MDM2-BCL-X_L PROTACs enable degradation of BCL-X_L and stabilization of p53

Highlights

- The first MDM2-Bcl-X_L PROTACs were developed.
- Compound BMM4 potently degraded Bcl-X_L and stabilized p53.
- The combination of BMM4 with ABT-199 exhibited synergic anticancer activity.

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In brief

BMM4, the first MDM2-Bcl-X_L PROTAC, showed unique antiproliferative activity and may serve as a potential anti-cancer agent for further development.
MDM2-BCL-X\textsubscript{L} PROTACs enable degradation of BCL-X\textsubscript{L} and stabilization of p53

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ABSTRACT

Inhibition or degradation of the anti-apoptotic protein BCL-X\textsubscript{L} is a viable strategy for cancer treatment. Despite the recent development of PROTACs for degradation of BCL-X\textsubscript{L}, the choice of E3 ligase has been restricted to VHL and CRBN. Herein, we report the development of MDM2-BCL-X\textsubscript{L} PROTACs using MDM2 as an E3 ligase for degradation of BCL-X\textsubscript{L}. Three MDM2-BCL-X\textsubscript{L} PROTACs derived from the MDM2 inhibitor Nutlin-3, which also upregulates p53, and the BCL-2/BCL-X\textsubscript{L} inhibitor ABT-263 with different linker lengths were designed, synthesized and evaluated in vitro. BMM4 exhibited potent, selective degradation activity against BCL-X\textsubscript{L}, and stabilized the tumor suppressor p53 in U87, A549 and MV-4-11 cancer cell lines. Moreover, the combination of BMM4 and the BCL-2 inhibitor ABT-199 showed synergistic antiproliferative activity. These unique bifunctional PROTACs offer an alternative strategy for targeted protein degradation.

Keywords: BCL-X\textsubscript{L}, MDM2 E3 ligase, PROTAC, targeted protein degradation

1. INTRODUCTION

Evasion of cellular apoptosis, a hallmark of cancer, is largely mediated by pro- and antiapoptotic proteins [1]. BCL-X\textsubscript{L} and BCL-2 are the main members of the anti-apoptotic BCL-2 family [2-5]. Upregulation of the antiapoptotic BCL-2 family proteins induces evasion of apoptosis and leads to tumor initiation, progression, and resistance to chemo- and targeted therapies [2-5]. BCL-X\textsubscript{L} and BCL-2 are well-validated cancer targets [2, 6]. Small-molecule inhibitors of these proteins promote Bax/Bak oligomerization, thus leading to permeabilization of the mitochondrial outer membrane, cytochrome c release and caspase activation, and ultimately apoptosis [2].

Navitoclax (ABT-263) is an orally bioavailable small-molecule drug that inhibits BCL-2/BCL-X\textsubscript{L} selectively and potently, thus resulting in single-agent efficacy in a preclinical model of small cell lung cancer (Figure 1) [7]. ABT-263 inhibition of BCL-X\textsubscript{L} causes on-target and dose-limiting thrombocytopenia, thus preventing its clinical use [8, 9]. To address this issue, ABT-263 derived PROTACs using cereblon (CRBN) and von Hippel–Lindau (VHL) ligands as E3 recruitment components have been developed by Zhou and Zheng, and have shown diminished platelet toxic effects and potent BCL-X\textsubscript{L} selective anti-cancer activity [10-14]. E3 ligands have been suggested to play key roles in degradation activity and selectivity [15-17]. Different E3 ligases may define different band regions of surface lysine involved in the regulation of PROTAC-induced target ubiquitination and degradation [14]. Some E3 ligases have a narrower range of activity, and narrower lysine and substrate selectivity, whereas others have a broader range of activity and less defined lysine and substrate selectivity [17, 18]. Therefore, the development of new BCL-2/X\textsubscript{L} degraders using different E3 ligases may enable unique degradation abilities [19].

Mouse double minute 2 homolog (MDM2) is an E3 ubiquitin ligase that binds and results in the degradation of the tumor suppressor p53 through the ubiquitin pathway [20, 21]. Compared with other E3 ligases (VHL or CRBN) used in PROTACs, MDM2 is unique in that its endogenous substrate, p53, plays a major role in tumor suppression [22]. In response to cellular stress, DNA damage and hypoxia, p53 is upregulated, and subsequently induces pathways causing cell-cycle arrest, DNA repair, cellular senescence, differentiation and apoptosis [23, 24]. Overexpression of MDM2 decreases the expression of p53 through a negative-feedback
pathway [25-27]. Inhibition of the MDM2 protein blocks MDM2-p53 interaction, up-regulates the expression of p53 and thus exerts antitumor activity [19, 28, 29]. Several small-molecule MDM2-p53 inhibitors have entered clinical trials [30, 31].

Given the important roles of BCL-2 and BCL-X L and MDM2 in cancer, we designed PROTACs through incorporating an MDM2 inhibitor into a BCL-2/X L inhibitor (e.g., ABT-263) via a linker. These unique PROTACs can degrade BCL-X L and/or BCL-2 while inhibiting MDM2 and stabilizing p53 expression. Herein, we report the development of bifunctional PROTACs that selectively degrade BCL-X L and stabilize p53.

2. RESULTS

2.1 Chemistry
Nutlin-3 (Figure 1b) is a potent MDM2 inhibitor [27]. We developed a homo-PROTAC by connecting two derivatives of the Nutlin-3 ligand (racemic forms used in our studies) with appropriate linkers for the degradation of MDM2 [32]. ABT-263 was used as a ligand in the design of CRBN/VHL-based BCL-X L-specific degraders [10, 11]. On the basis of the structural information, we designed Nutlin-3- and ABT-263-derived PROTAC BMMs via a linear linker connecting two solvent-exposure sites in the two structures (Figure 1). Because degradation activity is affected by the structure, particularly the length of the linker, we synthesized differently sized linkers and examined them in bioactivity studies.

The route for the synthesis of the designed PROTACs is illustrated in Scheme 1. ABT-263 derivative 12 was prepared as previously described [33]. Briefly, commercially available aspartic acid derivative 1 was reduced by NaBH₄, thus yielding alcohol 2, which was then converted to thioether 3. The reduction of ester 3 to aldehyde 4 by DIBAL-H at –78°C was followed by reductive amination with Troc-protected piperazine (5), thus yielding compound 6. Deprotection of Boc compound 6 yielded compound 7, which underwent aromatic nucleophilic substitution with 4-fluoro-3-((trifluoromethyl)sulfonyl)benzene sulfonamide 8 and formed aniline 9. The key intermediate 12 was obtained via amide condensation with compound 10 and subsequent deprotection. Coupling of piperazine 12 with linkers of various lengths ranging from six to ten carbons, and
deprotection of Boc, yielded amide 15. Finally, the amidation of amine 15 with MDM2 inhibitor 16 yielded the target structures BMM2–4.

2.2 Biological evaluation

2.2.1 BMM2–4 PROTAC degradation activity in the U87 glioblastoma cell line. BMM PROTACs were designed for the degradation of Bcl-XL. Their degradation ability in cancer cells was evaluated through immunoblotting. The glioblastoma cell line U87 was selected for initial evaluation of BMM PROTACs, given the roles of Bcl-XL in brain cancer [34]. U87-MG cells were treated with 1.0 or 10.0 μM of BMM for 24 h. Very limited degradation activity was observed at 1.0 μM for all three PROTACs (Figure 2). However, at 10.0 μM, BMM4 induced substantial degradation of Bcl-XL. These observations were consistent with those for VHL-based BCL-XL PROTACs [11, 12].

The BMM PROTACs used the derivative of Nutlin-3 as a ligand to recruit E3 ligase. Nutlin-3 is also an MDM2 inhibitor [27]. Inhibition of MDM2 protein blocks MDM2-p53 interaction and up-regulates the expression of p53, and consequently exerts antitumor activity [19, 28, 29]. Therefore, we analyzed p53 and p21 expression. After 24 h, immunoblotting in U87 cells treated with 10.0 μM BMM2, BMM3 or BMM4 revealed a significant increase in p53 and p21 (Figure 2). These results suggested that our designed compounds also inhibited MDM2, because the derivative of Nutlin-3 kept the biological active structure, and the solvent-exposed site for linker connection had limited effects on its activity.

Further protocol optimization revealed that the degradation activity of the PROTACs was dependent on concentration and incubation time, and BMM4 at 10.0 μM showed the best degradation ability and p53 stabilization (Figure 3a). We then studied the relationship between degradation and incubation time with 10 μM BMM4. Incubation times >16 h resulted in greater degradation (Figure 3c). Interestingly, the degradation activity did not lead to substantial cell apoptosis (Figure 3d). The difference in Bcl-XL-degradation results between BMM3 and BMM4 suggested that the linker plays a crucial role in PROTAC design.

2.2.2 Degradation activity in A549 cells. To demonstrate the sensitivity and broad applicability of the
BMM PROTACs, we explored Bcl-X\textsubscript{L}-degradation activity in A549 non-small cell lung cancer cell lines. The overexpression of Bcl-X\textsubscript{L} protein in A549 critically inhibits apoptosis, thus resulting in poor prognosis [35, 36]. We found that 10.0 \( \mu \)M BMM4 effectively degraded Bcl-X\textsubscript{L} in A549 cells (Figure 4a). Furthermore, increases in p53 and p21 levels were observed. However, in contrast to the results obtained in U87 cells, degradation of Bcl-X\textsubscript{L} markedly decreased the viability of A549 cells (IC\textsubscript{50} = 4.99 \( \mu \)M, Figure 4b). Together, these results indicated that BMM4 substantially degraded Bcl-X\textsubscript{L} in different cancer cell lines.

2.2.3 Synergic anticancer activity of BMM4 in combination with the Bcl-2 inhibitor ABT-199. Previous studies have shown that most acute leukemias express wild-type p53, and defects in the p53 pathway block p53's tumor-suppression activity [37]. MV-4-11, a commonly used acute myeloid leukemia cell line, is sensitive to
the Bcl-2 inhibitor ABT-199 [38]. We therefore asked whether combining BMM4 with ABT-199 might result in more effective cytotoxic activity than BMM4 alone in MV-4-11 cells. Western blotting indicated that 10.0 μM BMM4 effectively degraded BCL-XL and increased p53 levels (Figure 5a). Cell viability assays revealed substantial synergistic effects of co-administration of ABT-199 with BMM4, thus resulting in extensive cell apoptosis (Figure 5b). Because the Bcl-2 family proteins have important roles in inhibiting tumor apoptosis, we performed flow cytometric analysis to evaluate the antitumor activity of BMM4 (Figure 5c and 5d). Compound BMM4 at 10.0 μM induced 26.4% apoptosis (Q3+Q4) in MV-4-11 cells after 48 h treatment, whereas only 10.82% apoptosis (Q3+Q4) was observed in the control group. In addition, ABT-199 and BMM4 in combination increased the apoptosis (Q3+Q4) to 40.35% under the same conditions. These results suggested that BMM4 induced MV-4-11 cell apoptosis, and combining BMM4 with ABT-199 yielded more potent cytotoxic activity.

3. DISCUSSION AND CONCLUSION

Given the critical roles of Bcl family proteins and MDM2 in cancers, we developed a new class of PROTACs for targeting Bcl-XL. To our knowledge, this study reports the first exploration of MDM2 as an E3 ligase for degradation of Bcl-XL. Three Nutlin-ABT-263-derived PROTACs were designed, synthesized and evaluated. Compound BMM4 was identified as the most promising degrader.

In contrast to the previously reported Bcl-targeting PROTACs, the MDM2 PROTAC BMM4 exhibited a unique dual activity by selectively degrading Bcl-XL and stabilizing and enhancing p53 and p21 activity, even in the presence of ABT-263, a potent inhibitor of both Bcl-2 and Bcl-XL. These results suggested that BMM4 acts as not only an E3 ligase recruiter but also an MDM2 inhibitor. In addition, BMM4 showed high and broad sensitivity toward cancer cells, as shown in the U87, A549 and MV-4-11 cancer cell lines. Furthermore, in comparison with treatment with BMM4 alone, combination treatment with BMM4 and the BCL-2 inhibitor ABT-199 showed more potent antiproliferative activity. This finding may be attributable to the Bcl-2 and Bcl-XL proteins, both of which are involved in tumor survival. Moreover, the linker was found to have important roles in degradation activity. In a recent study, Zhou et al. [14] changed the linker position and achieved dual degradation of Bcl-2 and Bcl-XL instead of single degradation of Bcl-XL. Further studies must be conducted to establish dual-degradation PROTACs for both Bcl-2 and Bcl-XL via modification of the linker structures, which might influence the selectivity and activity toward Bcl-2 and Bcl-XL. Our future studies will follow this research direction.

4. EXPERIMENTAL METHODS

4.1 General information

Commercially available reagents were purchased from Sigma Aldrich, Matrix Chemical, AKSci, Alfa Aesar,
TCI and Chem Cruz, and were used as received unless otherwise noted. Merck 60 silica gel was used for chromatography, and Whatman silica gel plates with an F254 fluorescence indicator were used for thin-layer chromatography analysis. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance 400 spectrometer. Chemical shifts in ¹H NMR spectra are reported in parts per million (ppm) relative to residual chloroform (7.26 ppm) or
Na₂SO₄ and concentrated under vacuum. The residue was triturated under vacuum and used directly for the next step without purification (quantitative). LC–MS (ESI): m/z 1200.4 [M + H]⁺.

N-((4-(((2R)-4-((2-4-((4-(tert-Butyl)-2-ethoxyphenyl)-4,5-bis(4-chlorophenyl)-4,5-dihydro-1H-imidazole-1-carbonyl)piperazin-1-yl)acetamido)undecanoyl)piperazin-1-yl)-1-(phenylthio)butan-2-yl)amino)-3-(( trifluoromethyl)sulfonyl)phenyl)sulfonyl)-4-(4-((4'-chloro-4,4-dimethyl-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)methyl)piperazin-1-yl)benzamide (BMM3). The preparation of BMM3 was similar to that of compound BMM2. ¹H NMR (500 MHz, methanol-d₄) δ 8.28 (d, J = 2.2 Hz, 1H), 8.06 (dd, J = 9.3, 2.3 Hz, 1H), 7.77 (d, J = 8.7 Hz, 2H), 7.68 (d, J = 8.1 Hz, 1H), 7.42–7.37 (m, 2H), 7.36–7.30 (m, 4H), 7.25 (d, J = 8.3 Hz, 2H), 7.23–7.27 (m, 4H), 7.17–7.11 (m, 5H), 7.04 (d, J = 8.2 Hz, 2H), 6.98 (d, J = 8.9 Hz, 2H), 6.93 (d, J = 9.5 Hz, 1H), 6.26 (d, J = 11.2 Hz, 1H), 6.00 (d, J = 11.2 Hz, 1H), 4.36–4.29 (m, 2H), 4.14–4.07 (m, 3H), 3.69 (s, 2H), 3.51–3.37 (m, 6H), 3.29–3.13 (m, 8H), 3.12–3.06 (m, 2H), 2.88–2.50 (m, 10H), 2.43–2.37 (m, 2H), 2.30 (t, J = 7.4 Hz, 2H), 2.16–2.04 (m, 3H), 1.91–1.81 (m, 1H), 1.80–1.73 (m, 2H), 1.72–1.66 (m, 2H), 1.62–1.53 (m, 4H), 1.50 (t, J = 7.0 Hz, 4H), 1.47–1.44 (m, 1H), 1.41 (s, 9H), 1.34–1.25 (m, 8H), 1.06 (s, 6H). LC–MS (ESI): m/z 1746.6 [M + H]⁺.

(4-((4-(4-(7-Aminoheptanoyl)piperazin-1-yl)-1(phenylthio)butan-2-yl)amino)-3-(( trifluoromethyl)sulfonyl)phenyl)sulfonyl)-4-(4-((4'-chloro-4,4-dimethyl-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)methyl)piperazin-1-yl)benzamide (BMM4). The preparation of BMM4 was similar to that of compound BMM1. ¹H NMR (500 MHz, methanol-d₄) δ 8.29 (d, J = 2.2 Hz, 1H), 8.06 (dd, J = 9.3, 2.3 Hz, 1H), 7.77 (d, J = 8.7 Hz, 2H), 7.68 (d, J = 8.1 Hz, 1H), 7.42–7.37 (m, 2H), 7.36–7.30 (m, 4H), 7.25 (d, J = 8.3 Hz, 2H), 7.23–7.27 (m, 4H), 7.17–7.11 (m, 5H), 7.04 (d, J = 8.2 Hz, 2H), 6.98 (d, J = 8.9 Hz, 2H), 6.93 (d, J = 9.5 Hz, 1H), 6.26 (d, J = 11.2 Hz, 1H), 6.00 (d, J = 11.2 Hz, 1H), 4.36–4.29 (m, 2H), 4.14–4.07 (m, 3H), 3.69 (s, 2H), 3.51–3.37 (m, 6H), 3.29–3.13 (m, 8H), 3.12–3.06 (m, 2H), 2.88–2.50 (m, 10H), 2.43–2.37 (m, 2H), 2.30 (t, J = 7.4 Hz, 2H), 2.16–2.04 (m, 3H), 1.91–1.81 (m, 1H), 1.80–1.73 (m, 2H), 1.72–1.66 (m, 2H), 1.62–1.53 (m, 4H), 1.50 (t, J = 7.0 Hz, 4H), 1.47–1.44 (m, 1H), 1.41 (s, 9H), 1.34–1.25 (m, 8H), 1.06 (s, 6H). LC–MS (ESI): m/z 1746.6 [M + H]⁺.
4.2 Cell culture
U87-MG cells and A549 were maintained in Dulbecco’s modified Eagle’s medium (cat. No. 10-013-cv) containing 10% FBS, penicillin at 100 units/mL and streptomycin at 100 μg/mL (cat. No. 30-002-CI). The cells were grown at 37°C with 5% CO₂. MV-4-11 cells were maintained in RPMI 1640 containing 10% FBS.

4.3 Cell viability assays
Tumor cells were plated in 96-well plates with 6.0 x 10³ cells per well and subsequently incubated for 24 h in a humidified atmosphere of 5% CO₂ and 37 °C. Then different concentrations of test compounds or vehicles were added to triplicate wells. After incubation for an additional 72 h, 10 μL CCK-8 solution (Dojindo Molecular Technologies, cat. No. CK04-11) was added to each well, and the plates were incubated for 4 h at 37°C. The absorbance was read at 450 nm on a microplate reader (Molecular Devices). The IC₅₀ values were calculated with the Logit method in GraphPad software.

4.4 Antibodies
Anti-Bcl-xl was purchased from Thermo Fisher Scientific (cat. No. 66020-1-IG). Anti-Bcl-2 was purchased from Thermo Fisher Scientific (cat. No. MA5-11757). Anti-GAPDH was purchased from Sigma-Aldrich (cat. No. G8795). Anti-p53 was purchased from Sigma-Aldrich (cat. No. P6874). Anti-p21 was purchased from Cell Signaling Technology (cat. No. 2947T). All primary antibodies were used at the suggested dilutions in 5% non-fat milk in PBST buffer for western blot assays. All secondary antibodies were used at a 1:4000 dilution in 5% non-fat milk in phosphate-buffered saline with 0.1% Tween-20 (PBST) buffer for western blot assays. All primary antibodies were used at the suggested dilutions in 5% non-fat milk in phosphate-buffered saline with 0.1% Tween-20 (PBST) buffer for western blot assays.

4.5 Immunoblot assays
Cells were lysed in RIPA buffer supplemented with protease inhibitors. The lysates (40–60 μg protein) were then resolved on 4%–12% Mini Protein Gels (Thermo Fisher, cat. No. NP0322BOX) at 70 V for 10 min and 150 V for 40 min. Then the proteins were transferred from the gels to PVDF membranes (Bio-Rad, cat. No. 1620177) at 20 V for 120 min. The membrane was incubated with primary antibody at 4°C overnight, washed three times with PBST, incubated with secondary antibody in 5% nonfat milk for 60 min at room temperature and then washed three times with PBST.

4.6 Apoptosis assays
MV-4-11 cells (5 x 10⁴ cells/well) were placed in six-well transparent plates and then treated with test compounds and vehicle in a humidified atmosphere of 5% CO₂ at 37°C for 48 h. Annexin V-fluorescein isothiocyanate (FITC, 5 μL, BioLegend) was then added to the resuspended cell solution, which was incubated for 15 min at room temperature. After addition of 10 μL PI (BioLegend), the treated cells were incubated for another 15 min in the dark at room temperature. The analysis of stained cells was performed with a flow cytometer.

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CONFLICTS OF INTEREST
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported herein.

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