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Design and Synthesis of Pin1-PROTACs as Potential Therapeutic Tools for Cancer Treatment

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Introduction

Peptidyl-prolyl isomerase NIMA-interacting-1 (Pin1) is a small two-domain protein member of the Peptidyl-Prolyl *cis-trans* Isomerases (PPIases) which catalyses the *cis-trans* isomerisation of Xaa-Proline amide ω-bonds in proteins [1]. Pin1 is structurally made up of two different domains, WW and PPIase, connected by a flexible linker loop region [2]. Pin1 differs from all others 30 PPIases through its unique substrate specificity for phosphorylated Serine/Threonine-Proline peptide bonds. Pin1 interacts with conformation-specific Pro-directed phosphatases and kinases to control common targets' stability, subcellular localization, and activity [3]. Pin1 is frequently overexpressed and/or overactivated in different types of cancer, and elevated Pin1 overexpression correlates with poor clinical prognosis [4]. Polymorphisms that under express Pin1 are linked with reduced tumour risk [5], and the depletion of Pin1 significantly inhibits tumorigenesis in mice models [6]. However, since Pin1 is not essential for cellular viability [7], its inhibition represents a potential strategy for cancer therapy.

Nevertheless, the development of Pin1 inhibitors remains challenging, despite decades of research, since Pin1 is considered "undruggable" [1]. Although many Pin1 inhibitors have been identified [8], most lack potency, specificity, cell permeability, and safety in clinical application [9]. To overcome this issue and, considering the rise of protein degradation as a promising therapeutic strategy [10], we decided to target the degradation of Pin1 as an alternative to inhibition. To reach our goal, we take advantage of PROTACs (PROteolysis TArgeting Chimeras) strategy. PROTACs [11] are bifunctional molecules made up of a ligand for the target protein of interest (POI) and a ligand for an E3 ubiquitin ligase (E3), joined by a flexible linker. Mechanistically, PROTACs promote the recruitment of the E3 ligase close to the POI, forming a ternary complex. This proximity enables E3 ligase-mediated ubiquitination of the POI, followed by its consecutive recognition and degradation by the Ubiquitin Proteasome System. Thanks to their unique catalytic mode of action, PROTACs present different advantages over small molecule-based inhibitors, including the capability to target "undruggable" protein [12]. In this work, we report the design, the synthesis, and the preliminary biological evaluation of the first series of Pin1-PROTACs. These degraders could have remarkable applications as potential therapeutic tools for cancer treatment. Pin1-PROTACs could also represent a helpful gear for investigating the complex biology of Pin1.

Results and Discussion

Designing and predicting the structure of a surely effective PROTAC is quite challenging, especially when the POI, as Pin1, has never been targeted for degradation using this technology before. Indeed, PROTAC's activity depends not only on the affinity of the ligand for the POI but, mainly, on its capability to form the ternary complex and, consequently, on its geometry. For this reason, we have synthesised four Pin1-PROTACs to maximise the probability of having an active degrader (Figure 1, **1a**-**4a**).

The scaffold of Pin1 ligands (**1b**-**4b**) was based on a versatile template that comprised the minimal peptide backbone length (three residues) and exploited the Pin1 preference for *C*-terminal aromatic amino acid and *N*-terminal aromatic moiety [13]. A terminal alkyne was incorporated at the *N*-terminal (**1b**, **2b**) or *C*-terminal (**3b**, **4b**) of the pseudopeptides, to allow the CuAAC (Copper-catalysed Alkyne-Azide Cycloaddition) with the flexible azide linker **5**. Both *N*-Fmoc protected (**1b**-**3b**) and the *N*-acetylated versions (**2b**-**4b**) of Pin1 ligands were synthesised in order to tune the solubility and affinity of the molecules. All the Pin1 ligands also bear a SATE (*S*-acyl-2-thioethyl) moiety, to mask

the anionic phosphoric group allowing the degraders to penetrate the cell membrane. Upon cell entry, SATE will be enzymatically removed, and the Pin1-PROTACs will be converted into their biologically active form. On the other side, a ligand of the E3 (**6**), accessorised with a terminal carboxylic acid, was synthesised. Between all the suitable E3 ligases, we decided to target Cereblon (CRBN) since it is present in the same type of cells where Pin1 is also expressed. CRBN ligand **6** was coupled with the amino PEG linker **5** *via* an amide bond formation reaction.

The synthetic pathway for the *N*-Fmoc protected Pin1 ligands (**1b** and **3b**) is reported in Scheme 1. The synthesis of **3b** started with the Fmoc-L-Ser(O*t*Bu)-OH (**7**), which reacted with NH_2-L-Pro-OBn under usual peptidic coupling conditions to obtain dipeptide **8** in excellent yield. After debenzylation with (Pd/C, H_2), the subsequent reaction with *O*-propargyl serotonin provided compound **9**. Deprotection of the *t*Bu group (TFA/DCM) and phosphorylation of the resulting alcohol gave the desired pseudopeptide **3b**. The synthesis of **1b** started with the benzyl deprotection (at *C*- terminal) of compound **8**. After that, the coupling with tryptamine, the Fmoc deprotection (at *N*- terminal), and the reaction with Fmoc-L-propargylglycine provided the pseudopeptide **10**. Deprotection of the *t*Bu group (TFA/DCM) and the subsequent phosphorylation gave Pin1 ligand **1b**. The acetylated version of **1b** and **3b** (**2b** and **4b**) were synthesised following a similar pathway, adding a deprotection (piperidine/DMF 20:80) and an acetylation (acetyl chloride/DCM) steps before the deprotection of *t*Bu group. The rest of the synthesis of Pin1-PROTAC **1a** is also reported in Scheme 1. After synthesising the E3 ligand scaffold starting from compound **11**, the carboxylic acid moiety was inserted to obtain the thalidomide analogue **12**. The reaction of **12** with the 11-azido-3,6,9-trioxaundecan-1-amine **5** in presence of HATU and DIPEA provided the azide **13**. Finally, copper catalysed click chemistry was employed to obtain Pin1-PROTAC **1a**. Pin1-PROTACs **2a**, **3a** and **4a** were synthesised following the same pattern of **1a** starting from the appropriate Pin1 ligands.

In order to check if the presence of the terminal alkyne on Pin1 ligands scaffold affected the binding affinity of the ligands for the protein, we evaluated their K_d. The affinity constants were calculated by NMR, using the CSP (Chemical Shift Perturbation) method. Pin1 was titrated with a ligand until saturation, and after each addition, a ^1H-^{15}N HSQC (500 MHz, 298 K) was recorded. Following the changes in the chemical shifts of Pin1 NMR spectrum, we were able to evaluate the K_d of **1b** (37 ± 0.4 μM), **2b** (285 ± 8 μM) and **4b** (373 ± 31 μM) for Pin1 (WW domain). **3b** was not soluble in water and we were unable to perform the CSP NMR experiment and calculate its affinity constant. We compared the affinity constants of **1b**, **2b**, **4b** with the ones of Fmoc-pSer-Pro-Tryptamine (26 ± 6 μM) and Ac-pSer-Pro-Tryptamine (32 ± 6 μM), two Pin1 inhibitors previously developed in our laboratory. As a result, we were able to conclude that the insertion of the alkyne moiety at *C*-terminal of the pseudopeptide (**4b**) is detrimental for the affinity. At the same time, the scaffold modification at *N*-terminal is relatively well tolerated (**1b-2b**).

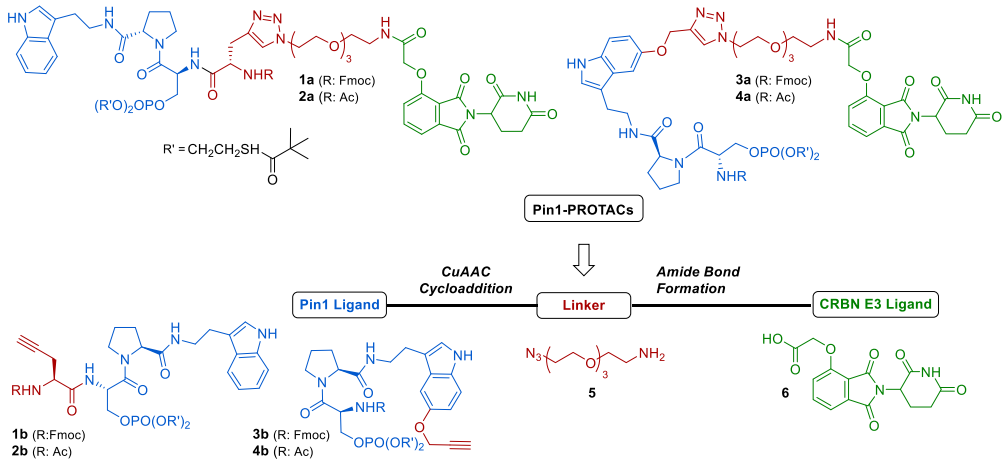
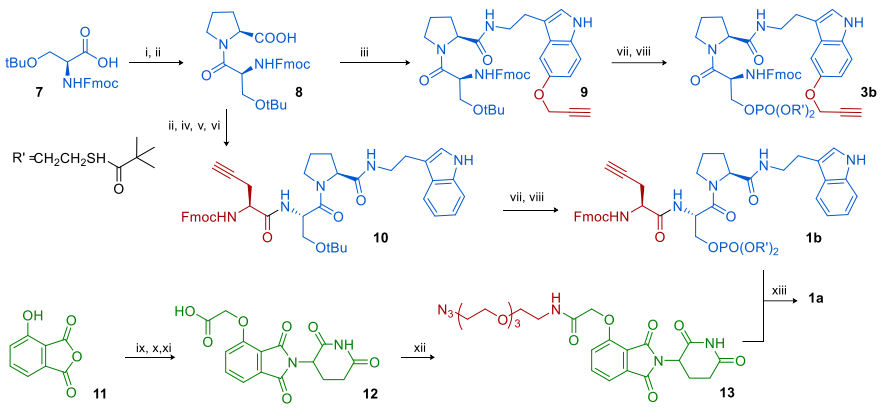


Fig. 1. Pin1-PROTACs scaffolds.



*Scheme 1. Synthesis of Pin1-PROTACs **1a**. Conditions: i) H-Pro-OBzl·HCl, DIPEA, EDC, HOBt; ii) H_2, Pd/C; iii) O-propargyl serotonin, DMAP, EDC, HOBt; iv) Tryptamine, DMAP, EDC, HOBt; v) DEA / DCM; vi) Fmoc-propargylglycine, DMAP, EDC, HOBt; vii) TFA / DCM; viii) $(iPr_2N)P(OR')_2$ 5-ethylthio-H-tetrazole, tBuOOH; ix) 3-Aminopiperidine-2,6-dione·HCl, pyridine; x) Ethyl 2-bromoacetate, K_2CO_3; xi) TFA / DCM; xii) 11-azido-3,6,9-trioxaundecan-1-amine, HATU, DIPEA; xiii) **1b**, $CuSO_4$, Na-ascorbate.*

Finally, preliminary biological results were performed to confirm the presence and the localization of Pin1 and E3 CRBN inside the tumour cell lines that will be employed for the Pin1-PROTACs degradation assay (IGROV1 ovarian adenocarcinoma cell lines). Confocal microscopy analysis of IGROV1 shows the presence of both Pin1 and CRBN in the cytoplasm and nucleus.

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