Homo-PROTAC mediated suicide of MDM2 to treat non-small cell lung cancer

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Received 5 August 2020; received in revised form 6 November 2020; accepted 17 November 2020

Abstract The dose-related adverse effects of MDM2–P53 inhibitors have caused significant concern in the development of clinical safe anticancer agents. Herein we report an unprecedented homo-PROTAC strategy for more effective disruption of MDM2–P53 interaction. The design concept is inspired by the capacity of sub-stoichiometric catalytic PROTACs enabling to degrade an unwanted protein and the dual functions of MDM2 as an E3 ubiquitin ligase and a binding protein with tumor suppressor P53. The new homo-PROTACs are designed to induce self-degradation of MDM2. The results of the investigation have shown that PROTAC 11a efficiently dimerizes MDM2 with highly competitive binding activity and induces proteasome-dependent self-degradation of MDM2 in A549 non-small cell lung cancer cells. Furthermore, markedly, enantiomer 11a-1 exhibits potent in vivo antitumor activity in A549 xenograft nude mouse model, which is the first example of homo-PROTAC with in vivo therapeutic potency. This study demonstrates the potential of the homo-PROTAC as an alternative chemical tool for tumorigenic MDM2 knockdown, which could be developed into a safe therapy for cancer treatment.

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1. Introduction

P53 is a crucial tumor suppressor that promotes the apoptosis of cancer cells and prevents tumor development. Moreover, P53 possesses the function of gene repair, which plays a protective role in human health. Notably, nearly 50% of human cancers are associated with abnormal P53 activity. The interaction between P53 and human murine double minute-2 (MDM2) is the main factor leading to the inactivation of the normal biological function of P53. MDM2 is one of the pivotal suppressors of P53, and overexpressed MDM2 can downregulate the expression of P53 through a negative feedback pathway. Inhibition or degradation of MDM2 protein blocks MDM2–P53 interaction, upregulates the expression of P53 and thus exerts antitumor activity. The development of antitumor agents targeting the MDM2–P53 interaction has become a promising strategy for cancer therapy. Several small-molecule inhibitors of MDM2–P53 have entered into the clinical trial stages. However, many years of efforts have not yielded a single clinically approved agent. One of the main reasons arises from various dose-related adverse effects of MDM2–P53 inhibitors have been observed during clinical studies, particularly the risk to cause hematological diseases. Overcoming this challenging issue may open a new avenue but requires a new paradigm for the design of conceptually distinct regulators to more safely mediate the MDM2–P53 interaction.

Emerging proteolysis targeting chimeras (PROTACs) offer new opportunities for exploiting undruggable or ineffective drug targets via the degradation of undesired proteins. Generally, a PROTAC is a heterobifunctional molecule with the capacity to specifically bind to both a target protein of interest and an E3 ubiquitin ligase, and induce rapid protein degradation without the necessity of modification of the target protein. Notably, several small-molecule PROTACs have entered the clinical trial stages to treat multiple cancer types. The unrivalled feature of this technology is a sustained cellular effect to degrade the target protein with only the addition of sub-stoichiometric amount of PROTACs. Therefore, the degradation concentration is orders of magnitude lower than the inhibitory concentration of its constitutive counterpart, and because only a very low dose is required, fewer side effects may result.

Blocking the MDM2–P53 interaction by the targeted degradation strategy is expected to achieve improved efficacy and sustained pharmacological effect. Until now, the widely used heterobifunctional PROTACs have been designed targeting the MDM2–P53 interaction. However, the heterobifunctional PROTAC is a double-edged sword. In addition to exerting the recruitment of the target protein, the heterobifunctional PROTAC also introduces the second target, E3 ubiquitin ligase. When binding to the E3 ubiquitin ligase, it may result in the inhibition of other proteins and thus generate adverse effects. For example, recent studies have shown that CRBN-based PROTACs degrade both the target protein and other proteins, causing off-target effects in the meantime. Therefore, it is highly desirable to design a PROTAC that specifically degrades MDM2 protein without introducing other targets and decreases the potential side effects.

Overexpression of E3 ubiquitin ligases, such as MDM2, is generally observed in human cancer cells and is associated with poor clinical prognosis or drug resistance. Due to the lack of enzymatic activity of E3 ubiquitin proteins, the inhibition of E3 ligase function is usually achieved by targeting protein–protein interactions. It is difficult to develop small-molecule inhibitors that can effectively decrease the efficacy of E3 ubiquitin ligases. We envision that designing tailored homo-PROTACs that recruit two identical molecules of the same E3 ligase could trigger its self-degradation by the ubiquitin–proteasome system. Although conceptual VHL- and CRBN-based homo-PROTACs have been reported for effective self-degradation of E3 ubiquitin ligase, the therapeutic application of the strategy remains unknown, and in vivo therapeutic potency has not been attained for any of them.

Considering the dual functions of MDM2 as an antitumor target and an E3 ubiquitin ligase, a homo-PROTAC targeting MDM2 may lead to a distinct, effective strategy in cancer therapy. Inspired by our previous efforts to design small molecules targeting MDM2–P53 interaction, herein the first homo-PROTACs targeting MDM2 have been successfully developed. They exhibit excellent MDM2 binding activity and induce proteasome-dependent self-degradation of MDM2 in non-small cell lung cancer cells. Furthermore, PROTAC 11a–11c exhibits potent in vivo antitumor activity in a xenograft mouse model using A549 human lung adenocarcinoma cells, which is the first example of a homo-PROTAC possessing in vivo therapeutic potency.

2. Results and discussion

2.1. Rational design of nutlin-based homo-PROTACs

cis-Biphenyl-substituted imidazoline compound 2 is a derivative of Nutlin-3 (1, a classic MDM2 inhibitor), which effectively inhibits MDM2 protein and up-regulated P53 protein by negative feedback regulation. MDM2 also belongs to a class of E3 ubiquitin ligase. We proposed to design a homo-PROTAC by connecting two MDM2 ligands through appropriate linkers. Herein compound 2 was selected as the MDM2 binding moiety for homo-PROTAC design. Analysis of the docking model of compound 2 with MDM2 revealed that the piperazine-containing N3 side chain on compound 2 was directly exposed to the solvent and may represent a suitable position for introducing a linker and another MDM2 ligand to obtain a homo-PROTAC (Fig. 1B). Also, various linkers bridging the N3 position of two molecules of compound 2 were investigated to probe the degradation efficiency (Supporting Information for chemical synthesis). It is expected that when the homo-PROTAC simultaneously binds to MDM2 (E3 ubiquitin ligase), MDM2 will be degraded by proteasome in a “suicide” cleavage manner (Fig. 1C).

2.2. Chemistry

The procedure for the synthesis of the key intermediates 5 and 7 is outlined in Scheme 1. Commercially available 1-Boc-piperatine and compound 3 were condensed via nucleophilic substitution reaction to obtain intermediate 4. After deprotection, key intermediate 5 was obtained. Intermediate 3 and 1-Boc-piperatine were reacted in the existence of Lewis base to obtain compound 6, which was subsequently subjected to ester hydrolysis reaction to give corresponding carboxylic acids 7. Reaction between intermediates 5 and 8 in the presence of K₂CO₃ in MeCN at 80 °C gave compound 9. Then, target compounds 10a–f were afforded by reacting intermediate 5 with commercially available compounds 12a–f in the condition of HBTU in DMF at room temperature (Scheme 2).
Finally, condensation reaction between compound 7 and commercially available reagents 13a–c yielded title compounds 11a–c (Scheme 3).

2.3. Study of structure—activity relationships

It was reported that the degradation efficiency of a PROTAC depended on its affinity to bind with the target protein. To investigate the binding affinity of homo-PROTACs, MDM2–P53 competitive binding activities were tested through a fluorescence polarization (FP) binding assay (Table 1 and Fig. 2A), and compound 1 was used as the positive control. Initially, compounds 9 and 10a–d containing alkyl linkers of different lengths were analyzed. With the increasing length of the alkyl linker, the MDM2–P53 competitive binding affinity was correspondingly decreased, demonstrating that the long alkyl linker might not be favorable for MDM2 binding. For example, compounds 10e and 10f, which possess a long linker, were almost inactive (Ki = 26 and 39 μmol/L, respectively). In contrast, compound 10a with a short linker showed the greatest MDM2–P53 competitive binding activity (Ki = 0.65 μmol/L). Exchanging the alkyl linker for a polyethylene glycol (PEG) type chain resulted in enhanced MDM2–P53 competitive binding activities (Ki of 10e = 1.5 μmol/L, Ki of 10f = 0.22 μmol/L). When the linker length of 10f was extended by adding a flexible imino group, PROTAC 11a exhibited increased MDM2–P53 competitive binding activity (Ki = 0.1 μmol/L) that was almost 1.5-fold more potent than that of positive control 1. Unlike the alkyl linker, decreasing the linker length of 11a resulted in a significant decrease in MDM2–P53 competitive binding activity (Ki of 11b = 1.8 μmol/L, Ki of 11c = 12 μmol/L).

2.4. Antitumor activities in vitro

We further evaluated the antiproliferative activities of all homo-PROTACs against four human cancer cell lines, A549 (lung cancer) cells, HepG2 (liver cancer) cells, HCT116 (colon cancer) cells, and MCF-7 (breast cancer) cells, by the CCK-8 (Cell Counting Kit-8) assay. As shown in Table 1, the in vitro antiproliferation efficacies were generally consistent with the MDM2–P53 competitive binding activities. For example, compounds 10a–b and 10e–f with higher MDM2–P53 competitive binding affinity exhibited more potent than that of compounds 9, 10c–d, and 11c. Moreover, compounds with interoxygenated chains exhibited increased in vitro antitumor activity as compared to those containing fat chains. For example, compounds containing an alkyl linker (9 and 10b–d) exhibited poor in vitro antiproliferative activities. The loss of antitumor activities was observed when the alkyl linker chain exceeded a certain length (e.g., compounds 10e–d). Compounds 10a, 10f, and 11a exhibited satisfactory antiproliferative activity with a broad spectrum, and were more potent than that of the positive control. Particularly, compound 11a, the most potent MDM2 binder, exhibited the highest antiproliferative activity against A549 cancer cells (IC50 = 1.0 μmol/L).

2.5. Effects of target compounds on MDM2 degradation

In consideration of molecular and cellular activities, compounds 10a, 10f, and 11a were chosen for further evaluation of the effects on MDM2 degradation and P53 expression in A549 cell line by analysis of Western blotting, and compound 1 was used as positive control (Fig. 2B). The results showed that compounds 10f and 11a dose-dependently induced MDM2 protein cleavage and increased the P53 protein expression in A549 cells. Particularly, compound 11a exhibited a higher potency for MDM2 degradation than that of compound 10f at the same concentrations. Compound 11a achieved degradation concentration DC50 (concentration causing 50% protein degradation) values of 1.01 μmol/L and induced >95% MDM2 degradation at a concentration of 2 μmol/L in A549 cells for 24 h (Fig. 2E). However, no degradation was observed in A549 cells when they were treated with compound 10f.

Figure 1  Homo-PROTAC design strategy. (A) Chemical structures of compounds 1 and 2; (B) The binding model of compound 2 with MDM2 (PDB: 4IPF); (C) Illustration of homo-PROTAC-induced protein ubiquitination and degradation.
10a. In addition, the cellular permeability assay was performed to assess the cellular permeability. The results showed that compound 11a had low permeability, which might be the reason for the moderate degradation efficiency in A549 cells (Supporting Information Table S1). Control assays clearly indicated that compound 1 at equimolar concentrations did not induce the degradation of MDM2 in A549 cells. Moreover, additional compounds (9, 10b–c, and 11b–c) were further assessed, although they failed to induce the degradation of MDM2 in A549 cells at 2.5 μmol/L for 24 h (Fig. 2F).

2.6. Mechanism used by compound 11a to degrade MDM2

To explore whether the degradation of MDM2 is dependent on the proteasome and results in the death of A549 cells, we used MG132, an inhibitor of the proteasome, to pre-treat A549 cells at 0.1 or 0.2 μmol/L for 8 h. Then, compound 11a was added to the pre-treated A549 cells for another 48 h. We predicted that MG132 would be able to effectively reduce growth inhibitory activity of 11a on A549 cells, but it would have no effect on the activity of compound 1. Indeed, pre-treatment with MG132 in a concentration-dependent manner reduced the inhibitory activity of 11a on A549 cells to the same level as that of compound 1 (Fig. 3A). These results verified that MDM2 degradation mediated by compound 11a was dependent on the proteasome, which further induced the death of A549 cells.

Given that MDM2 degradation is a well-known marker for A549 cells apoptosis, we performed flow cytometric analysis to evaluate the antitumor mechanism of compound 11a (Fig. 3B and C). Treatment with MG132 showed no toxic effect on the A549 cells at 0.2 μmol/L after incubation for 24 h. It was found that compound 11a induced 23.49% apoptosis in A549 cells at 5.0 μmol/L after 24 h treatment, whereas only 15.81% apoptosis were detected upon treatment with compound 11a and MG132 in combination. In addition, compound 1 and MG132 in combination (12.64% apoptosis) failed to decrease the A549 cell apoptosis compared to that of compound 1 alone (10.94% apoptosis) under the same conditions. The result suggested that compound 11a induced A549 cells apoptosis by the ubiquitin–proteasome system.

2.7. Biochemical evaluations of enantiomers of compound 11a

The enantiomers of compound 11a were isolated by chiral chromatography, and their values of optical rotation were determined through a polarimeter. In addition, three configurations of compound 11a enantiomers were identified by comparing the optical rotation values with those of compound 1 enantiomers (Supporting Information Fig. S1). The FP binding assay indicated that the enantiomers had different MDM2–P53 competitive binding activities (Fig. 4A). The mesomer 11a-2 had a Ki value of 0.12 μmol/L. Interestingly, enantiomer 11a-1 (Ki = 0.09 μmol/L, Table 2) exhibited an effectiveness that was almost 110-fold greater than that of enantiomer 11a-3 (Ki = 9.9 μmol/L), and was superior to compound 1 (Ki = 0.15 μmol/L).

Furthermore, CCK-8 assay was used to investigate the effect of enantiomers 11a on cell viability in the A549 and HCT116 cell line. Similar to MDM2–P53 inhibitor 1, PROTAC 11a exerted its antitumor activity by influencing the expression of P53 because enantiomers of 11a had better inhibitory activity against P53-wild HCT116 cells than the P53-deleted HCT116 cells (Table 2). In addition, consistent with the MDM2 competitive binding activity, enantiomer 11a-1 also exhibited the best anti proliferative activity among the three enantiomers. Particularly, enantiomer 11a-1 exhibited the best inhibitory activity with the IC50 value of 0.58 μmol/L for the A549 cells, which was more effective than that of racemate 11a (IC50 = 1.4 μmol/L) and reference compound 1 (IC50 = 9.9 μmol/L).

Next, the ability of 11a enantiomers for MDM2 degradation was evaluated by Western blotting analysis. Enantiomer 11a-1 dose-dependently induced the degradation of MDM2 and upregulated the expression of P53 through the negative feedback loop in A549 cells after 24 h whereas the enantiomer 11a-3 and mesomer 11a-2 could not induce the cleavage of MDM2 under the same conditions (Fig. 4C). These results demonstrated that the chiral center on the imidazole scaffold of 11a is important to MDM2 binding and degradation, and enantiomer 11a-1 was the preferential conformation of 11a. Next, the time course degradation experiment was performed to assess the kinetics of MDM2 degradation caused by 11a and 11a-1. As shown in Fig. 4D, compounds 11a and 11a-1 induced MDM2 degradation in a time-dependent manner and the depletion of
MDM2 was observed as early as 2 h after treatment. In order to further explore whether the degradation of MDM2 is based on proteasome pathway, the proteasome inhibitor MG132 was used to rescue the MDM2 degradation. As depicted in Fig. 4F, MG132 blocked the MDM2 degradation induced by 11a and 11a-1 in A549 cells. Finally, the mRNA level of MDM2 didn't exhibit obvious change after treatment with 11a-1 in A549 cells (Fig. 4E), which demonstrated that compound 11a-1 could not affect the MDM2 mRNA level and acted as a bona fide MDM2 degrader.

2.8. In vivo antitumor activity of compound 11a-1

A bottleneck in current PROTAC-based drug discovery has been created by the limited in vivo efficacy due to large molecular weights and unfavorable physicochemical properties. Thus, the in vivo antitumor activity of enantiomer 11a-1 was evaluated by the A549 xenograft model. The results demonstrated that compound 11a-1 effectively inhibited the A549 tumor growth in a concentration-dependent manner compared with vehicle control (Fig. 5A). Intraperitoneal injection of compound 11a-1 at a dose of 20 mg/kg twice-daily for 21 continuous days achieved tumor growth inhibition (TGI) of 45.6%, which was comparable to MDM2 inhibitor 1 under the same dose (TGI = 47.8%). When the dosage was increased to 30 mg/kg, treatment with compound 11a-1 achieved a TGI of 52.4%. Importantly, compound 11a-1 at both doses was well tolerated and caused minimal weight loss in mice (Fig. 5B). Thus, PROTAC 11a-1 showed potent in vivo efficacy in the induction of tumor regression at well-tolerated doses. Western blotting assay was used to detect the expression of P53 and MDM2 in tumor tissues. As shown in Fig. 5C, compound 11a-1 dose-dependently increased the expression of P53, which was more effective than that of compound 1. In contrast, the expression level of MM2 was decreased, indicating the efficient degradation of MDM2 induced by 11a-1 in mice. In addition, pharmacokinetic (PK) profiles of compound 11a-1 were evaluated in Sprague-Dawley (SD) rats administered intravenously (i.v.) at 2 mg/kg (Fig. 5D and E). The terminal half-life and area under the curve (AUC) of compound 11a-1 was approximately 9.6 h and 42,835 h-ng/mL, respectively. The PK data indicated that compound 11a-1 could achieved good plasma exposure in rats.

3. Conclusions

In summary, a series of novel homo-PROTACs have been designed and evaluated for self-degradation of MDM2. The biological assays uncovered that compound 11a possesses potent activities both in molecular and cellular level. Antitumor mechanism studies revealed that compound 11a induced MDM2 degradation and increased P53 expression in a dose-dependent manner. It significantly induced the apoptosis of A549 cell line via a ubiquitin–proteasome pathway. In particular, enantiomer 11a-1 exhibited potent in vivo antitumor activity in A549 xenograft model (TGI = 52%). Taken together, this study highlights the effectiveness of MDM2-based homo-PROTACs as a new cancer treatment strategy. PROTAC 11a represents a promising lead compound that can be used for further optimization and biological studies. The homo-PROTAC approach may offer an opportunity to overcome the challenge of the dose-related adverse effects of MDM2–P53 inhibitors that remains as an obstacle to be overcome in the field of clinical anticancer agent development.

4. Experimental

4.1. Chemistry

All reagents and solvents were obtained commercially and used without further purification. 1H NMR and 13C NMR spectra were collected by Bruker AVANCE300 or AVANCE600 spectrometers (Leipzig, Bruker Company, Germany), using tetramethylsilane (TMS) as an interior label and dimethyl sulfoxide (DMSO)-d6 as solvents. Chemical shifts (δ) are expressed in parts per million (ppm). The mass spectra (MS) were measured on an Esquire 3000 LC–MS mass spectrometer. The analysis of thin-layer chromatography (TLC) was performed with GF254 silica gel plates to detect reactions (Haiyang Chemical, Qingdao, China), and visualization of reactants occurred under 254 nm UV light. Silica gel column chromatography was carried out with Silica Gel 60 G (Haiyang Chemical). High-performance liquid chromatography (HPLC) analyses were performed on an Agilent Technologies 1206, instrument to determine purity, and water and methanol were used as the mobile phase.
4.1.1. Nonane-1,9-diylbis(piperazine-4,1-diyl)bis((2-(4-(tert-butyl)-2-ethoxyphenyl)-cis-4,5-bis(4-chlorophenyl)-4,5-dihydro-1H-imidazole-1-yl) methanone (9)

To a solution of compound 5 (200 mg, 0.35 mmol) in 10 mL MeCN, 1,9-dibromononane (50 mg, 0.18 mmol) and K$_2$CO$_3$ (120 mg, 0.86 mmol) were slowly added. After the reaction, the mixture was stirred at 80°C for 5 h. Then the resulting solution was cooled to room temperature and the organic solvent was removed under the reduced pressure distillation to obtain the impure product which was further purified by column chromatography (CH$_2$Cl$_2$:MeOH = 100:2) to obtain compound 9 (128 mg, 57%) as off-white solid. $^1$H NMR (DMSO-d$_6$, 150 MHz) δ: 1.18–1.30 (m, 14H), 1.29 (t, $J$ = 6.93 Hz, 6H), 1.32 (s, 18H), 1.63–1.76 (m, 8H), 1.99 (t, $J$ = 4.70 Hz, 2H), 2.85–3.06 (m, 8H), 4.02–4.20 (m, 4H), 5.50 (d, $J$ = 9.92 Hz, 2H), 5.65 (d, $J$ = 9.92 Hz, 2H), 6.94 (d, $J$ = 7.59 Hz, 4H), 6.99 (d, $J$ = 8.76 Hz, 4H), 7.05 (s, 2H), 7.08 (t, $J$ = 8.79 Hz, 6H), 7.13 (t, $J$ = 8.76 Hz, 4H), 7.48 (d, $J$ = 7.95 Hz, 2H). $^{13}$C NMR (DMSO-d$_6$, 150 MHz) δ: 14.9, 26.6, 27.1, 28.6, 29.1, 31.5, 32.4, 35.4, 45.8, 51.8, 64.0, 68.4, 71.0, 109.2, 117.5, 117.9, 127.9, 129.1, 130.1, 130.7, 131.5, 137.0, 137.9, 155.5, 157.0, 160.3. HRMS m/z Calcd. for C$_{73}$H$_{89}$Cl$_4$N$_8$O$_4$ [M+H]$^+$ 1283.5726, Found 1283.5719.

HPLC purity: 95.7%.

4.1.2. 1,7-Bis(4-(2-(4-(tert-butyl)-2-ethoxyphenyl)-cis-4,5-bis(4-chlorophenyl)-4,5-dihydro-1H-imidazole-1-carbonyl) piperazin-1-yl) heptane-1,7-dione (10a)

To a solution of heptanedioic acid (29 mg, 0.18 mmol) in 10 mL anhydrous DMF, HBTU (266 mg, 0.72 mmol), DIPEA (0.14 mL, 0.72 mmol) and 5 (202 mg, 0.35 mmol) were added. The mixture...
reaction was stirred at room temperature for 1.5 h. Then the reaction mixture was dissolved in EtOAc (10 mL) and washed successively with water (10 mL) and saturated NaCl solution (10 mL), the organic phase was dried with anhydrous Na2SO4. After filtration, the filtrate was further purified by column chromatography (CH2Cl2:MeOH = 100:2) to afford compound 10a (190 mg, 85% yield) as off-white solid. 1H NMR (DMSO-d6, 600 MHz) δ: 1.12–1.16 (m, 4H), 1.26 (s, 18H), 1.28 (t, J = 6.97 Hz, 6H), 1.30 (m, J = 5.09 Hz, 2H), 2.11 (t, J = 7.41 Hz, 4H), 2.67–2.85 (m, 8H), 2.86–3.00 (m, 8H), 4.06 (t, J = 13.37 Hz, 7.21 Hz, 4H), 5.51 (d, J = 9.26 Hz, 2H), 5.65 (d, J = 8.34 Hz, 4H), 7.04 (s, 2H), 7.05–7.09 (m, 6H), 7.14 (d, J = 8.13 Hz, 4H), 7.52 (d, J = 7.85 Hz, 2H); 13C NMR (DMSO-d6, 150 MHz) δ: 14.9, 24.9, 25.3, 26.1, 31.4, 32.6, 35.4, 44.3, 45.9, 46.3, 64.1, 68.3, 71.0, 109.2, 117.4, 117.9, 127.9, 129.1, 130.2, 130.9, 131.5, 131.6, 136.9, 137.8, 155.7, 155.9, 157.1, 160.2, 171.1. HRMS m/z Calcd. for C71H81Cl4N8O6 [M+H]+ 1319.5341, Found 1319.5326. HPLC purity: 95.5%.

The synthesis procedure of target compounds 10b–d were similar to that of compound 10a.

4.1.4. Nonane-1,9-diylbis(piperazine-4,1-diyl)) bis((2-(4-(tert-butyl)-2-ethoxyphenyl)-cis-4,5-chlorophenyl)-4,5-dihydro-1H-imidazole-1-yl) methanone (10e)

1H NMR (DMSO-d6, 600 MHz) δ: 1.15–1.22 (m, 10H), 1.29 (s, 18H), 1.32 (t, J = 6.93 Hz, 6H), 1.33–1.40 (m, 2H), 2.15 (t, J = 7.43 Hz, 6H), 2.68–2.85 (m, 8H), 2.86–3.05 (m, 8H), 4.09 (dd, J = 13.63 Hz, 6.71 Hz, 4H), 5.54 (d, J = 9.90 Hz, 2H), 5.68 (d, J = 9.90 Hz, 2H), 6.97 (d, J = 7.79 Hz, 4H), 7.03 (d, J = 8.33 Hz, 4H), 7.05 (s, 2H), 7.09 (d, J = 8.32 Hz, 4H), 7.16 (d, J = 8.46 Hz, 4H), 7.55 (d, J = 7.94 Hz, 2H); 13C NMR (DMSO-d6, 150 MHz) δ: 14.9, 25.1, 29.1, 29.2, 31.3, 32.6, 35.4, 44.3, 45.9, 46.3, 64.1, 68.3, 71.0, 109.2, 117.4, 117.9, 127.9, 129.1, 130.2, 130.9, 131.5, 131.6, 136.9, 137.8, 155.7, 155.9, 157.1, 160.2, 171.1. HRMS m/z Calcd. for C77H89Cl4N8O6 [M+H]+ 1337.5654, Found 1337.5698. HPLC purity: 96.3%.

4.1.5. 1,5-Bis(4-(2-(4-(tert-butyl)-2-ethoxyphenyl)-cis-4,5-chlorophenyl)-4,5-dihydro-1H-imidazole-1-yl) pentane-1,3-dione (10f)

1H NMR (DMSO-d6, 600 MHz) δ: 1.19–1.25 (m, 20H), 1.27–1.30 (t, J = 10.02 Hz, 6H), 1.29 (s, 18H), 1.34–1.38 (m, 4H), 2.15 (t, J = 7.32 Hz, 4H), 2.52–3.01 (m, 16H), 4.01–4.12 (m, 4H), 5.53 (t, J = 9.76 Hz, 2H), 5.68 (t, J = 9.76 Hz, 2H),
6.96 (t, J = 7.50 Hz, 4H), 7.00 (e7.05 (m, 4H), 7.06 (s, 2H), 7.09 (d, J = 8.22 Hz, 6H), 7.16 (d, J = 8.58 Hz, 4H), 7.55 (d, J = 7.95 Hz, 2H). 13C NMR (DMSO-d6, 150 MHz): δ: 14.9, 25.2, 29.1, 29.3, 29.4, 29.5, 31.2, 31.4, 32.6, 35.4, 44.3, 45.4, 45.7, 46.3, 64.1, 68.3, 71.1, 109.2, 117.4, 117.9, 127.9, 129.1, 130.2, 130.9, 131.5, 131.7, 136.9, 137.8, 155.7, 155.9, 157.1, 160.2, 171.1. HRMS m/z Calcd. for C80H99Cl4N8O6 [M+H]+ 1407.6436, Found 1407.6403. HPLC purity: 95.7%.

4.1.6. 2,2'-((Ethane-1,2-diylbis(oxy)) bis(1H-imidazole-1-carbonyl) piperazin-1-yl) ethan-1-one (10e)

To a solution of 2,2'-((ethane-1,2-diylbis(oxy)) diacetic acid (32 mg, 0.18 mmol) was added HBTU (266 mg, 0.72 mmol), DIPEA (0.14 mL, 0.72 mmol) followed by dropwise addition of 5 (200 mg, 0.35 mmol) in anhydrous DMF (10 mL). After the reaction, the mixture reaction was stirred at room temperature for
Table 2  MDM2–P53 competitive binding and in vitro antitumor activities of enantiomers 11a.

| Compd. | IC50 (μM/L) | A549 | HCT116 (P53+) | HCT116 (P53-) |
|--------|-------------|------|---------------|---------------|
| 11a    | 0.1 ± 0.2   | 1.4 ± 0.31 | 1.9 ± 0.67   | >50           |
| 11a-1  | 0.09 ± 0.06 | 0.58 ± 0.11 | 0.31           | 21 ± 8.3     |
| 11a-2  | 0.12 ± 0.04 | 8.3 ± 0.22  | 9.9 ± 0.10    | >50           |
| 11a-3  | 9.9 ± 2.3   | 11.1 ± 1.4  | 8.7 ± 0.22    | >50           |
| 1      | 0.15 ± 0.02 | 9.9 ± 1.3   | 8.6 ± 1.0     | 19 ± 0.91    |

Values expressed as mean ± SD in parallel three times. P53+; P53-wild; P53–; P53-deleted.

4.1.7. 2,2'-((Oxybis(ethane-2,1-diyl))bis(oxo))oxoyl(ethane-2,1-diyl)-bis(2,2-(4-(4-((tert-butyl)-2-ethoxyphenyl)-cis-4,5-bis(4-chlorophenyl)-4,5-dihydro-1H-imidazole-1-carbonyl)-2-oxopiperazin-1-yl)acetamide) (10f)

H NMR (DMSO-d6, 600 MHz): d = 1.31 (s, 18H), 1.32 (d, J = 7.07 Hz, 6H) 2.71–2.85 (m, 8H), 2.86–2.94 (m, 8H), 3.46 (d, J = 6.23 Hz, 8H), 4.02 (t, 4H, 6.01 (d, J = 12.87 Hz, 7.06 Hz, 4H), 5.57 (d, J = 9.89 Hz, 2H), 5.69 (d, J = 9.89 Hz, 2H), 6.97 (d, J = 7.64 Hz, 4H), 7.04 (d, J = 8.01 Hz, 4H), 7.05–7.13 (m, 8H), 7.17 (d, J = 7.64 Hz, 4H), 7.56 (d, J = 7.87 Hz, 2H), 13C NMR (DMSO-d6, 150 MHz): d = 14.8, 31.3, 35.4, 43.7, 45.9, 45.1, 64.1, 68.3, 69.7, 70.1, 70.1, 109.2, 117.4, 117.8, 127.9, 127.9, 129.2, 130.3, 130.9, 131.1, 131.6, 131.7, 136.8, 137.7, 155.7, 155.9, 157.1, 160.3, 167.7. HRMS m/z. Calcd. for C39H39Cl2N4O6 [M+H]⁺ 1299.4769, Found 1299.4769. HPLC purity: 95.2%.

The synthesis proceed of target compound 10f was similar to that of compound 10e.

4.1.10. N,N'-(Oxybis(ethane-2,1-diyl))bis(2,2-((4-((tert-butyl)-2-ethoxyphenyl)-cis-4,5-bis(4-chlorophenyl)-4,5-dihydro-1H-imidazole-1-carbonyl)-2-oxopiperazin-1-yl)acetamide) (11e)

H NMR (DMSO-d6, 600 MHz): d = 1.28 (t, J = 6.74 Hz, 6H), 1.32 (s, 18H), 2.28 (s, 4H), 3.16–3.22 (m, 6H), 3.36 (t, J = 6.21 Hz, 4H), 3.60 (d, J = 17.37 Hz, 2H), 3.67–3.72 (m, 4H), 3.81 (d, J = 16.08 Hz, 2H), 4.03–4.13 (m, 5H), 5.61 (d, J = 9.84 Hz, 2H), 5.70 (d, J = 9.84 Hz, 2H), 6.98 (d, J = 8.04 Hz, 4H), 7.04 (d, J = 8.30 Hz, 6H), 7.09 (d, J = 8.56 Hz, 2H), 7.10 (d, J = 8.69 Hz, 4H), 7.15 (d, J = 8.43 Hz, 4H), 7.55 (d, J = 7.78 Hz, 2H), 7.90 (t, J = 5.45 Hz, 2H), 13C NMR (DMSO-d6, 150 MHz): d = 14.8, 31.4, 35.4, 38.9, 42.7, 46.9, 49.0, 49.3, 64.1, 68.4, 69.1, 71.5, 109.3, 117.6, 117.8, 127.9, 128.0, 129.2, 130.3, 130.8, 131.6, 131.8, 136.7, 154.8, 155.8, 156.6, 160.4, 164.8, 167.7. HRMS m/z. Calcd. for C39H39Cl2N4O6 [M+Na]⁺ 1369.4937, Found 1369.4931. HPLC purity: 96.1%.

4.2. In vitro antitumor assay

A549 cells were placed in 96-well plates with 6.0 × 10³ cells in each well and subsequently incubated for 24 h in a moist atmosphere of 5% CO₂ and 37 °C. Then, different concentrations of test compounds or vehicle were added to triplicate wells. After incubation for an additional 72 h, 10% of CCK-8 in 100 µL final volume of culture medium was added to every well, then the plates were incubated for 0.5–2.0 h. The absorbance (OD value) was read at 450 nm on a spectrophotometer (Biotek Synergy H2). The values of IC50 were calculated by the Logit method. All experimental results were measured in parallel three times.
4.3. Immunoblotting assay

A549 cells were cultured (3.5 × 10^5 cells/well) in a 6-well transparent plate for 24 h. Different concentrations of test compounds and vehicle were added to the A549 cells in the plates, and then, the plates were incubated for another 12 or 24 h. The cells were subsequently washed with cold PBS and 70 μL of cold lysis RIPA buffer containing protease inhibitors and phosphatase inhibitors for 15 min. After placing the 6-well plate on ice for 15 min, the cells were scraped off from the plate and centrifuged at 12,000 rpm at 4°C to collect the protein lysate. Then the lysate concentrations were tested by protein test assay. The protein extract was denatured and separated onto a 10%–15% SDS-PAGE gels.

The protein in the gels was electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The PVDF membrane containing the proteins was rinsed with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) for 2 min, then blocked with 5% bovine serum albumin (BSA) buffer at room temperature for 1.5 h. The blocking solution was removed by aspiration, and the diluted primary antibody was immediately added. The membrane was incubated overnight at 4°C. After recovering the primary antibody solution, the PVDF membrane was washed 3 times with TBST, for 5 min per time. The secondary antibody was added to the membrane, which was incubated at room temperature for 1.5 h in the dark. After recovering the secondary antibody, the PVDF membrane was washed 3 times with TBST, for 5 min per time. The blot was scanned using the LI-COR Odyssey imaging system. Taking vehicle control as the standard, the expression level of protein was quantified by analysis of the gray values of the band in the obtained image. The antibodies of Western blotting were: MDM2 (Abcam, ab16895), P53 (Abcam, ab26), GADH (Abcam, ab8245), and secondary antibody (Abcam, ab150115).

4.4. Apoptosis assay

A549 cells with a density of 4.0 × 10^5 cells/well were placed in 6-well transparent plates and then treated with test compounds and vehicle in a moist atmosphere of 5% CO2 at 37°C for 48 h. The A549 cells were collected by trypsinization without EDTA and washed with pre-cold PBS. A549 cells were resuspended with 200 μL of 1× binding buffer after centrifugation and removal the supernatants. Annexin V-fluorescein isothiocyanate (FITC, 5 μL) was added to the resuspended cell solution, which was then incubated for 15 min at room temperature. After adding 10 μL PI, the treated cells were incubated for another 15 min in the dark at room temperature. The analysis of stained cells was performed by a flow cytometer (BD Accuri C6, USA).

4.5. In vivo A549 xenograft assay

The animal experiments procedures, animal use, and animal care were approved through the Animals of Committee on Ethical Use of Second Military Medical University. The in vivo antitumor activity was evaluated by A549 tumor xenograft by subcutaneously injecting A549 cells (5.0 × 10^6 cells/animal) into the flank of the BALB/c nude female mouses with an average weight of 18–20 g (certificate SCXK-2007-0005, 6–7 weeks old, Shanghai Experimental Animal Center). The mice were randomized into four groups (5 mice per group) when the tumor volumes reached an average value of 100 mm^3. Three treatment groups were continuously treated for 21 days with compounds 11a-1 (20 or 30 mg/kg, twice daily) and I (20 mg/kg, twice daily), which were administered by intraperitoneal injection. The vehicle control group was treated with an equal volume of normal saline. Tumor volumes were measured every three days with a vernier caliper, and the body weight of every mouse was recorded at the meantime. The
volume of tumor was determined by the length and width of the tumor tissue. Eq. (1) is used to calculate the tumor volume:

\[ V \ (\text{mm}^3) = \left( A \times B^2 \right) / 2 \]

where \( A \) and \( B \) represent length and width of the tumor tissues, respectively. The antitumor efficacy is represented by TGI as Eq. (2):

\[ \text{TGI} \ (%) = \left( 1 - \frac{V_{\text{Treat}}}{V_{\text{Control}}} \right) \times 100 \]

Analysis of the data was performed by one-way ANOVA test. Statistical significance was considered at \( P < 0.05 \).

Acknowledgments

This work was supported by National Natural Science Foundation of China (Grant Nos. 82030105, 21738002 and 21807113), the National Key R&D Program of China (Grant No. 2020YFA0509100), and the Innovation Program of Shanghai Municipal Education Commission (Grant No. 2019-01-00-07-E00073, China).

Author contributions

Shipeng He designed and completed most biological and cell-based assays. Junhui Ma designed and synthesized most of the target compounds. Yuxin Fang, Ying Liu and Shanchao Wu participated in research design and conducted experiments. Guoqiang Dong, Wei Wang and Chunquan Sheng proposed the project, performed data analysis and contributed to the writing—review&editing of the manuscript. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.11.022.

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