Hijacking the MDM2 E3 Ligase with novel BRD4-Targeting PROTACs in Pancreatic Cancer Cells

Mihaela P. Ficu,[a] Dan Niculescu-Duvaz,[a,b] Mohammed Aljarah,[a,b] Christopher S. Kershaw,[a] and Caroline J. Springer*[,a,b]

[a]Drug Discovery Unit, Cancer Research UK Manchester Institute, University of Manchester, Alderley Park, Macclesfield, SK10 4TG, United Kingdom

[b]Oncodrug Ltd, Alderley Park, Macclesfield, SK10 4TG, United Kingdom

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ABSTRACT: The phenotypic effect induced by a Proteolysis-Targeting Chimera (PROTAC) can depend on several factors, including the E3 ligase recruited. For the discovery of a first-in-class PROTAC for a target of interest, the E3 ligases commonly hijacked remain the Von Hippel-Lindau (VHL) and Cereblon (CRBN) since potent and accessible ligands are readily available to recruit them. Mouse double minute 2 (MDM2) E3 ligase stands out because it regulates p53 levels to maintain cellular homeostasis. However, the synthesis of the most potent MDM2 ligands remains very complex. Here we report the discovery of novel MDM2-recruiting PROTACs incorporating *rac*-Nutlin-3 as a ligand with an easier synthetic tractability, further demonstrating its potential in this technology. The most promising degrader, PROTAC **3**, showed preferential degradation of the BRD4 short isoform and c-Myc compared with MZ1, a validated VHL-based PROTAC.

Novel strategies and technologies in the oncology drug discovery field have been directed towards tackling the complexities of cancer progression, particularly to overcome and potentially prevent undesired side effects of existing first-line cancer therapies.[1] One exciting such technology that expanded rapidly over the past 20 years is the Targeted Protein Degradation (TPD) field, which encompasses molecules that hijack the cellular machinery to degrade proteins involved in disease progression.[2] Its tremendous potential has been demonstrated by 18 degraders in clinical trials, one of them already used in a Phase III trial.[2]

Protein degradation strategies can involve the recruitment of the component proteins from the Ubiquitin-Proteasome System (UPS) such as an E3 ligase,[3] an E2 enzyme,[4] or even the proteasome.[5] Examples include Proteolysis-Targeting Chimeras (PROTACs),[3] molecular glues[6, 7] or hydrophobic tagging.[8] The Autophagy-Lysosome pathway has also been hijacked with Autophagy-Targeting Chimeras (AUTACs),[9] Lysosome-Targeting Chimeras (LYTACs),[10] Autophagosome-Tethering Compounds (ATTECs)[11] and others.[3]

An effective PROTAC molecule needs to simultaneously bind to a protein of interest (POI) and an E3 ubiquitin ligase to induce the tagging of the POI for proteasomal destruction, through the formation of a productive ternary complex[12] and efficient ubiquitination.[13] PROTACs are heterobifunctional molecules incorporating ligands that bind to the POI and the E3 ligase and a linker between the two, attached to solvent-exposed functional groups.[3]

With over 800 E3 ubiquitin ligases that dictate the substrate recognition and specificity of the UPS, having the tools to hijack any E3 ligase could provide the opportunity to discover tissue-selective or cell type-specific degraders.[14, 15] Although there is active research leveraging chemoproteomics approaches, such as activity-based protein profiling, to expand the E3 ligase toolbox,[16, 17] the Von Hippel-Lindau (VHL) and Cereblon (CRBN) remain the most commonly recruited E3 ligases for PROTAC development with well-validated ligands.[3]

Mouse double minute 2 (MDM2) E3 ligase stands out because it regulates the tumor suppressor p53 by inducing its degradation to maintain cellular homeostasis.[18] Since cancer cells rely on p53 inactivation for survival through MDM2 overexpression or by gaining p53 mutations, targeting the MDM2-p53 protein-protein interaction has emerged as a therapeutic strategy to rescue wild type p53.[19] The nine MDM2 inhibitors evaluated in clinical trials[20] mimic three key amino acid residues in the p53 pocket recognized by MDM2, and hence contain several chiral centers with poor synthetic tractability for the extensive trial-and-error process necessary to discover a degrader.

A small number of MDM2-based PROTACs have been developed by using either Nutlin-3a or Idasanutlin (RG7388) to degrade the androgen receptor (AR) in cervical carcinoma HeLa cells transiently expressing AR,[21] bromodomain-containing protein 4 (BRD4) in colorectal cancer cell line HCT116,[22] poly (ADP-ribose) polymerase 1 (PARP1) in breast cancer cell line MDA-MB-231,[23] and MDM2 itself in non-small cell lung cancer cell line A549 (**Figure 1A, B**).[24]

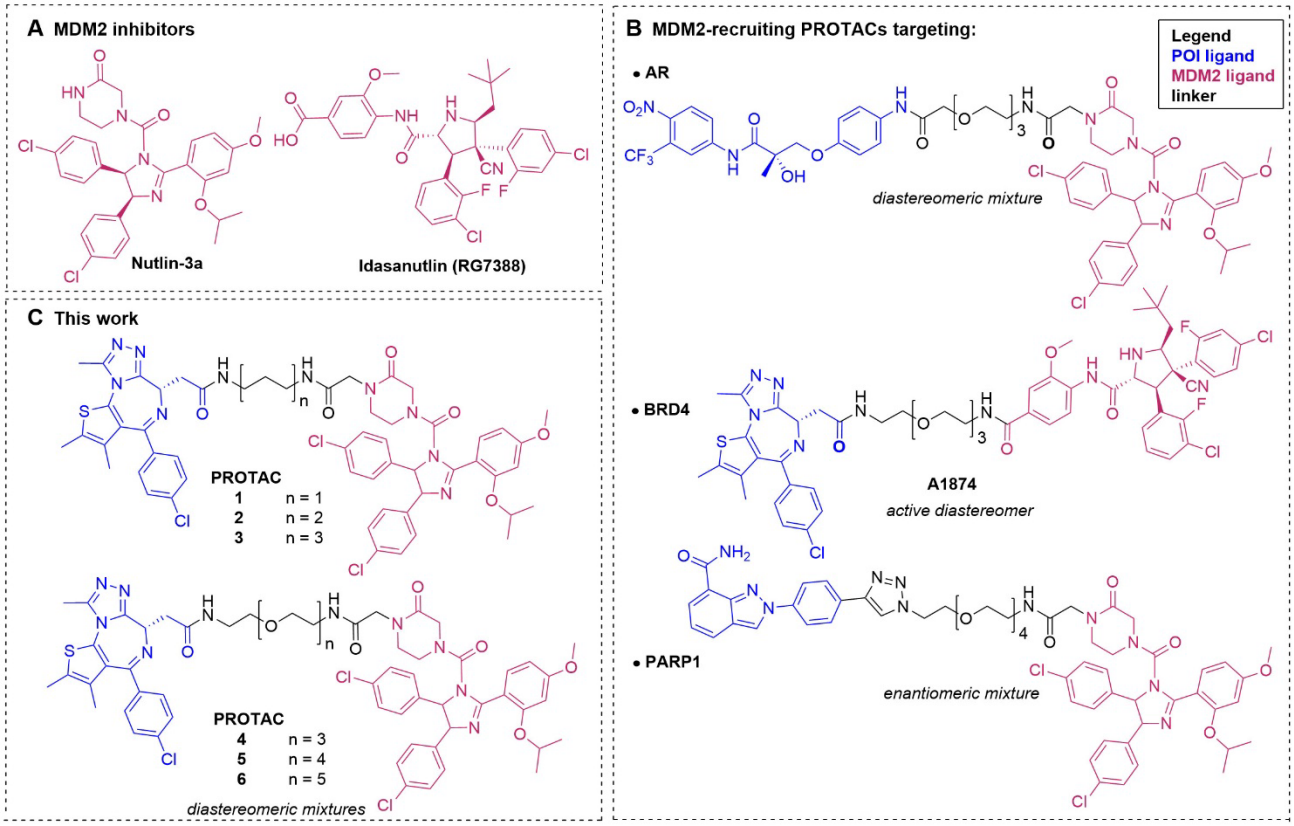


Figure 1. Chemical structures of: A) representative MDM2 inhibitors,[25, 26] B) previously reported MDM2-recruiting PROTACs,[21-23] and C) the library of MDM2-recruiting PROTACs described in this work.

The progress of the MDM2 inhibitors in the clinic has been slow due to a lack of efficacy, even with compounds such as Idasanutlin with very promising preclinical data, highlighting the need for a different strategy to rescue p53 in cancer.[20]

Given that MDA-MB-231 was the only cell line with mutant p53 status among the models used in the literature to test MDM2-recruiting PROTACs, we aimed to expand the application of MDM2-based degraders to include an *in vitro* pancreatic cancer model and to strengthen the evidence behind *racemic* Nutlin-3 as an accessible ligand for the generation of PROTAC molecules.

A library of six *rac*-Nutlin-3-based PROTACs with different linkers based on methylene units and polyethylene glycol (PEG) units was designed and synthesized to target BRD4, an extensively studied POI for proof of concept studies in the TPD field (**Figure 1C**).[27] BRD4 inhibition also sensitized the pancreatic cancer cell line MIA PaCa-2 to gemcitabine, which is the first-line treatment for pancreatic ductal adenocarcinoma.[28] In addition, inhibitors with pan-affinity against bromodomain and extra-terminal domain (BET) proteins have shown efficacy in preclinical pancreatic tumor models.[29, 30] However, side effects due to toxicity were reported in the clinic for other indications demonstrating the need to obtain selective inhibitors.[30]

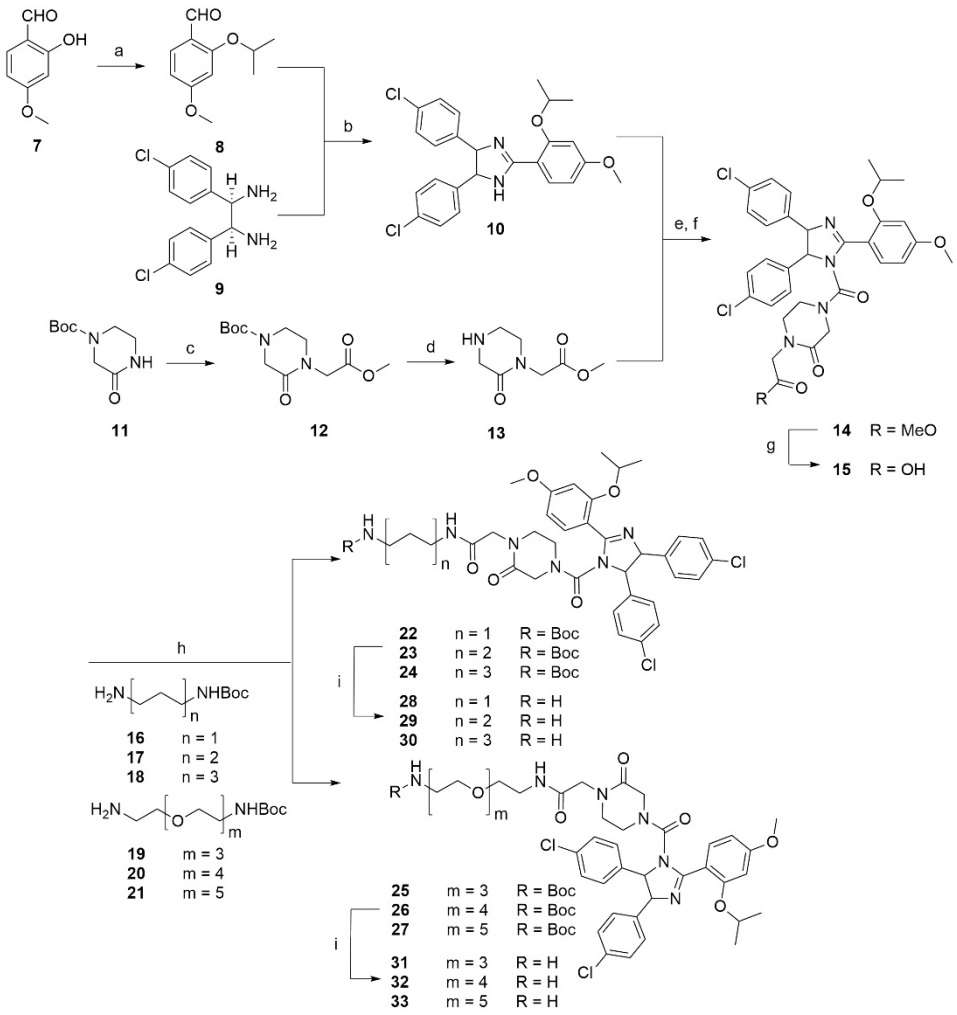
Isoform selectivity was achieved with PROTACs, such as MZ1, which degrades BRD4 and is selective over BRD2 and BRD3, two other BET isoforms.[31] In addition, a proof of concept study recruiting an E2 ligase for the development of PROTACs has shown selectivity for BRD4 short isoform (BRD4 S) over BRD4 long isoform (BRD4 L) in breast cancer cells.[4] BRD4 isoforms have been reported to have opposing roles in breast cancer development, with the short isoform being oncogenic, whilst the long isoform is tumor-suppressive.[32] Whether BRD4 isoforms play the same roles in pancreatic cancer progression remains unknown, and the development and evaluation of BRD4 isoform-selective PROTACs could be very useful to answer this question.

A study describing Idasanutlin-based PROTAC A1874 (**Figure 1B**) offered a synergistic response in HCT116 *via* simultaneous degradation of BRD4 and an increase in p53 and p21 levels, being superior to a VHL-based counterpart.[22] Inspired by this study, the validated well-characterized VHL-based PROTAC MZ1, along with its negative control, *cis*MZ1, were used in this work to compare the effect of the E3 ligase in the chosen model.

Nutlin-3a or (–)-Nutlin-3 is the active enantiomer and has an 150-fold higher binding potency against MDM2 over the other enantiomer, Nutlin-3b or (+)-Nutlin-3, as determined by surface plasmon resonance (SPR).[25] Due to the high cost to isolate the pure enantiomer, the racemic mixture of Nutlin-3 was used in this study to further validate the applicability of this ligand for PROTAC development (**Figure 1C**). As indicated by a crystal structure, the imidazoline core replaces the helical backbone of p53 and displaces the Phe[19], Leu[26] and Trp[23] p53 residues with the phenyl rings,[25] leaving the piperazinone moiety solvent exposed and suitable for further derivatization and linker attachment.[33]

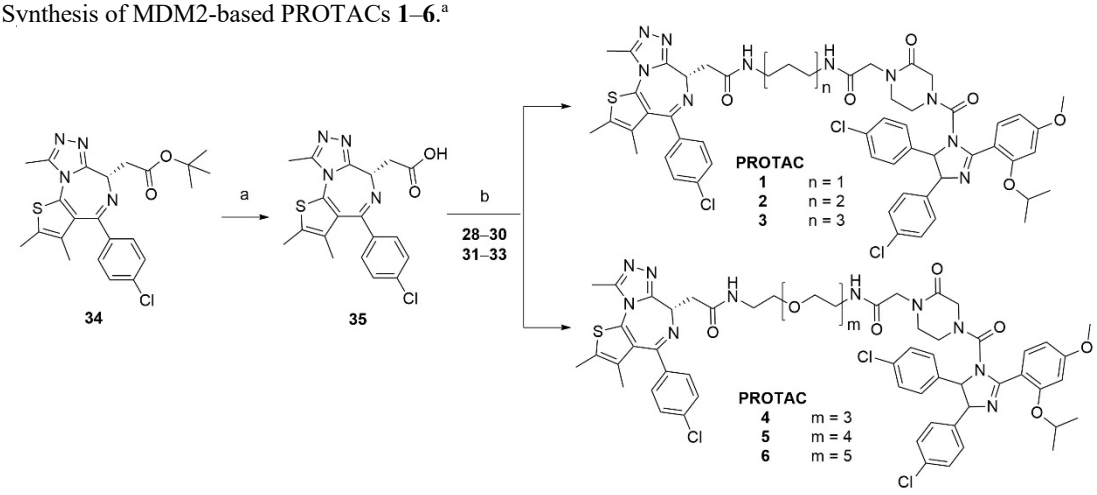
Several synthetic routes to prepare Nutlin-3a have been published, particularly for large-scale synthesis.[34, 35] Starting from

Scheme 1. Synthesis of intermediates **28–30** and **31–33**.[a]



[a]Reagents and conditions: a) K_2CO_3, ^{i}PrBr, Bu_4NBr, THF, 70ºC, 24 h, 69%.; b) NBS, DCM, 0ºC–rt, 16 h, 44–82%; c) NaH, methyl bromoacetate, THF, 0ºC–rt, 18 h, 80–96%; d) 4 M HCl/1,4-dioxane, rt, 18 h, 65–99%.; e) triphosgene, Et_3N, DCM, 0ºC–rt, 18 h; f) **13**, DCM, rt, 2–18 h, 71–88% (over 2 steps); g) LiOH•H_2O, THF, MeOH, H_2O, rt, 18 h, 44%–quant.; h) corresponding amine **16–21**, HATU, DIPEA, DCM, DMF, rt, 18–60 h, 30–58%; i) 4 M HCl/1,4-dioxane, 0°C, 1–7 h, 67%–quant.

Scheme 2. Synthesis of MDM2-based PROTACs **1–6**.[a]



[a]Reagents and conditions: a) HCOOH, rt, 48 h, 96%; b) HATU, DIPEA, DMF, rt, 1–16 h, 14–43%.

commercially available reagents, the synthesis of the desired six compounds was completed in 10 steps, as described in **Schemes 1** and **2**. The *O*-alkylation of aldehyde **7** was performed in DMF at 40ºC in the presence of potassium carbonate, following a literature procedure.[36] This route was optimized for scale-up by replacing DMF with THF and by adding tetrabutylammonium bromide (Bu_4NBr), which is a phase-transfer catalyst used to aid solubilization.[37] The *cis*-imidazoline **10** was then synthesized *via* a condensation reaction between aldehyde **8** and the *meso*-diamine **9**. Then, intermediate **13** was synthesised in 2 steps from the Boc-protected piperazinone **11**. Next, imidazoline **10** was subjected to a urea formation in the presence of triphosgene, which proceeded by the initial formation of the corresponding carbamoyl chloride *in situ*, followed by the isolation of carbamate **14** upon addition of amine **13**. Following the ester hydrolysis of **14**, carboxylic acid **15** was used to couple the selected linkers (**Scheme 1**). Then, intermediates **22–24** and **25–27** were Boc-deprotected and coupled to the hydrolyzed (+)-JQ1 reagent **35** to obtain PROTACs **1–6** (**Scheme 2**).

The library of PROTACs was screened at different concentrations after 24 h and 48 h treatment (**Figure S1**). No correlation between the linker length and the degradation profile was observed (**Figure S1**). PROTAC **3** emerged as the most potent PROTAC against both BRD4 isoforms (BRD4 L and S) with the most pronounced effect after 48 h treatment (**Figure S1**) and was selected for further investigation (**Figure 2**).

Interestingly, an increase in BRD4 L levels was observed upon treatment with 0.5 μM and 1 μM PROTAC **3** after 24 h (**Figure 2A**). This could potentially indicate that PROTAC **3** behaves as an inhibitor at lower concentrations, before starting to act as a degrader. There are previous reports which showed that (+)-JQ1 can induce a dose-dependent BRD4 accumulation in Burkitt's lymphoma and lung cancer cell lines.[38, 39] Similar observations were reported with non-degrading inhibitors of BCL6, indicating that cancer cells can offer a target-dependent distinct feedback mechanism for specific proteins.[40]

Then, at higher concentrations of up to 15 μM PROTAC **3**, a similar degradation pattern of both BRD4 isoforms could be observed (**Figure 2A**). The third band appearing at around 100 kDa can be attributed to partially degraded BRD4, as it is not present in the DMSO lane or the ones corresponding to the negative controls (**Figure 2A**).

The levels of c-Myc, an oncogenic transcription factor whose expression is regulated by BRD4, were also evaluated (**Figure 2**).[41] PROTAC **3** at 15 μM induced ~77% BRD4 L degradation and ~83% BRD4 S degradation, leading to ~86% decrease in c-Myc levels (**Figure 2B**). Interestingly, these results showed that PROTAC **3** was more potent against BRD4 S and induced a higher decrease in c-Myc levels than MZ1 at the same concentration of 20 μM (**Figure 2A**).

Encouraged by these results, a time course experiment with PROTAC **3** and MZ1 was performed next (**Figure 3A**). The data revealed that their effect on BRD4 S can be observed after 18 h (**Figure 3A**). However, the most pronounced activity was achieved after 24 h, and this time point was selected for the next experiments.

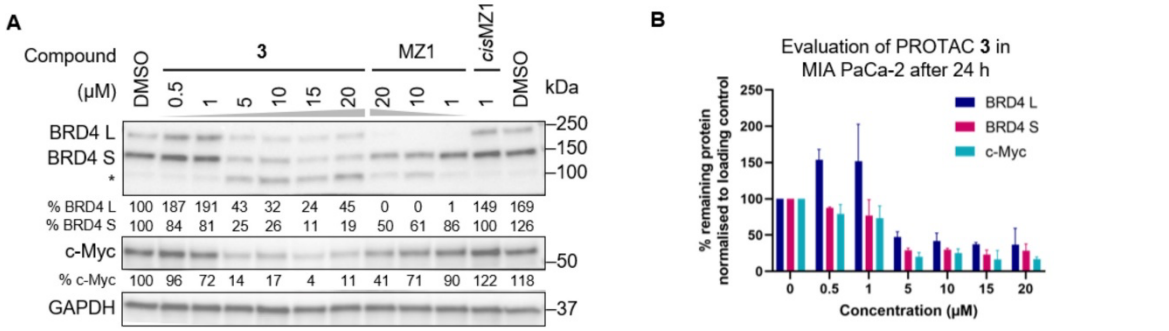


Figure 2. Novel MDM2-BRD4 PROTACs gave a more pronounced effect on BRD4 S and c-Myc than MZ1. A) MIA PaCa-2 cells were treated for 24 h with selected PROTAC **3**, MZ1, and *cis*MZ1 at the indicated concentrations. B) Quantified data are shown as mean ± SD from three independent biological replicates (quantified with ImageJ and analysed in GraphPad Prism 8.2.0, n = 3, except **3** at 0.5 μM, n = 2). The value for each condition was divided by the loading control (GAPDH), then the result was normalised against the left DMSO value to obtain the % remaining of the corresponding protein. *The third band on the BRD4 membrane could indicate partially degraded BRD4.

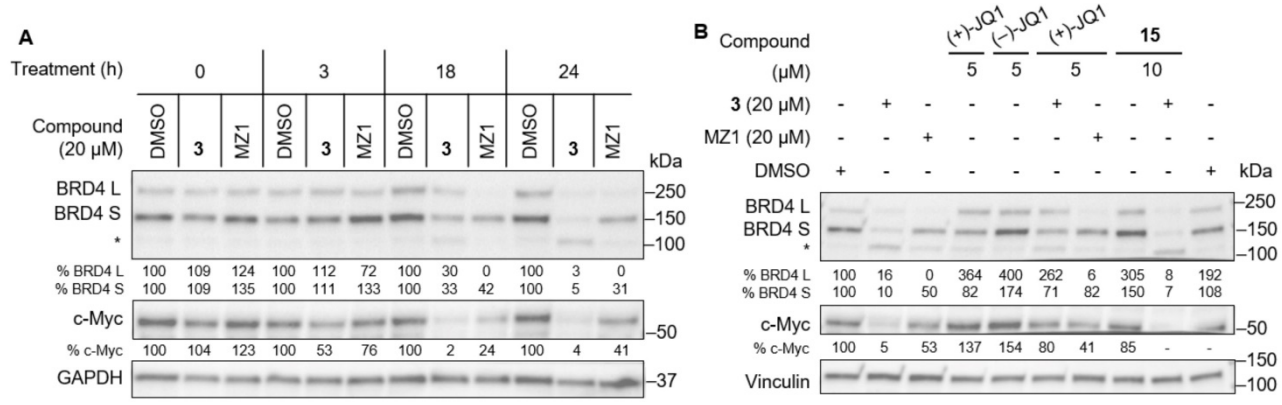


Figure 3. Investigation of the mode of action of PROTAC **3** in MIA PaCa-2. A) Time course experiment: MIA PaCa-2 cells were treated with PROTAC **3** or MZ1 at 20 μM for the indicated periods (n = 1). B) Competition study: MIA PaCa-2 cells were co-treated with PROTACs

3 or MZ1 and (+)-JQ1 or **15** at the indicated concentrations for 24 h (n = 1). (–)-JQ1 and *cis*MZ1 were used as controls. The BRD4 membrane was stripped and re-probed with an anti-vinculin antibody. *The third band on the BRD4 membrane could indicate partially degraded BRD4. Data quantified with ImageJ. The value for each condition was divided by the loading control (GAPDH or vinculin), then the result was normalised against the corresponding DMSO value to obtain the % remaining of the protein.

Next, a competition study between PROTAC **3** and its parent ligands, (+)-JQ1 and *rac*-Nutlin-acetic acid **15**, was conducted (**Figure 3B**). An increase in the levels of BRD4 was observed with both (+)-JQ1 (BRD4 L) and (–)-JQ1 (BRD4 L and S) at 5 μM (**Figure 3B**). This effect propagated downstream through an increase in the levels of c-Myc (**Figure 3B**).

Co-treatment with (+)-JQ1 and PROTAC **3** rescued the levels of the BRD4 isoforms, indicating that the effect of PROTAC **3** is dependent on binding to BRD4 (**Figure 3B**). In contrast, the MDM2 ligand, compound **15**, did not rescue the degradation of the BRD4 isoforms in the same experiment (**Figure 3B**). Interestingly, (+)-JQ1 only partially restored the levels of BRD4 S following its co-treatment with MZ1 (**Figure 3B**).

Proteasome inhibitors, such as MG132 or Bortezomib,[42] and the NEDD8-activating enzyme inhibitor MLN4924, which blocks the Cullin E3 ligase activity,[43] are routinely used in the TPD field to probe proteasome-driven degradation. Pretreatment with MG132 or MLN4924 did not rescue the degradation of the BRD4 isoforms induced by PROTAC **3** under the conditions attempted, and these observations warrant further investigation of the mechanism of action of MDM2-based PROTACs (**Figure S2**).

To summarise, the design and syntheses of six novel *rac*-Nutlin-3-based PROTACs were reported along with their assessment *in vitro* in a pancreatic cancer model. Selected MDM2-recruiting PROTAC **3** has shown a higher potency to degrade BRD4 S than MZ1, a validated VHL-based PROTAC incorporating the same BRD4 ligand, (+)-JQ1.

To the best of our knowledge, this is the first example of an MDM2-based PROTAC evaluated in the pancreatic cancer cell line MIA PaCa-2. The results presented here reinforce the potential of hijacking MDM2 as the E3 ligase for the development of novel PROTACs to degrade key overexpressed proteins in pancreatic cancer. In addition, this work provides a different perspective on the mode of action of MDM2-based degraders, which could provide new therapeutic strategies for distinct cancer types.

ASSOCIATED CONTENT

Supporting Information

Additional immunoblotting experiments, experimental procedures for the synthesized compounds and for the biological assays, characterization data for the lead compound, and full scans of the blots. (PDF)

AUTHOR INFORMATION

Corresponding Author

*Caroline J. Springer - Oncodrug Ltd, Alderley Park, Macclesfield, SK10 4TG, United Kingdom

Authors

Mihaela P. Ficu – Drug Discovery Unit, Cancer Research UK Manchester Institute, University of Manchester, Alderley Park, Macclesfield, SK10 4TG, United Kingdom; orcid.org/0000-0002-6702-4037

Caroline J. Springer – Oncodrug Ltd, Alderley Park, Macclesfield, SK10 4TG, United Kingdom; orcid.org/0000-0002-0650-4002

Dan Niculescu-Duvaz – Oncodrug Ltd, Alderley Park, Macclesfield, SK10 4TG, United Kingdom; orcid.org/0000-0002-8251-136X

Mohammed Aljarah – Oncodrug Ltd, Alderley Park, Macclesfield, SK10 4TG, United Kingdom

Christopher S. Kershaw – Drug Discovery Unit, Cancer Research UK Manchester Institute, University of Manchester, Alderley Park, Macclesfield, SK10 4TG, United Kingdom

Author Contributions

Conceptualization: D.N.-D., M.P.F, C.J.S.; Methodology: M.P.F., D.N.-D.; Validation: M.P.F.; Formal Analysis: M.P.F.; Investigation: M.P.F., Data Curation: M.P.F., University of Manchester, Cancer Research UK; Writing – original draft: M.P.F.; Writing – Review & Editing: M.P.F., D.N.-D., C.J.S., M.A., C.S.K.; Visualization: M.P.F.; Supervision: D.N.D., M.A., C.S.K., C.J.S.; Project Administration: D.N.-D., C.J.S., M.A., C.S.K.; Funding Acquisition: C.J.S. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no conflict of interest.

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ABBREVIATIONS

TPD, Targeted Protein Degradation; PROTAC, Proteolysis Targeting Chimera; VHL, Von Hippel-Lindau; CRBN, Cereblon; MDM2, Mouse double minute 2; UPS, Ubiquitin-Proteasome System; AUTAC, Autophagy-Targeting Chimera; LYTAC, Lysosome-Targeting Chimera; ATTEC, Autophagosome-Tethering Compound; POI, protein of interest; AR, androgen receptor; BRD4, bromodomain-containing protein 4; BRD4 S, bromodomain-containing protein 4 short isoform; BRD4 L, bromodomain-containing protein 4 long isoform; PARP1, poly (ADP-ribose) polymerase 1; rac, racemic; Phe^{19}, Phenylalanine 19; Leu^{26}, Leucine 26; Trp^{23}, Tryptophan; DMF, dimethylformamide; THF, Tetrahydrofuran; Boc, *tert*-butyloxycarbonyl, c-Myc, cellular Myelocytomatosis; HRMS, high-resolution mass spectrometry.

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