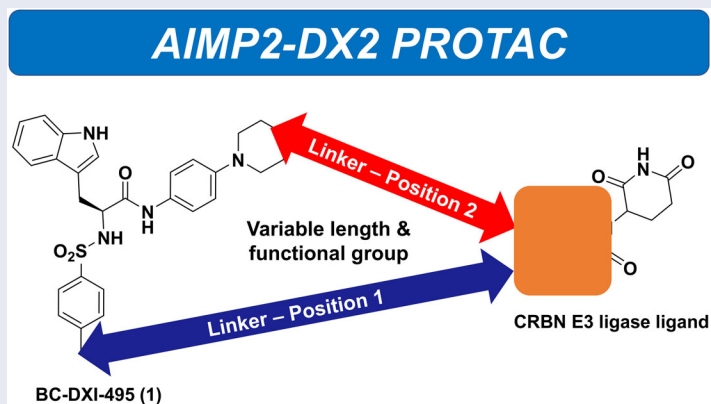
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### ABSTRACT

ARS-interacting multifunctional proteins 2 (AIMP2) is known to be a powerful tumour suppressor. However, the target AIMP2-DX2, AIMP2-lacking exon 2, is often detected in many cancer patients and cells. The predominant approach for targeting AIMP2-DX2 has been attempted *via* small molecule mediated inhibition, but due to the lack of satisfactory activity against AIMP2-DX2, new therapeutic strategies are needed to develop a novel drug for AIMP2-DX2. Here, we report the use of the PROTAC strategy that combines small-molecule AIMP2-DX2 inhibitors with selective E3-ligase ligands with optimised linkers. Consequently, candidate compound **45** was found to be a degrader of AIMP2-DX2. Together, these findings demonstrate that our PROTAC technology targeting AIMP2-DX2 would be a potential new strategy for future lung cancer treatment.

### GRAPHICAL ABSTRACT



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### KEYWORDS

PROTAC; AIMP2-DX2;  
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### Introduction

Multi-tRNA synthetase complex (MSC) plays an essential role in charging of tRNA with the cognate amino acid for synthesising peptide. It consists of nine types of Aminoacyl-tRNA Synthetases (ARS) and three types of ARS-interacting multifunctional proteins (AIMPs). Among the three AIMPs, AIMP2, which consists of four exons, is known as a powerful tumour suppressor by mediating various transduction systems<sup>1–4</sup>. A splicing variant of AIMP2, AIMP2 lacking exon 2 (AIMP2-DX2), is established as an oncogene and is highly expressed in lung cancer cells and lung cancer patient tissues<sup>5–7</sup>. This is because AIMP2-DX2 binds to its binding partners FBP, TRAF2, and p53 and competitively inhibits the

anticancer function of AIMP2 to induce cancer and also stabilises the cancer driver gene KRAS<sup>5,7,8</sup>. Recently, Lim et al.<sup>9</sup> discovered that the level of AIMP2-DX2 in cells can be regulated by Siah1 protein, a specific E3 ligase of AIMP2-DX2, *via* an ubiquitination-mediated degradation sequences. However, when 70 kDa heat shock proteins (HSP70s) selectively bind to AIMP2-DX2, it blocks the binding of AIMP2-DX2 and Siah1 protein, resulting in an increase in AIMP2-DX2 level<sup>9</sup>. Although many studies have been conducted so far to identify small molecules targeting AIMP2-DX2 itself using conventional methods, the development of new lung cancer drug that is fine-tuned to chemically inhibit the interaction of HSP70 and AIMP2-DX2 and increase the accessibility of Siah1 and AIMP2-DX2 has not been attempted<sup>4,9–13</sup>. To the best of our

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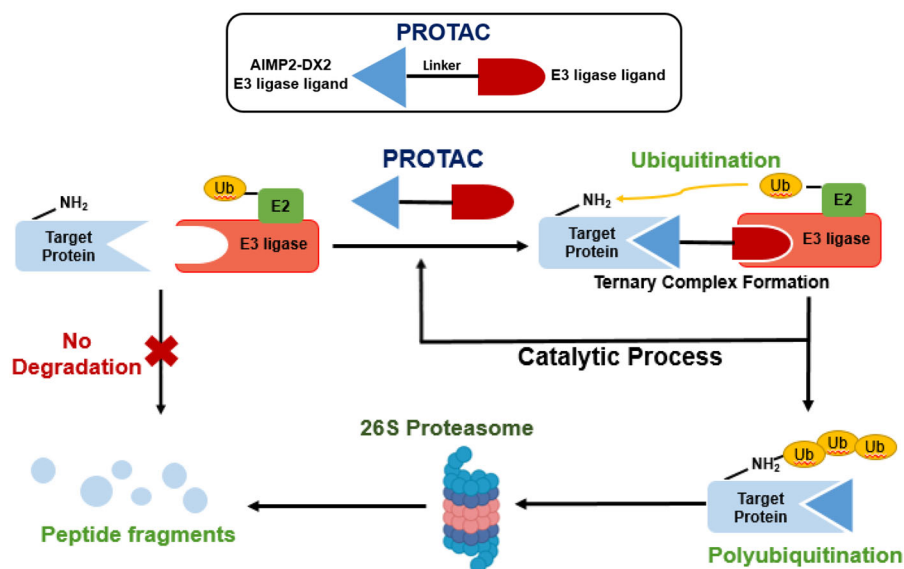


Figure 1. PROTAC composition and mechanism of PROTAC.

knowledge, a comprehensive study of E3 ligase ligand and linker length activity relationships with a PROTAC targeting AIMP-DX2 has not been forthcoming in the literature. Accordingly, this study presents the PROTAC technique as a promising alternative approach against AIMP2-DX2 targeting lung cancer.

Proteolysis targeting chimera (PROTAC) technology has received great attention in drug discovery and development areas over the past few years as the clinical results of anticancer drugs applied with PROTAC technology were positively announced. PROTAC is a heterobifunctional molecules and consists of three parts: target ligand, linker, and E3 ligase (Figure 1). This new platform technology follows a well-known ubiquitin-proteasome system. PROTAC can recruit E3 ubiquitin ligase to a target protein of interest, resulting in polyubiquitination of the protein of interest which is subsequently degraded *via* 26S proteasome<sup>14</sup>. In addition, PROTAC can act as a catalyst, making it reusable. It can exert the desired pharmacological effect at a much lower concentration compared to conventional small molecule inhibitors, thereby reducing the toxicity problem of small molecule inhibitors<sup>15</sup>.

In this study, we describe our efforts in the discovery of potent AIMP2-DX2 degraders utilising CRBN E3 ligases and various linkers, which resulted in a promising compound with anticancer activities. These data demonstrate the importance and potential for developing PROTAC-based therapeutic molecules for the AIMP2-DX2 targeted lung cancer drug.

## Experimental section

### General chemistry

All commercially available chemicals were of reagent grade and were used without further purification. Solvents were dried with standard procedures. All of the reactions were purified by flash column chromatography using CombiFlash<sup>®</sup> Rf 200 system (Teledyne Isco, Lincoln, NE). Additionally, thin-layer chromatography was performed on 0.25 mm silica gel 60-F254 plates (Merck, Kenilworth, NJ). Visualisation was accomplished with 254 nm of UV light or potassium permanganate staining followed by heating. The proton nuclear magnetic resonance (<sup>1</sup>H-NMR) spectra were determined on a Varian (400 and 100 MHz) or Bruker (400 and

100 MHz). <sup>1</sup>H NMR (at 400 MHz) was reported on a Varian 400 MHz spectrometer and are reported in ppm using solvent as an internal standard (DMSO-d<sub>6</sub> at 2.50, Acetone-d<sub>6</sub> at 2.05, at 3.31 for <sup>1</sup>H NMR). Data reported as (s = singlet, d = doublet, t = triplet, q = quartette, m = multiplet, br = broad; coupling constant(s) in Hz; integration). LC/MS data were obtained using a Waters 2695-ZQ2000 system and Waters ACQUITY UPLC-SQ Detector 2.

### Specific experimental procedures and product characterisation

#### General procedure for the click reaction

(+)-Sodium *L*-ascorbate (0.15 equiv.) and copper (II) sulphate pentahydrate (0.05 equiv.) were added to a stirred solution of azide (1equiv.) and alkyne (1 equiv.) in DMSO at 25 °C. The mixture was stirred at 25 °C for overnight before being quenched with aq. NaHCO<sub>3</sub> and extracted with dichloromethane. The combined organic phases were washed with water and brine then dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and concentrated under reduced pressure. The residue thus obtained was subjected to column chromatography to give the desired product.

(2S)-2-((4-((4-((2-((2-((2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)ethoxy)ethoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)sulfonamido)-3-(1H-indol-3-yl)-N-(4-morpholinophenyl)propanamide (**13**)

The title compound was prepared from azide **11** and alkyne **12a** according to general procedure as a light green solid (14.0 mg, 27%). <sup>1</sup>H NMR (400 MHz, Acetone-d<sub>6</sub>) δ 9.93 (s, 1H), 9.90 (s, 1H), 8.96 (s, 1H), 7.84 (s, 1H), 7.77 (t, *J* = 8.8 Hz, 1H), 7.58 (d, *J* = 8.0 Hz, 2H), 7.49–7.44 (m, 3H), 7.42–7.30 (m, 3H), 7.14 (d, *J* = 8.0 Hz, 2H), 7.10–7.06 (m, 2H), 6.95 (t, *J* = 7.4 Hz, 1H), 6.88 (t, *J* = 9.2 Hz, 2H), 6.81–6.88 (m, 1H), 5.55 (s, 2H), 5.11 (dd, *J* = 5.2 and 12.4 Hz, 1H), 4.60 (s, 2H), 4.34 (t, *J* = 4.8 Hz, 2H), 4.17 (brs, 1H), 3.83 (t, *J* = 4.8 Hz, 2H), 3.77–3.75 (m, 4H), 3.73–3.71 (m, 2H), 3.66–3.63 (m, 2H), 3.26 (dd, *J* = 5.2 and 12.4 Hz, 1H), 3.10–3.05 (m, 4H), 3.03–2.71 (m, 4H), 2.22–2.10 (m, 1H); LCMS (ESI) *m/z* 960 [M + H]<sup>+</sup>.

(2S)-2-((4-((4-((2-((2-((2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)ethoxy)ethoxy)ethoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)sulfonamido)-3-(1H-indol-3-yl)-N-(4-morpholinophenyl)propanamide (**14**)

The title compound was prepared from azide **11** and alkyne **12b** according to general procedure as a pale yellow solid (3.0 mg, 6%). <sup>1</sup>H NMR (400 MHz, Acetone-d<sub>6</sub>) δ 9.93 (s, 1H), 9.91 (s,

1H), 8.95 (s, 1H), 7.83 (s, 1H), 7.77 (t,  $J=8.4$  Hz, 1H), 7.60 (d,  $J=7.6$  Hz, 2H), 7.48–7.42 (m, 3H), 7.43–7.30 (m, 3H), 7.15 (d,  $J=8.0$  Hz, 2H), 7.10–7.06 (m, 2H), 6.95 (t,  $J=7.6$  Hz, 1H), 6.89–6.86 (m, 2H), 6.80 (d,  $J=8.4$  Hz, 1H), 5.55 (s, 2H), 5.10 (dd,  $J=5.2$  and 12.8 Hz, 1H), 4.59 (s, 2H), 4.35 (t,  $J=4.8$  Hz, 2H), 4.17–4.16 (m, 1H), 3.86 (t,  $J=4.8$  Hz, 2H), 3.76 (t,  $J=4.8$  Hz, 4H), 3.69–3.67 (m, 2H), 3.62–3.55 (m, 6H), 3.24 (dd,  $J=6.0$  and 14.8 Hz, 1H), 3.08 (t,  $J=4.8$  Hz, 4H), 3.03–2.74 (m, 4H), 2.22–2.17 (m, 1H); LCMS (ESI)  $m/z$  1002 [M-H]<sup>-</sup>.

(2S)-2-((4-((13-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)-2,5,8,11-tetraoxatridecyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)sulfonamido)-3-(1H-indol-3-yl)-N-(4-morpholinophenyl)propanamide (**15**)

The title compound was prepared from azide **11** and alkyne **12c** according to general procedure as a pale-yellow solid (2.0 mg, 4%). <sup>1</sup>H NMR (400 MHz, Acetone- $d_6$ )  $\delta$  9.91 (brs, 2H), 8.96 (brs, 1H), 7.84 (s, 1H), 7.79 (t,  $J=8.4$  Hz, 1H), 7.60 (d,  $J=8.4$  Hz, 2H), 7.49–7.42 (m, 3H), 7.34–7.30 (m, 3H), 7.15 (d,  $J=8.0$  Hz, 2H), 7.08 (dd,  $J=8.4$  and 6.4 Hz, 2H), 6.95 (dd,  $J=8.0$  and 6.8 Hz, 1H), 6.88 (d,  $J=8.8$  Hz, 2H), 6.79 (brs, 1H), 5.56 (s, 2H), 5.10 (dd,  $J=5.6$  and 12.4 Hz, 1H), 4.59 (s, 2H), 4.36 (t,  $J=4.8$  Hz, 2H), 4.16 (brs, 1H), 3.86 (t,  $J=4.8$  Hz, 2H), 3.76 (t,  $J=4.8$  Hz, 4H), 3.70–3.67 (m, 2H), 3.61–3.51 (m, 10H), 3.24 (dd,  $J=6.2$  and 14.6 Hz, 1H), 3.08 (t,  $J=4.8$  Hz, 4H), 3.05–2.74 (m, 4H), 2.21–2.10 (m, 1H); LCMS (ESI)  $m/z$  1046 [M-H]<sup>-</sup>.

(2S)-2-((4-((16-((2-(2,6-dioxopiperidin-3-yl)-1,3-Dioxoisindolin-4-yl)oxy)-2,5,8,11,14-pentaoxahexadecyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)sulfonamido)-3-(1H-indol-3-yl)-N-(4-morpholinophenyl)propanamide (**16**)

The title compound was prepared from azide **11** and alkyne **12d** according to general procedure as a pale-yellow solid (22.0 mg, 37%). <sup>1</sup>H NMR (400 MHz, Acetone- $d_6$ )  $\delta$  9.92 (brs, 2H), 8.98 (brs, 1H), 7.85 (s, 1H), 7.79 (t,  $J=8.8$  Hz, 1H), 7.61 (d,  $J=8.4$  Hz, 2H), 7.50–7.42 (m, 3H), 7.34–7.31 (m, 3H), 7.16 (d,  $J=8.4$  Hz, 2H), 7.08 (t,  $J=7.2$  Hz, 2H), 6.95 (t,  $J=7.2$  Hz, 1H), 6.87 (d,  $J=12.4$  Hz, 2H), 6.83 (brs, 1H), 5.56 (s, 2H), 5.10 (dd,  $J=5.2$  and 12.8 Hz, 1H), 4.59 (s, 2H), 4.37 (t,  $J=4.8$  Hz, 2H), 4.16 (t,  $J=7.6$  Hz, 1H), 3.87 (dd,  $J=4.8$  Hz, 2H), 3.77–3.75 (m, 4H), 3.70–3.68 (m, 2H), 3.62–3.51 (m, 14H), 3.25 (dd,  $J=5.8$  and 14.6 Hz, 1H), 3.10–3.07 (m, 4H), 3.05–2.73 (m, 4H), 2.23–2.19 (m, 1H); LCMS (ESI)  $m/z$  1090 [M-H]<sup>-</sup>.

(2S)-2-((4-((4-((3-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-3-oxopropoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)sulfonamido)-3-(1H-indol-3-yl)-N-(4-morpholinophenyl)propanamide (**18**)

The title compound was prepared from azide **11** and alkyne **17a** according to general procedure as a pale yellow solid (15.8 mg, 31%); <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.12 (s, 1H), 10.71 (s, 1H), 9.86 (s, 1H), 9.67 (s, 1H), 8.51 (d,  $J=8.4$  Hz, 1H), 8.16 (brs, 1H), 8.03 (s, 1H), 7.82 (t,  $J=7.6$  Hz, 1H), 7.61–7.56 (m, 3H), 7.41 (d,  $J=7.6$  Hz, 1H), 7.28 (d,  $J=8.0$  Hz, 1H), 7.19–7.14 (m, 4H), 7.06–7.01 (m, 2H), 6.91 (t,  $J=7.6$  Hz, 1H), 6.84 (d,  $J=8.8$  Hz, 2H), 5.53 (s, 2H), 5.13 (dd,  $J=5.6$  and 12.8 Hz, 1H), 4.60 (s, 2H), 4.09 (t,  $J=6.8$  Hz, 1H), 3.78–3.71 (m, 6H), 3.07–3.03 (m, 5H), 2.88–2.83 (m, 2H), 2.72 (t,  $J=6.0$  Hz, 2H), 2.63–2.53 (m, 2H), 2.07–2.04 (m, 1H); LCMS (ESI)  $m/z$  943 [M+H]<sup>+</sup>.

(2S)-2-((4-((4-((3-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-3-oxopropoxy)ethoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)sulfonamido)-3-(1H-indol-3-yl)-N-(4-morpholinophenyl)propanamide (**19**)

The title compound was prepared from azide **11** and alkyne **17b** according to general procedure as a pale yellow solid (23.0 mg, 43%); <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.12 (s, 1H), 10.71 (s, 1H), 9.86 (s, 1H), 9.67 (s, 1H), 8.51 (d,  $J=8.4$  Hz, 1H), 8.16 (brs, 1H), 7.99 (s, 1H), 7.81 (t,  $J=7.6$  Hz, 1H), 7.59 (t,  $J=8.4$ , 3H), 7.41 (d,

$J=7.6$  Hz, 1H), 7.29 (d,  $J=8.0$  Hz, 1H), 7.19–7.14 (m, 4H), 7.06–7.02 (m, 2H), 6.91 (t,  $J=7.6$  Hz, 1H), 6.84 (d,  $J=9.2$  Hz, 2H), 5.52 (s, 2H), 5.13 (dd,  $J=5.6$  and 12.8 Hz, 1H), 4.51 (s, 2H), 4.09 (t,  $J=7.2$  Hz, 1H), 3.73–3.71 (m, 6H), 3.59 (s, 4H), 3.07–3.03 (m, 5H), 2.94–2.83 (m, 2H), 2.69 (t,  $J=6.0$  Hz, 2H), 2.63–2.55 (m, 2H), 2.09–2.05 (m, 1H); LCMS (ESI)  $m/z$  987 [M+H]<sup>+</sup>.

(2S)-2-((4-((4-((2-(3-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-3-oxopropoxy)ethoxy)ethoxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)phenyl)sulfonamido)-3-(1H-indol-3-yl)-N-(4-morpholinophenyl)propanamide (**20**)

The title compound was prepared from azide **11** and alkyne **17c** according to general procedure as a yellow solid (15.5 mg, 28%); <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.12 (s, 1H), 10.71 (s, 1H), 9.85 (s, 1H), 9.66 (s, 1H), 8.53 (d,  $J=8.4$  Hz, 1H), 8.15 (d,  $J=8.8$  Hz, 1H), 8.00 (s, 1H), 7.81 (t,  $J=7.6$  Hz, 1H), 7.58 (t,  $J=8.0$  Hz, 3H), 7.40 (d,  $J=8.0$  Hz, 1H), 7.28 (d,  $J=8.0$  Hz, 1H), 7.19–7.14 (m, 4H), 7.05–7.01 (m, 2H), 6.91 (t,  $J=7.2$  Hz, 1H), 6.83 (d,  $J=8.8$  Hz, 2H), 5.53 (s, 2H), 5.13 (dd,  $J=5.6$  and 12.8 Hz, 1H), 4.48 (s, 2H), 3.72–3.71 (m, 6H), 3.55–3.52 (m, 9H), 3.07–3.02 (5H), 2.93–2.82 (m, 2H), 2.68 (t,  $J=6.0$  Hz, 2H), 2.58–2.54 (m, 2H), 2.07–2.05 (m, 1H); LCMS (ESI)  $m/z$  1029 [M-H]<sup>-</sup>.

1-(1-(4-(N-((S)-3-(1H-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)benzyl)-1H-1,2,3-triazol-4-yl)-N-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)-2,5,8,11-tetraoxatetradecan-14-amide (**21**)

The title compound was prepared from azide **11** and alkyne **17d** according to general procedure as a brown solid (4.5 mg, 9%); <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.11 (s, 1H), 10.71 (s, 1H), 9.85 (s, 1H), 9.66 (s, 1H), 8.54 (d,  $J=8.4$  Hz, 1H), 8.15 (brs, 1H), 8.01 (s, 1H), 7.84 (t,  $J=7.6$  Hz, 1H), 7.62–7.56 (m, 3H), 7.40 (d,  $J=8.0$  Hz, 2H), 7.28 (d,  $J=8.0$  Hz, 1H), 7.19–7.14 (m, 4H), 7.05–7.01 (m, 2H), 6.91 (t,  $J=7.2$  Hz, 1H), 6.83 (d,  $J=8.8$  Hz, 2H), 5.53 (s, 2H), 5.13 (dd,  $J=5.6$  and 12.8 Hz, 1H), 4.50 (s, 2H), 4.09 (brs, 1H), 3.72–3.71 (m, 6H), 3.52–3.41 (m, 11H), 3.04–3.02 (m, 5H), 2.93–2.82 (m, 2H), 2.70–2.67 (m, 2H), 2.58–2.50 (m, 2H), 2.07–2.05 (m, 1H); LCMS (ESI)  $m/z$  1073 [M-H]<sup>-</sup>.

1-(1-(4-(N-((S)-3-(1H-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)benzyl)-1H-1,2,3-triazol-4-yl)-N-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)-2,5,8,11,14-pentaoxaheptadecan-17-amide (**22**)

The title compound was prepared from azide **11** and alkyne **17e** according to general procedure as a light brown solid (12.5 mg, 25%); <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.12 (s, 1H), 10.71 (s, 1H), 9.86 (s, 1H), 9.67 (s, 1H), 8.54 (d,  $J=8.4$  Hz, 1H), 8.16 (brs, 1H), 8.02 (s, 1H), 7.82 (t,  $J=7.6$ , 1H), 7.59 (dd,  $J=8.0$  and 9.6 Hz, 3H), 7.41 (d,  $J=8.0$  Hz, 1H), 7.28 (d,  $J=7.6$  Hz, 1H), 7.19–7.15 (m, 4H), 7.06–7.02 (m, 2H), 6.91 (t,  $J=7.2$  Hz, 1H), 6.84 (d,  $J=8.4$  Hz, 2H), 5.54 (s, 2H), 5.14 (dd,  $J=4.4$  and 12.4 Hz, 1H), 4.52 (s, 2H), 4.09 (brs, 1H), 3.73 (brs, 6H), 3.55–3.45 (m, 16H), 3.04 (m, 5H), 2.94–2.83 (m, 2H), 2.69 (brs, 2H), 2.64–2.55 (m, 2H), 2.09–2.07 (m, 1H); LCMS (ESI)  $m/z$  1117 [M-H]<sup>-</sup>.

1-(1-(4-(N-((S)-3-(1H-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)benzyl)-1H-1,2,3-triazol-4-yl)-N-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)-2,5,8,11,14,17-hexaoxaicosan-20-amide (**23**)

The title compound was prepared from azide **11** and alkyne **17f** according to general procedure as a yellow solid (9.2 mg, 22%); <sup>1</sup>H NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.12 (s, 1H), 10.71 (s, 1H), 9.86 (s, 1H), 9.67 (s, 1H), 8.54 (d,  $J=8.4$  Hz, 1H), 8.16 (brs, 1H), 8.02 (s, 1H), 7.83 (dd,  $J=7.2$  and 8.0 Hz, 1H), 7.61–7.57 (m, 3H), 7.41 (d,  $J=7.6$  Hz, 1H), 7.29 (d,  $J=8.0$  Hz, 1H), 7.17 (dd,  $J=9.2$  and 8.4 Hz, 4H), 7.06–7.02 (m, 2H), 6.91 (t,  $J=7.6$  Hz, 1H), 6.84 (d,  $J=8.4$  Hz, 1H), 5.54 (s, 2H), 5.13 (dd,  $J=5.2$  and 12.4 Hz, 1H), 4.52 (s, 2H),

4.10–4.04 (m, 1H), 3.73 (brs, 6H), 3.56–3.45 (m, 20H), 3.04 (brs, 5H), 2.94–2.82 (m, 2H), 2.69–2.68 (m, 2H), 2.64–2.51 (m, 2H), 2.09–2.06 (m, 1H); LCMS (ESI)  $m/z$  1161 [M-H]<sup>-</sup>.

#### General procedure for amide coupling

To a solution of acid (1 equiv.), amine (1.2 equiv.), HATU (1.2 equiv.) in DMF, DIPEA (1.2 equiv.) was added. The mixture was stirred at 25 °C for overnight. The reaction mixture was quenched with water and extracted with ethyl acetate. The combined organic phases were washed with brine and dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and concentrated under reduced pressure. The residue thus obtained subjected to column chromatography to give the desired product.

4-(*N*-((*S*)-3-(1*H*-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)-*N*-(4-(2-(2-(2-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetamido)butyl)benzamide (**25**)

The title compound was prepared from **24** according to general procedure as a yellow solid (10.0 mg, 12%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 11.09 (brs, 1H), 10.74 (s, 1H), 9.71 (s, 1H), 8.48 (t, *J* = 5.6 Hz, 1H), 8.31 (brs, 1H), 7.98 (t, *J* = 5.6 Hz, 1H), 7.81–7.76 (m, 3H), 7.68 (d, *J* = 8.4 Hz, 2H), 7.48 (d, *J* = 7.2 Hz, 1H), 7.40 (t, *J* = 8.4 Hz, 2H), 7.25 (d, *J* = 8.0 Hz, 1H), 7.17 (d, *J* = 8.8 Hz, 2H), 7.07 (d, *J* = 2.0 Hz, 1H), 7.01 (t, *J* = 7.0 Hz, 1H), 6.91 (t, *J* = 7.0 Hz, 1H), 6.79 (d, *J* = 8.0 Hz, 2H), 5.11 (dd, *J* = 5.2 and 12.8 Hz, 1H), 4.77 (s, 2H), 4.14 (t, *J* = 7.2 Hz, 1H), 3.71 (t, *J* = 5.2 Hz, 4H), 3.27–3.19 (m, 4H), 3.09–2.99 (m, 5H), 2.89–2.84 (m, 2H), 2.67–2.57 (m, 2H), 2.04–2.01 (m, 1H), 1.53–1.51 (m, 4H); LCMS (ESI)  $m/z$  933 [M + H]<sup>+</sup>.

4-(*N*-((*S*)-3-(1*H*-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)-*N*-(2-(2-(2-(2-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)acetamido)ethoxy)ethoxy)ethyl)benzamide (**26**)

The title compound was prepared from **24** according to general procedure as a yellow solid (30 mg, 39%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 11.10 (s, 1H), 10.72 (s, 1H), 9.72 (s, 1H), 8.51 (t, *J* = 5.4 Hz, 1H), 8.30 (d, *J* = 8.4 Hz, 1H), 7.99 (t, *J* = 5.4 Hz, 1H), 7.81–7.73 (m, 3H), 7.66 (d, *J* = 8.4 Hz, 2H), 7.48 (d, *J* = 7.2 Hz, 1H), 7.39 (t, *J* = 8.8 Hz, 2H), 7.25 (d, *J* = 8.0 Hz, 1H), 7.17 (d, *J* = 8.8 Hz, 2H), 7.07 (d, *J* = 2.0 Hz, 1H), 7.01 (t, *J* = 7.4 Hz, 1H), 6.90 (t, *J* = 7.2 Hz, 1H), 6.79 (d, *J* = 8.8 Hz, 2H), 5.10 (dd, *J* = 5.6 and 12.8 Hz, 1H), 4.77 (s, 2H), 4.16–4.10 (m, 1H), 3.71–3.69 (m, 4H), 3.55–3.45 (m, 8H), 3.33–3.30 (m, 2H), 3.01–2.88 (m, 5H), 2.86–2.82 (m, 2H), 2.61–2.54 (m, 2H), 2.05–1.98 (m, 1H), 1.23 (brs, 2H); LCMS (ESI)  $m/z$  993 [M + H]<sup>+</sup>.

4-(*N*-((*S*)-3-(1*H*-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)-*N*-(2-(2-(2-(2-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethyl)benzamide (**28**)

The title compound was prepared from acid **24** and amine **27a** according to general procedure as a yellow solid (27.4 mg, 55%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 11.10 (s, 1H), 10.71 (s, 1H), 10.36 (s, 1H), 9.69 (s, 1H), 8.70 (d, *J* = 8.4 Hz, 1H), 8.58 (t, *J* = 5.6 Hz, 1H), 8.28 (brs, 1H), 7.86 (t, *J* = 7.6 Hz, 1H), 7.75 (d, *J* = 8.4 Hz, 2H), 7.67–7.61 (m, 3H), 7.40 (d, *J* = 7.6 Hz, 1H), 7.24 (d, *J* = 8.0 Hz, 1H), 7.17 (d, *J* = 8.8 Hz, 2H), 7.06 (d, *J* = 2.0 Hz, 1H), 7.00 (t, *J* = 7.2 Hz, 1H), 6.90 (t, *J* = 7.2 Hz, 1H), 6.79 (d, *J* = 9.2 Hz, 2H), 5.11 (dd, *J* = 5.6 and 12.8 Hz, 1H), 4.24 (s, 2H), 4.14–4.09 (m, 1H), 3.78 (t, *J* = 5.6 Hz, 2H), 3.70 (d, *J* = 4.4 and 4.8 Hz, 4H), 3.58 (dd, *J* = 5.6 and 11.6 Hz, 2H), 3.16 (d, *J* = 5.2 Hz, 1H), 3.08–2.99 (m, 5H), 2.90–2.81 (m, 2H), 2.70–2.56 (m, 1H), 2.03–2.00 (m, 1H); LCMS (ESI)  $m/z$  905 [M + H]<sup>+</sup>.

4-(*N*-((*S*)-3-(1*H*-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)-*N*-(2-(2-(2-(2-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-2-oxoethoxy)ethoxy)ethyl)benzamide (**29**)

The title compound was prepared from acid **24** and amine **27b** according to general procedure as a yellow solid (24.2 mg, 47%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 11.12 (s, 1H), 10.70 (s, 1H),

10.35 (s, 1H), 9.70 (s, 1H), 8.69 (d, *J* = 8.4 Hz, 1H), 8.48 (t, *J* = 5.6 Hz, 1H), 8.28 (brs, 1H), 7.84 (t, *J* = 7.6 Hz, 1H), 7.72 (d, *J* = 8.8 Hz, 2H), 7.65–7.59 (m, 3H), 7.39 (d, *J* = 8.0 Hz, 1H), 7.24 (d, *J* = 8.0 Hz, 1H), 7.17 (d, *J* = 8.8 Hz, 2H), 7.06 (d, *J* = 2.4 Hz, 1H), 7.00 (t, *J* = 7.2 Hz, 1H), 6.90 (t, *J* = 7.2 Hz, 1H), 6.78 (d, *J* = 9.2 Hz, 2H), 5.15 (dd, *J* = 5.6 and 12.8 Hz, 1H), 4.20 (s, 2H), 4.14–4.09 (m, 2H), 3.79–3.77 (m, 2H), 3.71–3.69 (m, 5H), 3.58 (t, *J* = 6.0 Hz, 2H), 3.17 (d, *J* = 5.2 Hz, 2H), 3.08–2.99 (m, 5H), 2.91–2.82 (m, 2H), 2.67–2.54 (m, 2H), 2.08–2.04 (m, 1H); LCMS (ESI)  $m/z$  949 [M + H]<sup>+</sup>.

4-(*N*-((*S*)-3-(1*H*-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)-*N*-(2-(2-(2-(2-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-2-oxoethoxy)ethoxy)ethoxy)ethyl)benzamide (**30**)

The title compound was prepared from acid **24** and amine **27c** according to general procedure as a yellow solid (17.2 mg, 32%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 11.13 (s, 1H), 10.71 (s, 1H), 10.33 (s, 1H), 9.70 (s, 1H), 8.70 (d, *J* = 8.4 Hz, 1H), 8.47 (t, *J* = 5.6 Hz, 1H), 8.28 (brs, 1H), 7.84 (t, *J* = 7.6 Hz, 1H), 7.73 (d, *J* = 8.4 Hz, 2H), 7.66–7.60 (m, 3H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.24 (d, *J* = 8.4 Hz, 1H), 7.17 (d, *J* = 9.2 Hz, 2H), 7.06 (d, *J* = 2.0 Hz, 1H), 7.00 (t, *J* = 7.2 Hz, 1H), 6.90 (t, *J* = 7.2 Hz, 1H), 6.79 (d, *J* = 9.2 Hz, 2H), 5.14 (dd, *J* = 5.6 and 12.8 Hz, 1H), 4.18 (s, 2H), 4.14–4.09 (m, 2H), 3.75–3.69 (m, 6H), 3.67–3.65 (m, 2H), 3.56–3.50 (m, 3H), 3.17 (d, *J* = 5.2 Hz, 4H), 3.08–2.99 (m, 5H), 2.93–2.83 (m, 2H), 2.67–2.54 (m, 2H), 2.10–2.05 (m, 1H); LCMS (ESI)  $m/z$  993 [M + H]<sup>+</sup>.

4-(*N*-((*S*)-3-(1*H*-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)-*N*-(17-(2-(2-(2-(2-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-17-oxo-3,6,9,12,15-pentaoxaheptadecyl)benzamide (**31**)

The title compound was prepared from acid **24** and amine **27d** according to general procedure as a yellow solid (28.1 mg, 48%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 11.13 (s, 1H), 10.73 (s, 1H), 10.35 (s, 1H), 9.72 (s, 1H), 8.72 (d, *J* = 8.8 Hz, 1H), 8.51 (t, *J* = 5.6 Hz, 1H), 8.32 (brs, 1H), 7.85 (t, *J* = 7.6 Hz, 1H), 7.75 (d, *J* = 8.4 Hz, 2H), 7.67–7.61 (m, 3H), 7.41 (d, *J* = 7.6 Hz, 1H), 7.25 (d, *J* = 8.0 Hz, 1H), 7.18 (d, *J* = 9.8 Hz, 2H), 7.06 (d, *J* = 2.0 Hz, 1H), 7.00 (t, *J* = 7.2 Hz, 1H), 6.90 (t, *J* = 7.2 Hz, 1H), 6.79 (d, *J* = 9.2 Hz, 2H), 5.16 (dd, *J* = 5.6 and 13.2 Hz, 1H), 4.20 (s, 2H), 4.13 (brs, 1H), 3.76–3.70 (m, 6H), 3.66–3.64 (m, 2H), 3.53–3.40 (m, 14H), 3.08–2.99 (m, 5H), 2.94–2.83 (m, 2H), 2.67–2.54 (m, 4H), 2.09–2.06 (m, 1H); LCMS (ESI)  $m/z$  [M + H]<sup>+</sup>.

4-(*N*-((*S*)-3-(1*H*-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)-*N*-(2-(2-(2-(2-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethyl)benzamide (**33**)

The title compound was prepared from acid **24** and amine **33** according to general procedure as a yellow solid (10.7 mg, 21%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 11.08 (s, 1H), 10.73 (s, 1H), 9.73 (s, 1H), 8.51 (t, *J* = 5.6 Hz, 1H), 8.32 (s, 1H), 7.75 (d, *J* = 8.4 Hz, 2H), 7.66 (d, *J* = 8.4 Hz, 2H), 7.56 (t, *J* = 8.0 Hz, 1H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.25 (d, *J* = 8.0 Hz, 1H), 7.17 (d, *J* = 8.8 Hz, 2H), 7.12–7.07 (m, 2H), 7.04–6.99 (m, 2H), 6.91 (t, *J* = 7.6 Hz, 1H), 6.80 (d, *J* = 9.2 Hz, 2H), 6.60 (t, *J* = 5.6 Hz, 1H), 5.05 (dd, *J* = 5.2 and 12.4 Hz, 1H), 4.13 (s, 1H), 3.72–3.70 (m, 4H), 3.62–3.53 (m, 8H), 3.46–3.41 (m, 4H), 3.08–2.99 (m, 5H), 2.91–2.82 (m, 2H), 2.67–2.54 (m, 2H), 2.02–1.94 (m, 1H); LCMS (ESI)  $m/z$  935 [M + H]<sup>+</sup>.

4-(*N*-((*S*)-3-(1*H*-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)-*N*-(2-(2-(2-(2-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethoxy)ethyl)benzamide (**34**)

The title compound was prepared from acid **24** and amine **34** according to general procedure as a yellow solid (12.6 mg, 16%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 11.08 (s, 1H), 10.73 (s, 1H), 9.73 (s, 1H), 8.51 (t, *J* = 5.6 Hz, 1H), 8.33 (brs, 1H), 7.75 (d, *J* = 8.4 Hz, 2H), 7.66 (d, *J* = 8.4 Hz, 2H), 7.56 (t, *J* = 8.0 Hz, 1H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.25 (d, *J* = 8.0 Hz, 1H), 7.17 (d, *J* = 9.2 Hz, 2H), 7.12 (d,

$J=8.8$  Hz, 1H), 7.07 (d,  $J=2.0$  Hz, 1H), 7.04–6.99 (m, 2H), 6.90 (t,  $J=7.6$  Hz, 1H), 6.79 (d,  $J=8.8$  Hz, 2H), 6.57 (t,  $J=5.6$  Hz, 1H), 5.04 (dd,  $J=5.6$  and 12.8 Hz, 1H), 4.12 (t,  $J=7.2$  Hz, 1H), 3.72–3.69 (m, 4H), 3.59 (t,  $J=5.6$  Hz, 2H), 3.62–3.53 (m, 11H), 3.08–2.99 (m, 6H), 2.91–2.82 (m, 2H), 2.67–2.54 (m, 2H), 2.03–1.96 (m, 1H), (three resonances obscured or overlapping); LCMS (ESI)  $m/z$  979 [M + H]<sup>+</sup>.

*N*-(4-(*N*-(*S*)-3-(1*H*-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)benzyl)-4-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)butanamide (**36**)

The title compound was prepared from amine **10** and acid **35a** according to general procedure as a yellow solid (10.2 mg, 41%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.09 (s, 1H), 10.73 (s, 1H), 9.68 (s, 1H), 8.38 (dd,  $J=8.0$  and 5.2 Hz, 1H), 8.11 (d,  $J=9.2$  Hz, 1H), 7.57–7.54 (m, 3H), 7.40 (d,  $J=8.0$  Hz, 1H), 7.28 (d,  $J=8.0$  Hz, 1H), 7.20–7.16 (m, 4H), 7.09 (d,  $J=8.8$  Hz, 1H), 7.03–7.01 (m, 3H), 6.94 (t,  $J=7.2$  Hz, 1H), 6.83 (d,  $J=8.8$  Hz, 2H), 6.65–6.62 (m, 1H), 5.04 (dd,  $J=5.2$  and 12.8 Hz, 1H), 4.22 (d,  $J=5.6$  Hz, 2H), 4.09 (dd,  $J=8.4$  and 16.4 Hz, 1H), 3.72–3.70 (m, 4H), 3.06–3.02 (m, 4H), 2.93–2.80 (m, 2H), 2.67–2.50 (m, 3H), 2.25 (t,  $J=7.2$  Hz, 2H), 2.07–2.00 (m, 1H), 1.88–1.80 (m, 2H), 1.26–1.23 (m, 2H); LCMS (ESI)  $m/z$  875 [M + H]<sup>+</sup>.

*N*-(4-(*N*-(*S*)-3-(1*H*-Indol-3-yl)-1-((4-morpholinophenyl)amino)-1-oxopropan-2-yl)sulfamoyl)benzyl)-10-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)decanamide (**37**)

The title compound was prepared from amine **10** and acid **35b** according to general procedure as a yellow solid (7.8 mg, 26%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.08 (s, 1H), 10.72 (s, 1H), 9.68 (s, 1H), 8.27 (dd,  $J=6.4$  and 5.4 Hz, 1H), 8.11 (brs, 1H), 7.59–7.56 (m, 3H), 7.39 (d,  $J=8.4$  Hz, 1H), 7.28 (d,  $J=7.6$  Hz, 1H), 7.17 (d,  $J=8.0$  Hz, 4H), 7.08 (d,  $J=8.8$  Hz, 1H), 7.05–7.00 (m, 3H), 6.93 (t,  $J=7.2$  Hz, 1H), 6.83 (d,  $J=8.8$  Hz, 2H), 6.53–6.50 (m, 1H), 5.04 (dd,  $J=5.2$  and 12.4 Hz, 1H), 4.21 (d,  $J=6.0$  Hz, 2H), 4.09 (brs, 1H), 3.73–3.70 (m, 4H), 3.07–3.01 (m, 4H), 2.92–2.80 (m, 2H), 2.67–2.56 (m, 3H), 2.12 (t,  $J=7.2$  Hz, 2H), 2.02–1.94 (m, 2H), 1.56–1.51 (m, 4H), 1.29–1.25 (m, 11H); LCMS (ESI)  $m/z$  959 [M + H]<sup>+</sup>.

(2*S*)-2-((4-(3-(2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)propanamido)methyl)phenyl)sulfonamido)-3-(1*H*-indol-3-yl)-*N*-(4-morpholinophenyl)propanamide (**39**)

The title compound was prepared from amine **10** and acid **38a** according to general procedure as a yellow solid (5.8 mg, 23%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.12 (s, 1H), 10.75 (s, 1H), 9.71 (s, 1H), 8.39 (dd,  $J=6.4$  and 5.2 Hz, 1H), 8.15 (brs, 1H), 7.59–7.55 (m, 3H), 7.40 (d,  $J=8.4$  Hz, 1H), 7.28 (d,  $J=8.0$  Hz, 1H), 7.19–7.11 (m, 5H), 7.05–7.01 (m, 3H), 6.93 (dd,  $J=7.2$  and 7.6 Hz, 1H), 6.83 (d,  $J=8.4$  Hz, 2H), 6.60–6.59 (m, 1H), 5.05 (dd,  $J=5.6$  and 12.4 Hz, 1H), 4.22 (d,  $J=5.6$  Hz, 2H), 4.10–4.06 (m, 1H), 3.71–3.66 (m, 6H), 3.58–3.56 (m, 3H), 3.45–3.44 (m, 2H), 3.06–3.01 (m, 4H), 2.89–2.81 (m, 2H), 2.67–2.59 (m, 2H), 2.43–2.40 (m, 2H), 2.04–1.97 (m, 1H); LCMS (ESI)  $m/z$  905 [M + H]<sup>+</sup>.

(2*S*)-2-((4-(3-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)propanamido)methyl)phenyl)sulfonamido)-3-(1*H*-indol-3-yl)-*N*-(4-morpholinophenyl)propanamide (**40**)

The title compound was prepared from amine **10** and acid **38b** according to general procedure as a yellow solid (3.9 mg, 15%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.08 (s, 1H), 10.72 (s, 1H), 9.68 (s, 1H), 8.35–8.33 (dd,  $J=6.4$  and 5.2 Hz, 1H), 8.11 (brs, 1H), 7.59–7.56 (m, 3H), 7.40 (d,  $J=8.4$  Hz, 1H), 7.28 (d,  $J=8.0$  Hz, 1H), 7.20–7.11 (m, 5H), 7.04–7.01 (m, 3H), 6.93 (dd,  $J=8.0$  and 6.4 Hz, 1H), 6.82 (d,  $J=8.8$  Hz, 2H), 6.60–6.58 (m, 1H), 5.05 (dd,  $J=5.6$  and 13.2 Hz, 1H), 4.23 (d,  $J=6.0$  Hz, 2H), 4.09 (brs, 1H), 3.72–3.70 (m, 4H), 3.66–3.59 (m, 4H), 3.55–3.46 (m, 4H), 3.45–3.44 (m, 3H), 3.03–3.00 (m, 4H), 2.86–2.80 (m, 2H), 2.67–2.60 (m, 2H), 2.40–2.37 (m, 2H), 2.05–1.97 (m, 1H); LCMS (ESI)  $m/z$  949 [M + H]<sup>+</sup>.

(2*S*)-2-((4-(14-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-3-oxo-6,9,12-trioxa-2-azatetradecyl)phenyl)sulfonamido)-3-(1*H*-indol-3-yl)-*N*-(4-morpholinophenyl)propanamide (**41**)

The title compound was prepared from amine **10** and acid **38c** according to general procedure as a yellow solid (13.7 mg, 49%); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.08 (s, 1H), 10.72 (s, 1H), 9.67 (s, 1H), 8.45 (dd,  $J=6.4$  and 5.2 Hz, 1H), 8.10 (brs, 1H), 7.59–7.56 (m, 3H), 7.40 (d,  $J=8.0$  Hz, 1H), 7.28 (d,  $J=7.6$  Hz, 1H), 7.20–7.12 (m, 5H), 7.04–7.01 (m, 3H), 6.93 (dd,  $J=8.0$  and 6.8 Hz, 1H), 6.83 (d,  $J=8.8$  Hz, 2H), 6.60–6.58 (m, 1H), 5.05 (dd,  $J=5.2$  and 13.2 Hz, 1H), 4.23 (d,  $J=5.6$  Hz, 2H), 4.10 (brs, 1H), 3.73–3.70 (m, 4H), 3.64–3.59 (m, 4H), 3.54–3.43 (m, 11H), 3.07–3.01 (m, 4H), 2.92–2.80 (m, 2H), 2.60–2.50 (m, 2H), 2.40–2.36 (m, 2H), 2.03–1.97 (m, 1H); LCMS (ESI)  $m/z$  993 [M + H]<sup>+</sup>.

(2*S*)-*N*-(4-(4-(4-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)butanoyl)piperazin-1-yl)phenyl)-3-(1*H*-indol-3-yl)-2-((4-methylphenyl)sulfonamido)propanamide (**44**)

The title compound was prepared from tosyl-L-tryptophan (**42**) and **43a** according to general procedure as an orange solid (7 mg, 43%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.09 (brs, 1H), 10.76 (s, 1H), 9.67 (s, 1H), 8.06 (brs, 1H), 7.58 (t,  $J=8.0$  Hz, 1H), 7.50 (d,  $J=8.0$  Hz, 2H), 7.39 (d,  $J=8.0$  Hz, 1H), 7.28 (d,  $J=8.0$  Hz, 1H), 7.21–7.17 (m, 3H), 7.10–7.07 (m, 3H), 7.05–7.01 (m, 2H), 6.92 (t,  $J=7.2$  Hz, 1H), 6.84 (d,  $J=8.4$  Hz, 2H), 6.68 (brs, 1H), 5.05 (dd,  $J=5.2$  and 13.2 Hz, 1H), 4.07 (brs, 1H), 3.56 (brs, 4H), 3.04–3.01 (m, 5H), 2.92–2.80 (m, 3H), 2.67–2.56 (m, 2H), 2.47–2.43 (m, 2H), 2.23 (s, 3H), 2.01 (brs, 2H), 1.83–1.82 (m, 2H); LCMS (ESI)  $m/z$  859 [M + H]<sup>+</sup>.

(2*S*)-*N*-(4-(4-(3-(2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)propanoyl)piperazin-1-yl)phenyl)-3-(1*H*-indol-3-yl)-2-((4-methylphenyl)sulfonamido)propanamide (**45**)

The title compound was prepared from tosyl-L-tryptophan (**42**) and **43b** according to general procedure as a yellow solid (13 mg, 31%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.10 (brs, 1H), 10.75 (s, 1H), 9.66 (s, 1H), 8.08 (brs, 1H), 7.57 (t,  $J=8.0$  Hz, 1H), 7.50 (d,  $J=8.4$  Hz, 2H), 7.40 (d,  $J=8.8$  Hz, 1H), 7.28 (d,  $J=8.0$  Hz, 1H), 7.18 (d,  $J=8.8$  Hz, 2H), 7.13 (d,  $J=8.8$  Hz, 1H), 7.10–6.97 (m, 5H), 6.92 (t,  $J=7.2$  Hz, 1H), 6.80 (d,  $J=8.8$  Hz, 2H), 6.58–6.57 (m, 1H), 5.05 (dd,  $J=6.7$  and 13.9 Hz, 1H), 4.07 (brs, 1H), 3.70 (t,  $J=6.4$  Hz, 2H), 3.61–3.57 (m, 4H), 3.47–3.32 (m, 2H), 3.06–2.98 (m, 5H), 2.92–2.80 (m, 2H), 2.64–2.62 (m, 4H), 2.61–2.53 (m, 2H), 2.19 (s, 3H), 2.2–1.99 (m, 1H); LCMS (ESI)  $m/z$  889 [M + H]<sup>+</sup>.

(2*S*)-*N*-(4-(4-(3-(2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)propanoyl)piperazin-1-yl)phenyl)-3-(1*H*-indol-3-yl)-2-((4-methylphenyl)sulfonamido)propanamide (**46**)

The title compound was prepared from tosyl-L-tryptophan (**42**) and **43c** according to general procedure as a yellow solid (19 mg, 51%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.10 (brs, 1H), 10.76 (s, 1H), 9.66 (s, 1H), 8.08 (brs, 1H), 7.57 (t,  $J=8.0$  Hz, 1H), 7.50 (d,  $J=8.4$  Hz, 2H), 7.40 (d,  $J=8.8$  Hz, 1H), 7.28 (d,  $J=8.0$  Hz, 1H), 7.19 (d,  $J=8.8$  Hz, 2H), 7.13 (d,  $J=8.8$  Hz, 1H), 7.10–6.97 (m, 5H), 6.92 (t,  $J=7.6$  Hz, 1H), 6.82 (d,  $J=8.8$  Hz, 2H), 6.62–6.59 (m, 1H), 5.05 (dd,  $J=6.7$  and 13.9 Hz, 1H), 4.07 (brs, 1H), 3.65–3.53 (m, 10H), 3.45–3.44 (m, 4H), 3.06–2.99 (m, 5H), 2.91–2.84 (m, 2H), 2.59–2.53 (m, 4H), 2.23 (s, 3H), 2.2–1.99 (m, 1H); LCMS (ESI)  $m/z$  933 [M + H]<sup>+</sup>.

(2*S*)-*N*-(4-(4-(3-(2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethoxy)propanoyl)piperazin-1-yl)phenyl)-3-(1*H*-indol-3-yl)-2-((4-methylphenyl)sulfonamido)propanamide (**47**)

The title compound was prepared from tosyl-L-tryptophan (**42**) and **43d** according to general procedure as an orange solid (5 mg, 37%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  11.07 (brs, 1H), 10.75 (s, 1H),

9.68 (s, 1H), 8.05 (brs, 1H), 7.57 (t,  $J=8.0$  Hz, 1H), 7.50 (d,  $J=8.0$  Hz, 2H), 7.39 (d,  $J=8.0$  Hz, 1H), 7.28 (d,  $J=7.6$  Hz, 1H), 7.19 (d,  $J=9.2$  Hz, 2H), 7.13 (d,  $J=8.0$  Hz, 1H), 7.14–7.01 (m, 5H), 6.91 (t,  $J=8.0$  Hz, 1H), 6.83 (d,  $J=8.0$  Hz, 2H), 6.60 (m, 1H), 5.05 (dd,  $J=5.2$  and  $12.8$  Hz, 1H), 4.06 (brs, 1H), 3.64–3.45 (m, 14H), 3.04 (m, 3H), 2.99 (m, 2H), 2.89–2.80 (m, 3H), 2.60–2.57 (m, 6H), 2.23 (s, 3H), 2.03 (brs, 2H); LCMS (ESI)  $m/z$  977  $[M+H]^+$ .

(2*S*)-*N*-(4-(4-(1-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-3,6,9,12-tetraoxapentadecan-15-oyl)piperazin-1-yl)phenyl)-3-(1*H*-indol-3-yl)-2-((4-methylphenyl)sulfonamido)propanamide (**48**)

The title compound was prepared from tosyl-L-tryptophan (**42**) and **43e** according to general procedure as a pale-yellow solid (5.0 mg, 25%).  $^1\text{H}$  NMR (400 MHz, Acetone- $d_6$ )  $\delta$  9.97 (brs, 1H), 9.90 (brs, 1H), 8.90 (brs, 1H), 7.60–7.54 (m, 3H), 7.41 (d,  $J=7.6$  Hz, 1H), 7.33–7.30 (m, 3H), 7.14–7.09 (m, 4H), 7.07–7.04 (m, 2H), 6.94 (t,  $J=8.0$  Hz, 1H), 6.87 (d,  $J=8.8$  Hz, 2H), 6.62–6.59 (m, 2H), 5.07 (dd,  $J=5.4$  and  $12.6$  Hz, 1H), 4.16–4.11 (m, 1H), 3.86–3.71 (m, 4H), 3.69–3.65 (m, 4H), 3.64–3.61 (m, 4H), 3.58–3.57 (m, 8H), 3.55–3.53 (m, 2H), 3.26–3.21 (m, 1H), 3.14–3.09 (m, 2H), 3.07–3.03 (m, 2H), 3.00–2.92 (m, 1H), 2.76–2.72 (m, 1H), 2.64 (t,  $J=6.4$  Hz, 2H), 2.26 (s, 3H), 2.24–2.19 (m, 1H), 2.17–2.09 (m, 1H), 1.61–1.59 (m, 1H); LCMS (ESI)  $m/z$  1019  $[M-H]^-$ .

(2*S*)-*N*-(4-(4-(3-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-3-oxopropyl)piperazin-1-yl)phenyl)-3-(1*H*-indol-3-yl)-2-((4-methylphenyl)sulfonamido)propanamide (MC\_009279) (**51**)

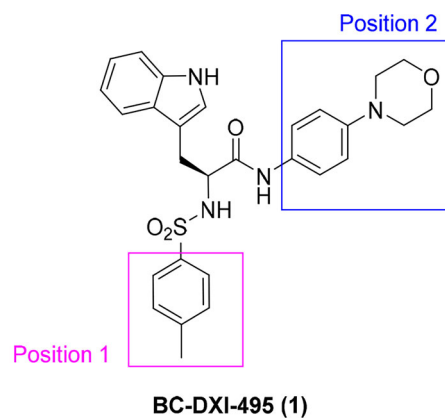
The title compound was prepared from tosyl-L-tryptophan (**42**) and 3-(4-(4-aminophenyl)piperazin-1-yl)-*N*-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)propanamide (**50a**) according to general procedure as a yellow solid (37.5 mg, 55%).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.12 (brs, 1H), 10.75 (s, 1H), 10.38 (s, 1H), 9.63 (s, 1H), 8.55 (d,  $J=8.4$  Hz, 1H), 8.04 (brs, 1H), 7.84 (dd,  $J=7.6$  and  $8.0$  Hz, 1H), 7.61 (d,  $J=7.2$  Hz, 1H), 7.51 (d,  $J=8.4$  Hz, 2H), 7.40 (d,  $J=7.6$  Hz, 1H), 7.29 (d,  $J=8.4$  Hz, 1H), 7.18 (d,  $J=8.8$  Hz, 2H), 7.11–7.02 (m, 4H), 6.93 (t,  $J=7.6$ , 1H), 6.80 (d,  $J=9.2$  Hz, 2H), 5.12 (dd,  $J=4.8$  and  $12.4$  Hz, 1H), 4.09–4.07 (m, 1H), 3.11–3.02 (m, 5H), 2.87–2.81 (m, 2H), 2.71–2.58 (m, 8H), 2.24 (s, 3H), 2.04–1.94 (m, 2H), 1.74 (brs, 1H); LCMS (ESI)  $m/z$  845  $[M+H]^+$ .

4-(4-(4-((*S*)-3-(1*H*-indol-3-yl)-2-((4-methylphenyl)sulfonamido)propanamido)phenyl)piperazin-1-yl)-*N*-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)butanamide (**52**)

The title compound was prepared from tosyl-L-tryptophan (**42**) and amine **50b** according to general procedure as a yellow solid (27.0 mg, 45%).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.13 (s, 1H), 10.74 (s, 1H), 9.70 (s, 1H), 9.60 (s, 1H), 8.49 (d,  $J=8.4$  Hz, 1H), 7.98 (d,  $J=9.2$  Hz, 1H), 7.82 (t,  $J=8.0$  Hz, 1H), 7.59 (d,  $J=7.2$  Hz, 1H), 7.51 (d,  $J=8.0$  Hz, 2H), 7.40 (d,  $J=7.6$  Hz, 1H), 7.29 (d,  $J=8.0$  Hz, 1H), 7.16 (d,  $J=9.2$  Hz, 2H), 7.11–7.02 (m, 4H), 6.92 (t,  $J=7.2$  Hz, 1H), 6.78 (d,  $J=9.2$  Hz, 2H), 5.13 (dd,  $J=5.2$  and  $12.4$  Hz, 1H), 4.13–4.04 (m, 1H), 3.07–3.01 (m, 5H), 2.94–2.81 (m, 3H), 2.67–2.58 (m, 2H), 2.40 (brs, 2H), 2.23 (s, 3H), 2.09–2.04 (m, 1H), 1.87–1.82 (m, 2H), (five resonance obscured or overlapping); LCMS (ESI)  $m/z$  857  $[M-H]^-$ .

5-(4-(4-((*S*)-3-(1*H*-indol-3-yl)-2-((4-methylphenyl)sulfonamido)propanamido)phenyl)piperazin-1-yl)-*N*-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)pentanamide (**53**)

The title compound was prepared from tosyl-L-tryptophan (**42**) and amine **50c** according to general procedure as a yellow solid (7.8 mg, 25%).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.14 (s, 1H), 10.75 (s, 1H), 9.70 (s, 1H), 9.62 (s, 1H), 8.47 (t,  $J=8.4$  Hz, 1H), 8.04 (brs, 1H), 7.83 (t,  $J=8.0$  Hz, 1H), 7.62 (d,  $J=7.2$  Hz, 1H), 7.50 (d,  $J=8.0$  Hz, 2H), 7.39 (d,  $J=7.6$  Hz, 1H), 7.28 (d,  $J=8.0$  Hz, 1H), 7.17 (d,  $J=8.8$  Hz, 2H), 7.11–7.01 (m, 4H), 6.92 (t,  $J=7.2$  Hz, 1H), 6.80 (d,  $J=9.2$  Hz, 2H), 5.14 (dd,  $J=5.2$  and  $12.8$  Hz, 1H), 4.09–4.06 (m, 1H), 3.04–3.01 (m, 5H), 2.94–2.86 (m, 2H), 2.67–2.54 (m, 2H), 2.34



**Figure 2.** Chemical Structures of BC-DXI-495 (**1**) and putative positions to link the E3 ligase ligand.

(dd,  $J=7.2$  and  $6.8$  Hz, 2H), 2.05 (s, 3H), 2.07–2.03 (m, 1H), 1.97–1.92 (m, 1H), 1.70–1.63 (m, 2H), 1.56–1.49 (m, 2H), (five resonances obscured or overlapping); LCMS (ESI)  $m/z$  871  $[M-H]^-$ .

6-(4-(4-((*S*)-3-(1*H*-indol-3-yl)-2-((4-methylphenyl)sulfonamido)propanamido)phenyl)piperazin-1-yl)-*N*-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)hexanamide (**54**)

The title compound was prepared from tosyl-L-tryptophan (**42**) and amine **50d** according to general procedure as a yellow solid (5.4 mg, 12%).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.14 (s, 1H), 10.75 (s, 1H), 9.70 (s, 1H), 9.61 (s, 1H), 8.48 (t,  $J=8.0$  Hz, 1H), 8.03 (d,  $J=8.0$  Hz, 1H), 7.83 (t,  $J=8.0$  Hz, 1H), 7.61 (d,  $J=7.2$  Hz, 1H), 7.51 (d,  $J=8.0$  Hz, 2H), 7.40 (d,  $J=8.0$  Hz, 1H), 7.28 (d,  $J=8.4$  Hz, 1H), 7.17 (d,  $J=9.2$  Hz, 2H), 7.11–7.01 (m, 4H), 6.92 (t,  $J=7.2$  Hz, 1H), 6.79 (d,  $J=9.2$  Hz, 2H), 5.15 (dd,  $J=5.6$  and  $12.8$  Hz, 1H), 4.10–4.06 (m, 1H), 3.06–3.01 (m, 5H), 2.94–2.80 (m, 2H), 2.67–2.54 (m, 2H), 2.31 (t,  $J=7.2$  Hz, 2H), 2.23 (s, 3H), 2.08–2.05 (m, 1H), 1.97–1.93 (m, 1H), 1.70–1.62 (m, 2H), 1.53–1.46 (m, 2H), 1.40–1.33 (m, 2H), (five resonances obscured or overlapping); LCMS (ESI)  $m/z$  887  $[M+H]^+$ .

## Results and discussion

### Design and synthesis of AIMP2-DX2 targeting PROTACs

We designed and synthesised AIMP2-DX2 PROTACs using the reported potent small-molecule, BC-DXI-495 (**1**) as the targeting protein ligase ligand (Figure 2). Based on the analysis of the docking model of compound **1** in the AIMP2-DX2-GST domain, the toluene part in position 1 and the morpholine part of position 2 have enough space to add linkers since the compound is less sterically hindered and exposed to the surface whereas the indole part of the molecule is embedded inside the protein<sup>9</sup>. Accordingly, the addition of various forms of the linkers and E3 ligase ligands were attempted in both positions 1 and 2 to find the best combination of the PROTAC.

Among a slew of E3 ubiquitin ligases, Siah1 protein is known to regulate the level of AIMP2-DX2 in cells using the ubiquitination-degradation process<sup>9</sup>. Therefore, in order to degrade the target protein AIMP2-DX2 through chemically induced degradation, it is necessary to find an appropriate E3 ligase ligand to bind this Siah1. Currently, two types of E3 ligase ligand are being used and studied extensively, i.e. von-Hippel-Lindau (VHL) (**2**) and Cereblon (CRBN). In the case of CRBN, 4-hydroxythalidomide (**3**) and pomalidomide (4-aminothalidomide, **4**) are the most used in research (Figure 3).

Thus, we used these compounds to find the suitable E3 ligase ligand for Siah1 and conducted the surface plasmon resonance

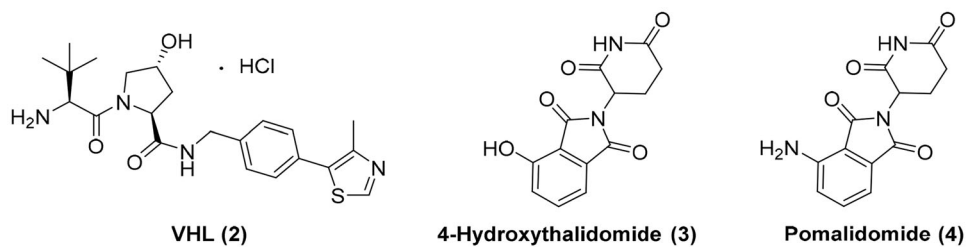


Figure 3. Representative E3 ligase ligands.

Input		Pull-down					
-	+	+	-	-	-		
-	-	-	-	+	+	-	<b>Biotin</b>
-	-	-	-	-	+	+	<b>Biotin-4-hydroxythalidomide (5)</b>
-	-	-	-	-	-	+	<b>Biotin-pomalidomide (6)</b>
-	+	-	+	-	+	-	<b>Siah1</b>
+	-	+	-	+	-	+	<b>AIMP2-DX2</b>
							<b>Siah1</b>
							<b>AIMP2-DX2</b>

Figure 4. *In vitro* pull-down assay representing final selection of E3 ligase ligand.

(SPR) assay. Both 4-hydroxythalidomide (**3**) and pomalidomide (**4**) showed some degree of binding affinity whereas VHL ligand has not shown any affinity with Siah1 (details are not reported). For further confirmation, biotin-attached 4-hydroxythalidomide (**5**) and pomalidomide (**6**) were synthesised (see details in [Supplementary Material](#)) and conducted the *in vitro* pull-down assay to see whether both CRBN types of E3 ligase ligands bind to Siah1. The result from the *in vitro* binding assay shows that Siah1 protein binds to both types of CRBN E3 ligase ligands but not to AIMP2-DX2 which means that 4-hydroxythalidomide (**3**) and pomalidomide (**4**) specifically bind to Siah1. Therefore, we decided to use both CRBN types of E3 ligase ligands for our study (Figure 4).

## Chemistry

The synthetic approaches used to generate AIMP2-DX2 PROTACs using BC-DXI-495 (**1**) derivatives and two types of CRBN-based E3 ligase ligands are described in [Schemes 1–5](#). Since the length of the linker plays a key role in the potency of PROTAC degraders, we systematically varied the linker length in compounds ranging from 4 to 22 atoms with the objective of determining the optimal linker length. The linker and E3 ligase ligand complexes were either purchased from Sigma-Aldrich® or synthesised based on literatures<sup>16–20</sup>. As mentioned above, BC-DXI-495 (**1**) derivative was synthesised according to the procedure shown in [Scheme 1](#). Starting from commercially available *L*-tryptophan (**7**) with 4-cyanobenzenesulfonyl chloride under basic conditions to give compound **8** and subsequent standard amide coupling conditions with 4-morpholinoaniline afforded compound **9**. Then, the nitrile moiety was reduced to amine **10** and further reaction with 2-azido-1,3-dimethylimidazolium hexafluorophosphate in dichloromethane gave the azide product **11**. Click reaction of compound **11** with alkyne **12a–d**<sup>16</sup> and **17a–f** afforded 4-hydroxy thalidomide-based PROTACs **13–16** and pomalidomide-based PROTACs **18–23**, respectively.

The preparation of AIMP2-DX2 degraders containing linear linkers instead of triazole-containing linkers was synthesised ([Scheme 2](#)). Thus, hydrolysis of nitrile **9** under basic conditions gave the corresponding acid **24**, which was then further treated with various forms of linker and E3 ligase ligand combinations for standard amination conditions to give PROTACs **25–26**, **28–31**, and **33–34** ([Scheme 2](#)).

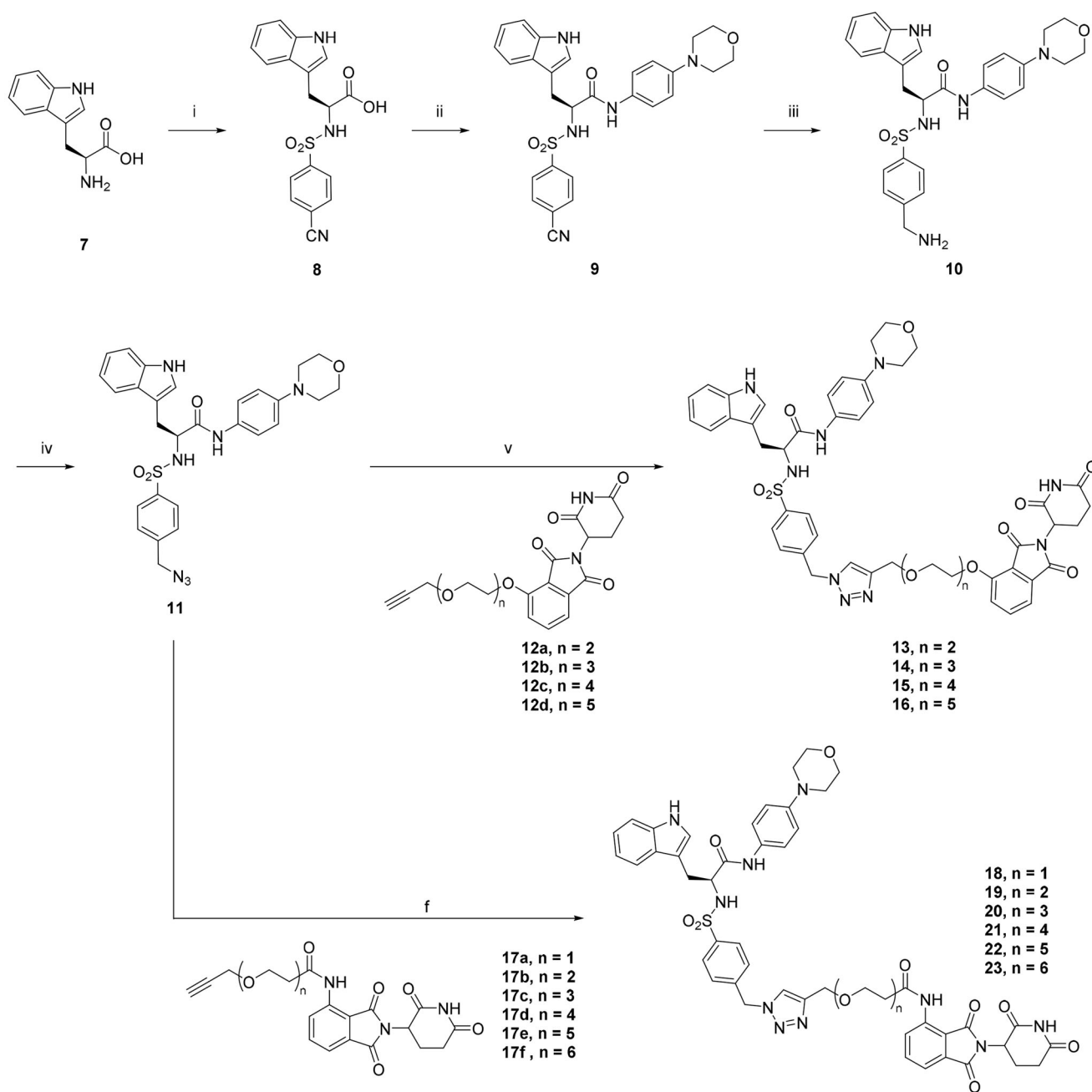
For other variations, amine **10** was treated with HATU, DIPEA and pomalidomide-based linkers **35a,b** and **38a–c** in DMF for overnight at room temperature affording the desired AIMP2-DX2 PROTACs **36–37** and **39–41**, respectively.

To prepare position 2 linked PROTACs **44–48**, the pomalidomide-based analogues used in [Scheme 3](#) were coupled with 4-(piperazin-1-yl) aniline, followed by another amination reaction with HATU and DIPEA to yield the desired compounds **44–48** ([Scheme 4](#)).

Lastly, E3 ligase linkers **49a–d**<sup>20</sup> were alkylated with 4-(piperazin-1-yl) aniline to afford corresponding compounds **50a–d**. These compounds were then further treated with compound **42** under basic conditions and gave the desired products **51–54**.

We then assessed inhibitory effects of all the designed PROTACs **13–16**, **18–23**, **25**, **26**, **28–31**, **33**, **34**, **36**, **37**, **39–41**, **44–48**, and **51–54** on the AIMP2-DX2-expressing A549 cell in treatment conditions at 4  $\mu$ M for 24 h and the results are shown in [Tables 1](#) and [2](#). For comparison, BC-DXI-495 (**1**) was used as the reference compound to compare the inhibitory activity. To be noted, linker length dependence was observed in AIMP2-DX2 degraders. PROTACs with longer linkers, such as **16**, **21**, **22**, **23**, and **31** possessed very poor inhibitory activities, which implied that these longer molecules were less preferred ([Table 1](#)). In addition, even if the same linker and E3 ligase ligand were used, the activity may vary depending on the location where the target molecule is linked. First, triazole containing 4-hydroxythalidomide-based PROTACs **13–16** were assessed for inhibitory activity. Compound **14** with triazole and 9 atoms in the linker was the most potent in this series with about 10% more potent than BC-DXI-495 (**1**). In the same series, either reducing or increasing the length of the linker decreases efficacy. These results indicate the optimal linker length is around 13 atoms. Switching to pomalidomide-based triazole-containing products, PROTACs **18–23** were tested against AIMP2-DX2. Interestingly, none of the above compounds have superior activity against AIMP2-DX2 compared to compound **1**. We then replaced the triazole linkage with an amine linkage. The 4-hydroxythalidomide-based amine-linked PROTACs **25** and **26** resulted in increased potency with 46.16% and 46.39% inhibition, respectively. However, when the E3 ligase ligand was changed to pomalidomide compounds **28–34**, the potency was dramatically decreased to the lowest of 14.84%. Considering that compounds **26** and **30** have the same length of the carbon atom linkers, it can be concluded that 4-hydroxythalidomide is preferable with the amide linear linkage. Reverse amide products **36–41** were also tested. Compared to compounds **36** and **37**, the inhibitory activity of compounds **39–41** was higher. Therefore, we suspect that the presence of oxygen atoms in the linkage is crucial for AIMP2-DX2 degraders.

To compare the position effect of the target, combinations of linkers and E3 ligase ligands were linked to position 2 of the target compound. Therefore, % inhibition of AIMP2-DX2 was measured for compounds **44–48**, **51–53**, and **54** ([Table 2](#)). Interestingly, AIMP2-DX2 degrader **44** showed more than 50%



**Scheme 1.** Synthesis of AIMP2-DX2 PROTACs **13–16**, **18–23**. Reagents and conditions: (i) 4-cyanobenzenesulfonyl chloride, TEA, THF:H<sub>2</sub>O, 0–25 °C, overnight; (ii) 4-morpholinoaniline, HATU, DIPEA, DMF, 25 °C, overnight; (iii) LiAlH<sub>4</sub>, THF, 0–25 °C, overnight; (iv) ADMP, DCM, DMAP, 25 °C overnight; (v) (+)-Sodium L-ascorbate, copper(II) sulphate pentahydrate, DMSO, 25 °C, overnight.

inhibitory activity although the length of the linker was only four atoms apart. Introducing one ether unit in the linker, the activity of compound **45** was further increased to 51.99%. However, longer linker lengths of the same series also led to a decrease in inhibitory activity similar to those attached to position 1. When carbonyl is presented next to an E3 ligase ligand rather than the target, the inhibitory activity of compounds **51–54** only presents a decrease in inhibitory activity with an increase in linker length. As a result, it was found that position 2 favours a shorter linker length, such as 4–6 atoms in length between the target and the E3 ligase ligand. In addition, like Position 1, the linker at position 2 also shows better activity with oxygen-containing linkers.

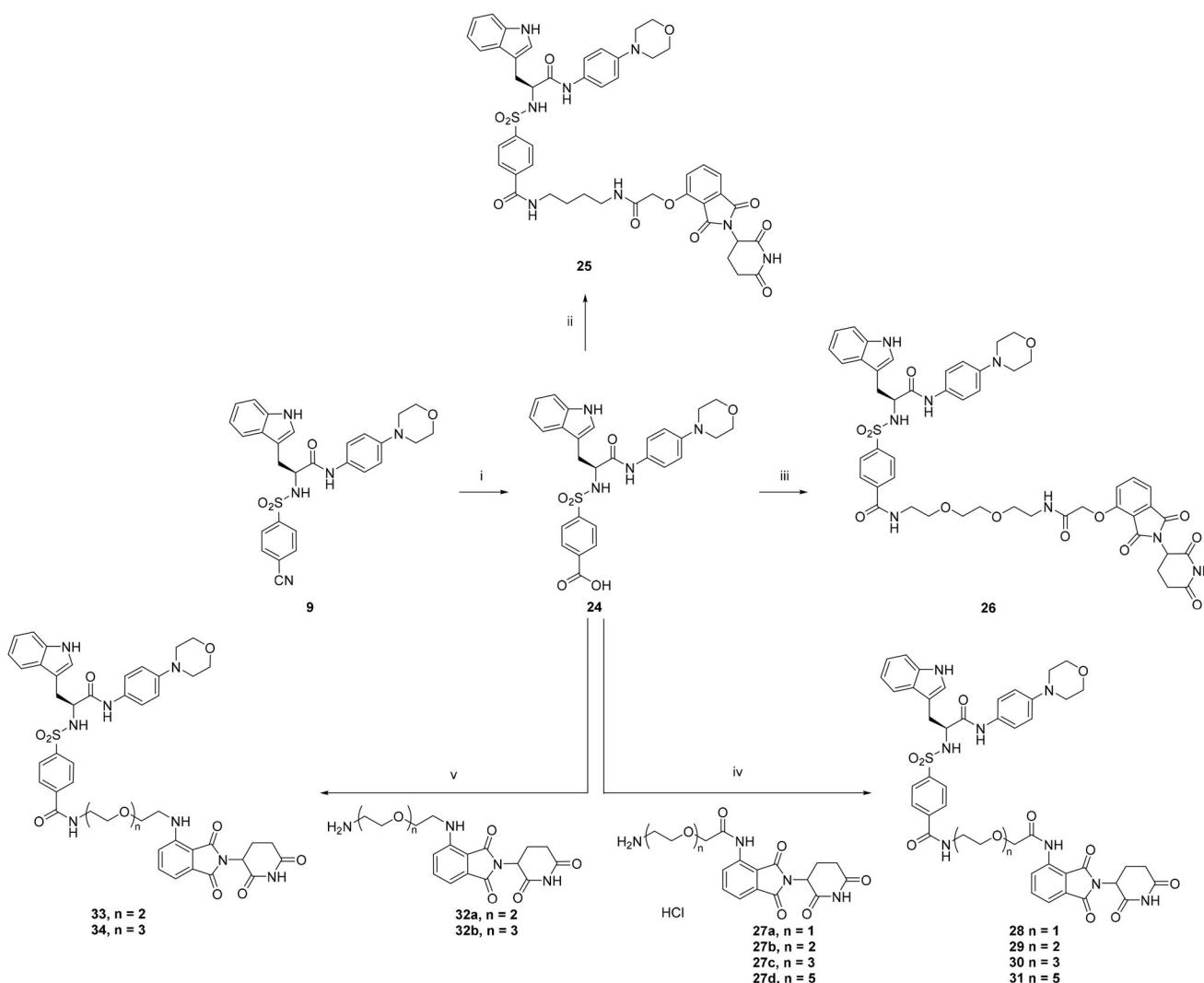
To confirm the inhibitory activity of compounds, **14** compounds with > 37% inhibitory activity were submitted for further

assay to determine the 50% inhibitory concentration (IC<sub>50</sub>). Among these PROTACs, compound **45** was the most potent AIMP2-DX2 degrader with an IC<sub>50</sub> value of 2.37 μM (Table 3). This indicated that the inhibitory activity of the PROTAC compound was slightly improved and comparable to the small-molecule inhibitor itself (**1**).

#### In vitro bioassays

Since PROTAC compound links the substrate and E3 ligase, AIMP2-DX2, and Siah1 in this study, respectively, we checked whether compound **45** affects the cellular binding of AIMP2-DX2 and Siah1. As a result, it was observed that treatment of compound **45** enhances the endogenous binding of Siah1 to AIMP2-





**Scheme 2.** Synthesis of AIMP2-DX2 PROTACs **25**, **26**, **28–31**, and **33–34**. Reagents and conditions: (i) KOH, 2-propanol, reflux, 18 h; (ii) HATU, DIPEA, DMF, 25 °C, overnight; (iii) HATU, DIPEA, DMF, 25 °C, overnight; (iv) HATU, DIPEA, DMF, 25 °C, overnight.

DX2 in a dose-dependent manner (Figure 5(A)). Then, we also determined the direct effect of compound **45** on binding of two proteins *via in vitro* pull-down assay (Figure 5(B)), implying that compound **45** could function as a PROTAC compound for connecting AIMP2-DX2 and Siah1 as a substrate and E3 ligase, respectively.

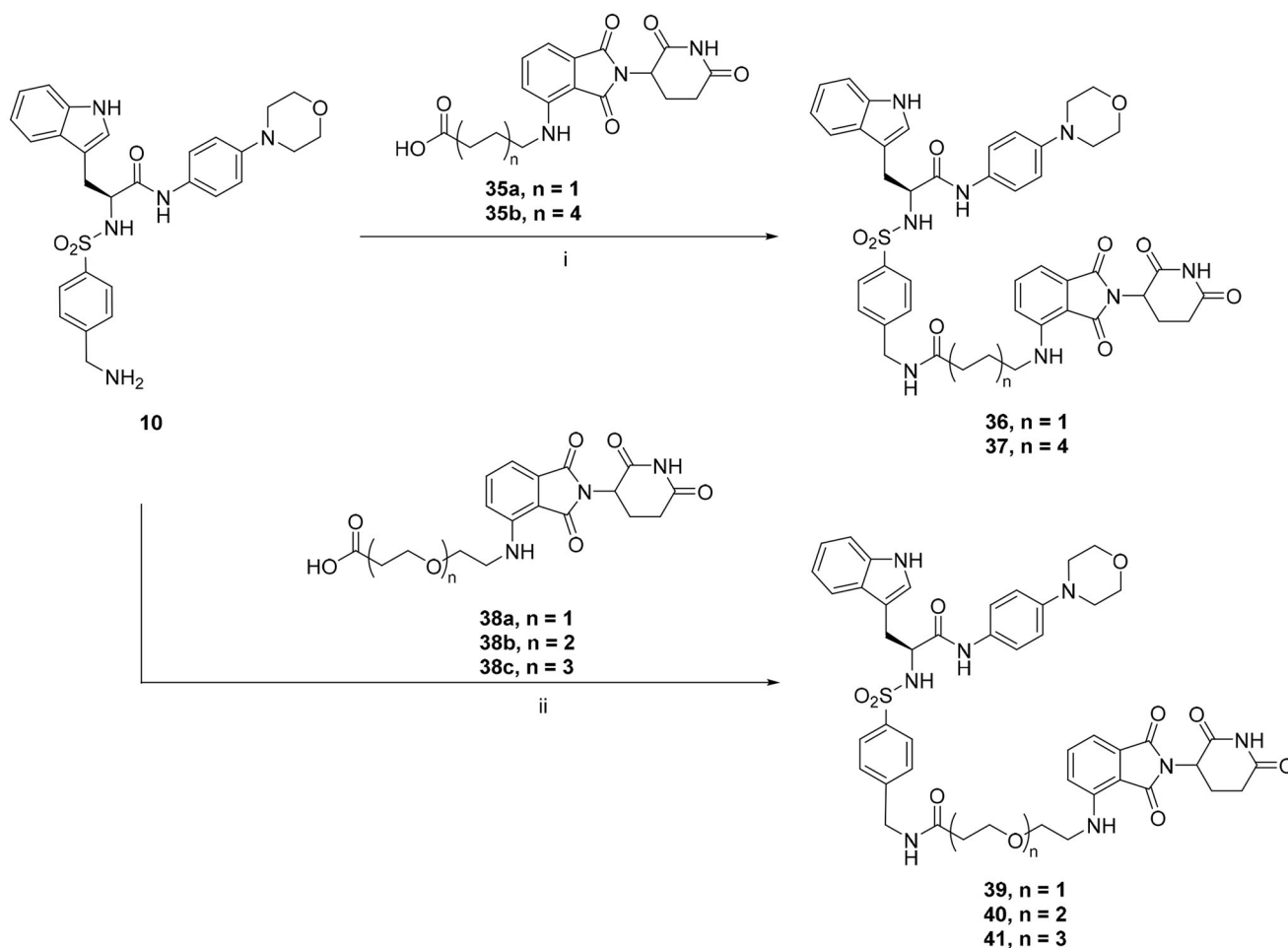
In addition, to check whether the PROTAC compound results in the degradation of AIMP2-DX2 *via* its binding to Siah1, we checked the protein and mRNA level of AIMP2-DX2. Compound **45** specifically decreased the level of AIMP2-DX2 protein, but not mRNA (Figure 6(A)), suggesting that compound **45** functions in the PROTAC system. The compound also selectively led to the degradation of AIMP2-DX2 without any effect on AIMP2, implying the specificity of compound **45**. We further confirmed that the ubiquitination of AIMP2-DX2 was induced by the treatment of compound **45** (Figure 6(B)). Altogether, it could be concluded that compound **45** acts as a PROTAC compound to degrade oncogenic protein, AIMP2-DX2.

To ensure whether compound **45** ultimately induces the decline of cell viability *via* degradation of AIMP2-DX2, we determined the anti-proliferative efficacy of compound **45**. Compound **45** efficiently reduced cell viability but there was no significant

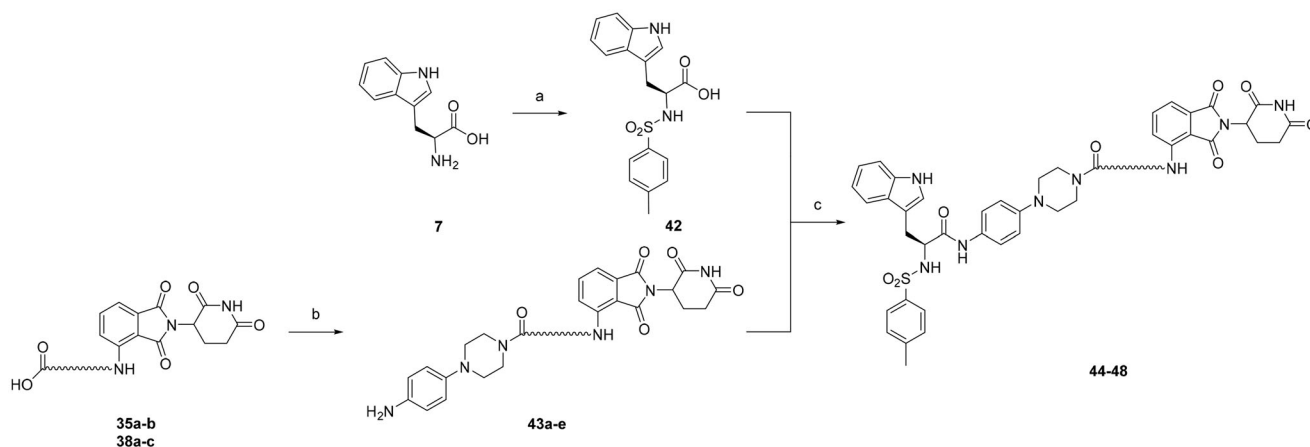
difference in cell viability, when Siah1 was knocked down using its specific si-RNA (Figure 7(A)), implying that compound **45** utilizes Siah1 as the E3 ligase. Compound **45** was further tested in several lung cancer cell lines, Calu6, HCC1588, and H226, showing different protein levels of AIMP2-DX2 and Siah1. Compound **45** efficiently declined the cell viability of only Calu6 which has a high level of both AIMP2-DX2 and Siah1 among the tested cell lines (Figure 7(B)). Combined together, compound **45** was unveiled to depend on the existence of both substrate and E3 ligase to function as a PROTAC. From the comparison of EC<sub>50</sub> with IC<sub>50</sub> of compound **45**, we also concluded that the anti-proliferative efficacy of the compound was mediated by degradation of AIMP2-DX2.

#### Prediction of binding modes of E3 ligases in the Siah1

We have also performed docking of the E3 ligase ligand pomalidomide (**4**) and its parent compound, thalidomide against the human E3 ubiquitin ligase Siah1 to predict the binding modes of E3 ligase ligands. The selected *homo sapiens* Siah1 PDB structure was 4CA121 with RMSD of 1.58 Å and the overall quality based on



**Scheme 3.** Synthesis of AIMP2-DX2 PROTACs **36**, **37**, and **39–41**. Reagents and conditions: (i), (ii) HATU, DIPEA, DMF, 25 °C, overnight.

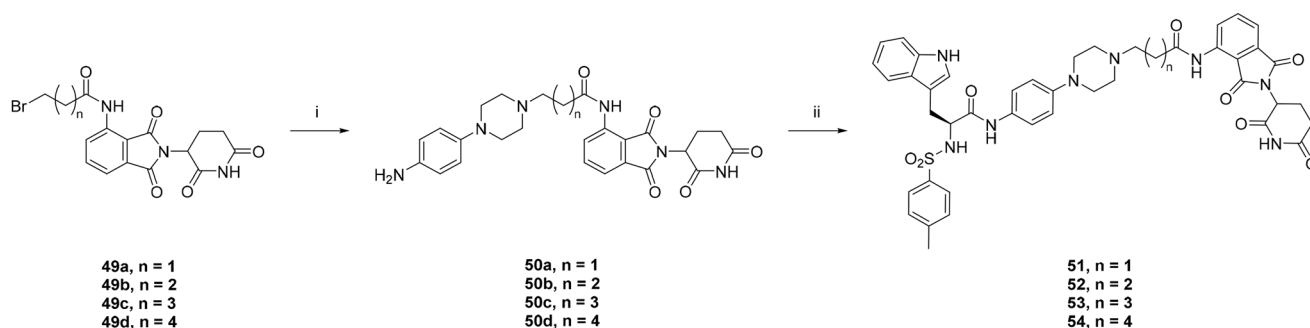


**Scheme 4.** Synthesis of AIMP2-DX2 PROTACs **45–49**. Reagents and conditions: (i) *p*-Toluenesulfonyl chloride,  $\text{NEt}_3$ , THF:H<sub>2</sub>O (10:1 v/v mixture) 0–25 °C, overnight; (ii) HATU, DIPEA, DCM, 25 °C, 1 h; (iii) 4-(piperazin-1-yl) aniline, HATU, DIPEA, DMF, 60 °C, 4 h.

wwPDB structure validation indicated a good quality x-ray structure. This homodimer protein structure was prepared for docking using Protein Preparation tool where all waters were removed, and the hydrogens were added. Putative binding sites in each chain were predicted using CASTp version 3.0 (Chicago, IL, USA)<sup>22</sup>. Predicted Pockets 1 and 3 were targeted for docking (Figure 8).

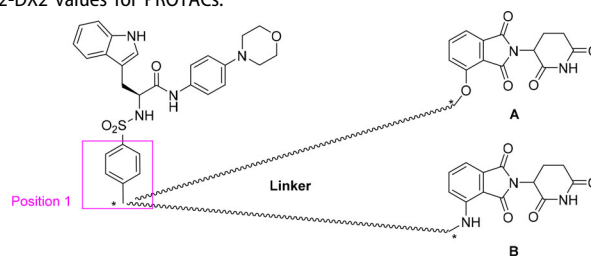
The program used for docking was Glide, Schrödinger (release version 2021–4), using the XP mode<sup>23</sup>. The putative binding sites were defined by selected residues constituting Pockets 1 and 3.

Selected Pockets 1 and 3 are exposed towards the surface area, which could be simulated that E3 ligase ligands would be connected with the linker and the target binder. The best docking poses were determined based on docking scores as well as binding modes. Based on the docking results, pomalidomide (**4**) was docked better than thalidomide in each of the designated Pockets 1 and 3. In general, pomalidomide (**4**) interacted more with residues due to its additional amino group (see [Supplementary Material](#) for more details, Figure S1). This is in correlation with the



**Scheme 5.** Synthesis of AIMP2-DX2 PROTACs 51–54. Reagents and conditions: (i) 4-(piperazin-1-yl) aniline,  $K_2CO_3$ , ME CN, reflux, overnight; (ii) compound 42, HATU, DIPEA, DCM, 25 °C, 1 h, overnight.

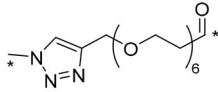
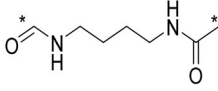
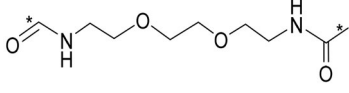
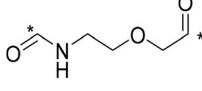
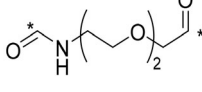
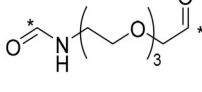
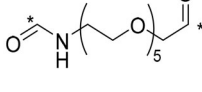
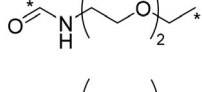
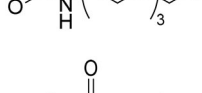
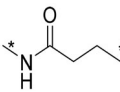
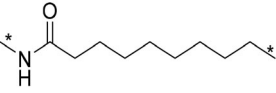
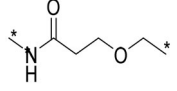
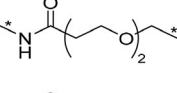
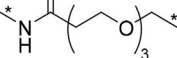
**Table 1.** Structures and the % inhibition AIMP2-DX2 values for PROTACs.



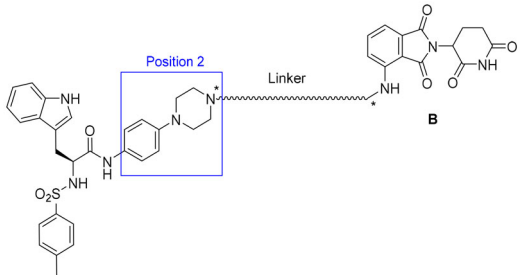
Compound	E3 ligand	Number of non-H linker atoms	Linker	% Inhibition of AIMP2-DX2 Luciferase <sup>a,b</sup>
1				37.67 ± 15.68
13	A	10		36.63 ± 15.82
14	A	13		47.09 ± 6.11
15	A	16		41.48 ± 1.88
16	A	18		32.97 ± 5.23
18	B	8		34.11 ± 5.44
19	B	11		38.13 ± 1.33
20	B	14		33.79 ± 3.20
21	B	17		24.03 ± 1.17
22	B	20		29.10 ± 8.91

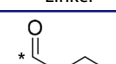



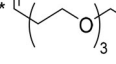
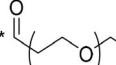
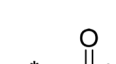

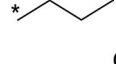
(continued)

Table 1. Continued.

Compound	E3 ligand	Number of non-H linker atoms	Linker	% Inhibition of AIMP2-DX2 Luciferase <sup>a,b</sup>
23	B	23		23.61 ± 18.96
25	A	8		46.16 ± 6.60
26	A	12		46.39 ± 5.37
28	B	6		35.88 ± 14.48
29	B	9		24.65 ± 5.14
30	B	12		24.37 ± 16.10
31	B	18		14.84 ± 14.91
33	B	9		32.40 ± 11.15
34	B	12		34.01 ± 5.36
36	B	4		37.50 ± 5.70
37	B	10		31.73 ± 5.42
39	B	6		40.03 ± 4.42
40	B	9		44.61 ± 4.77
41	B	12		37.80 ± 5.19

<sup>a</sup>The change of the luciferase-tagged AIMP-DX2 level upon treating chemicals was determined by detecting the luminescence signal. <sup>b</sup>4 μM, 24 h.

**Table 2.** Structures and the % inhibition AIMP2-DX2 values for PROTACs with different linkers.


Compound	E3 ligand	Number of non-H linker atoms	Linker	% Inhibition of AIMP2-DX2 Luciferase <sup>a,b</sup>
44	B	4		51.10 ± 3.69
45	B	6		51.99 ± 1.89
46	B	9		45.79 ± 6.04
47	B	12		39.74 ± 3.28
48	B	15		26.11 ± 4.25
51	B	3		25.50 ± 9.90
52	B	4		35.33 ± 4.64
53	B	5		44.44 ± 8.81
54	B	6		42.53 ± 5.94

<sup>a</sup>The change of the luciferase-tagged AIMP2-DX2 level upon treating chemicals was determined by detecting the luminescence signal.

<sup>b</sup>4 μM, 24 h.

**Table 3.** IC<sub>50</sub> value of compounds against AIMP2-DX2.

Compound number	IC <sub>50</sub> (μM)
1	2.65 ± 2.74
14	3.58 ± 0.40
15	3.31 ± 0.21
19	N.A. <sup>a</sup>
25	3.74 ± 0.11
26	3.53 ± 2.68
36	10.4 ± 2.42
39	N.A. <sup>a</sup>
40	N.A. <sup>a</sup>
44	2.75 ± 1.74
45	2.37 ± 0.09
46	3.96 ± 0.77
47	N.A. <sup>a</sup>
53	4.80 ± 1.89
54	4.41 ± 0.93

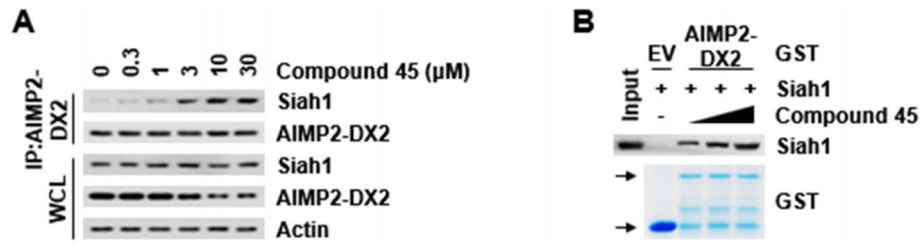
<sup>a</sup>N.A.: not active

SPR results where pomalidomide (**4**) was shown to be the most favourable E3 ligase of choice for Siah1. Overall, E3 ligase ligands docked better in Pocket 3.

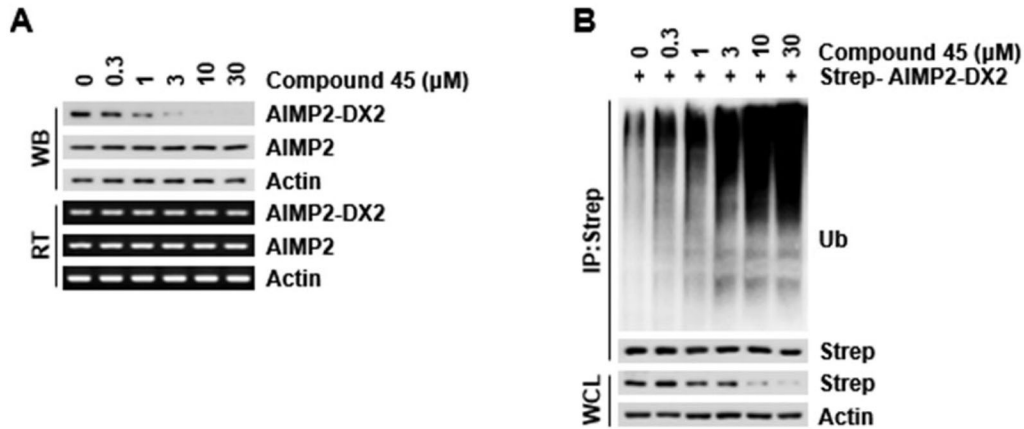
## Biological methods

### Cell culture and materials

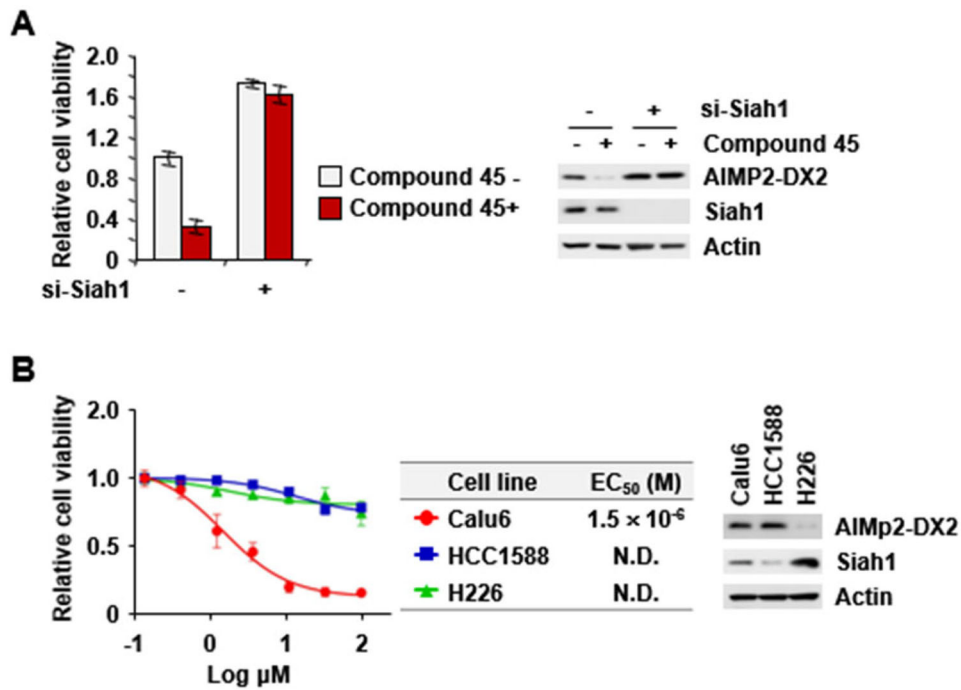
The H460, Calu6, HCC1588, H226, A549, and 293T cell lines were kindly gifted from Biocon (Medicinal Bioconvergence Research Centre, Yonsei University, Seoul, South Korea). The H460, Calu6, HCC1588, H226, and A549 cells were cultivated in RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin in 5% CO<sub>2</sub> at 37 °C. 293T cells were cultured in Dulbecco's modified Eagle's medium under the same conditions as above. AIMP2-DX2 was cloned into the EcoRI/XhoI sites of the pEXPR-IBA5 vector to



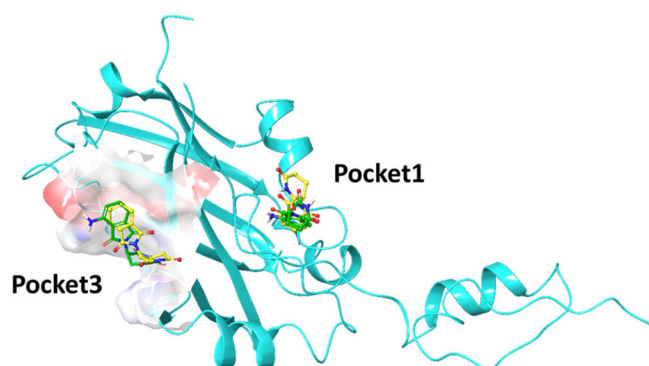
**Figure 5.** Compound 45 mediated induction of interaction between Siah1 and AIMP2-DX2. (A) Immunoprecipitation assay showing compound 45-dependent increased binding of two proteins. IP and WCL mean immunoprecipitation and whole cell lysates, respectively. Actin was used as a loading control. (B) *In vitro* pull-down assay confirming compound 45-mediated direct effect on binding of two proteins. GST proteins were detected by Coomassie staining. EV means empty vector.



**Figure 6.** Ubiquitination-mediated degradation of AIMP2-DX2 *via* compound 45. (A) Compound 45-mediated alteration of AIMP2-DX2 protein and mRNA level. Cells treated with compound 45 were subjected to western blotting (WB) and RT-PCR (RT). (B) Determination of compound 45-dependent ubiquitination of AIMP2-DX2. The cells expressing strep-AIMP2-DX2 were treated with compound 45 and MG132 and subjected to ubiquitination assay. Ub: ubiquitin.



**Figure 7.** Dependency of compound 45-mediated anti-proliferative efficacy on level of both AIMP2-DX2 and Siah1. (A) Siah1-dependency of the suppressed cell viability *via* compound 45. Siah1-knockdown cells *via* its specific si-RNA were treated with compound and subjected to MTT assay. The results were shown as a bar graph (left) and the level of proteins were checked by immunoblotting (right). (B) Compound 45-mediated cell viability in lung cancer cell lines with the different level of AIMP2-DX2 and Siah1. Cell viability was determined as above. N.D.: not determined. (A and B) The experiments were independently repeated three times.



**Figure 8.** Binding modes of pomalidomide (green atom-coloured stick) and thalidomide (yellow atom-coloured stick) in Pockets 1 and 3 of the human Siah1 protein (blue ribbon). The electron density surface is shown for Pocket 3.

express strep-tagged AIMP2-DX2. MG-132 (#474790) was purchased from Millipore. Specific antibodies against AIMP2-DX2 and AIMP2 were purchased from Curebio. The anti-Siah1 (#ab2237), -actin (#A1978), and ubiquitin (#sc-8017) antibodies were purchased from Abcam (Cambridge, UK), Sigma (St. Louis, MO), and Santa Cruz Biotechnology (Dallas, TX).

### Screening

A549 cells ( $4 \times 10^4$  cells/well) expressing luciferase-tagged AIMP2-DX2 were seeded in 96-well, flat-bottom white plates (#3903 Corning) and incubated for 12 h. The cells were treated with the compounds ( $4 \mu\text{M}$ ) for 24 h, and luminescence was detected following the manufacturer's protocol (Promega, Madison, WI). All experiments were independently repeated three times.

### Cell viability assay

Calu6, HCC1588, and H226 ( $4 \times 10^4$  cells/well) cells were cultured in 96-well, flat-bottom plates for 24 h. Serum-free media containing the diluted compounds was added to the cells and incubated for 96 h. Of  $10 \mu\text{L}$  of MTT solution ( $5 \text{ mg/mL}$ , Sigma, St. Louis, MO) was added to the cells and incubated for 1.5 h at  $37^\circ\text{C}$ . After discarding the culture media with the MTT solution, the precipitated formazan crystals in each well were dissolved with  $100 \mu\text{L}$  of DMSO (Duchefa, Haarlem, Netherlands). Absorbance was measured at 560 nm using a microplate reader (Sunrise, TECAN, Männedorf, Switzerland). All experiments were independently repeated three times.

### Ubiquitination assay

The 293T cells expressing strep-tagged AIMP2-DX2 were treated with the compounds with MG-132 ( $50 \mu\text{M}$ ) and lysed with  $50 \text{ mM}$  Tris-HCl (pH 7.4) lysis buffer containing  $100 \text{ mM}$  NaCl,  $0.5\%$  Triton X-100,  $0.5\%$  SDS,  $10\%$  glycerol,  $1 \text{ mM}$  EDTA, and protease inhibitor cocktail (Calbiochem). AIMP2-DX2 from total cell extracts were precipitated by using strep-tactin column. The precipitates were subjected to SDS-PAGE and immunoblotting using specific antibodies against the proteins of interest.

### Conclusions

In summary, a focussed library of AIMP2-DX2 degraders was developed. Various factors, including linker length, functional groups on the linker, type of E3 ligase ligands and target connection positions were systematically evaluated to understand AIMP2-DX2 PROTACs for deriving the best strategy for inhibiting AIMP2-DX2. As a result, the

PROTAC compound **45** targeting AIMP2-DX2 was identified. To the best of our knowledge, compound **45** is the first reported AIMP2-DX2 targeting PROTAC compound. Our data confirmed the applicability of PROTAC technology to target AIMP2-DX2, and this discovery of AIMP2-DX2 PROTAC compound **45** could potentially lead to future therapeutic opportunities to treat lung cancer.

### Disclosure statement

No potential conflict of interest was reported by the author(s).

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