Design, Synthesis, and Biological Evaluation of Novel Protopanoxadiol Derivatives Based PROTACs Technology for the Treatment of Lung Cancer

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Abstract:

Protopanoxadiol is a key active ingredient derived from Panax ginseng that is well-known to exhibit anti-tumor activity. Previous research focused on the natural protopanaxadiol derivative AD-1 has demonstrated that it possesses broad spectrum anti-tumor activities *in vitro* and *in vivo*. However, its limited activity, selectivity, and cell permeability have impeded its therapeutic application. Herein, a series of novel AD-1 derivatives were designed and synthesized based on proteolysis-targeting chimera (PROTAC) technology by linking AD-1 at the C-3 and C-12 positions with pomalidomide through linkers of alkyl chain of differing lengths to achieve the goal of improving the efficacy of the parent compound. Among these synthesized PROTACs, the representative compound **A05** exhibited the most potent anti-proliferative activity against A549 cells. Furthermore, mechanistic studies

revealed that compound **A05** was able to suppress MDM2 expression, disrupt interactions between p53 and MDM2 and readily induce apoptotic death via the mitochondrial apoptosis pathway. Moreover, the *in vivo* assays revealed that compound **A05** exhibited both anti-proliferative and anti-metastatic activities in the zebrafish tumor xenograft model with A549 cells. Together, our findings suggest that AD-1 based PROTACs associated with the degradation of MDM2 may have promising effects for the treatment of lung cancer and this work provide a foundation for future efforts to develop novel anti-tumor agents from natural products.

Keywords: Protopanoxadiol, PROTACs, Anti-tumor, MDM2

1. Introduction

Natural products have long been a primary source for compound screening in the design of novel anti-tumor drug candidates [1]. Ginseng is widely used to prevent and treat a range of diseases and disorders in many countries [2], reportedly exhibiting anti-tumor, anti-fatigue, immunostimulatory, and neuroprotective activity [3], with such activity being mediated primarily by ginsenosides, which are the primary active compounds present within ginseng roots [4]. Triterpenoids are a class of diverse natural compounds that are widely distributed in plants and exhibit varied biological effects [5], with dammarane skeleton-based natural triterpenoids reportedly exhibiting antioxidant, anti-tumor, anti-fatigue, and antiviral properties [6,7]. These reports have spurred efforts to modify the structures of dammarane triterpenoid parent compounds to enhance their pharmacological potency and yields. We have previously isolated and characterized ginseng-derived ginsenosides, leading to the discovery of the novel protopanaxadiol-type ginsenoside 20(*R*)-25-methoxyl-dammarane-3β,12β,20-triol (AD-1, Fig. 1), which exhibited potent *in vitro* and *in vivo* antitumor activity while causing minimal toxicity [8-11]. When used to treat twelve different cancer types including prostate, breast, pancreatic. and lung cancers, AD-1 exhibited low IC_{50} values [12]. Mechanistically, AD-1 was found to promote apoptotic dell death, G0/G1 cell cycle arrest, reactive oxygen species (ROS) production, the upregulation of p53,

and the concomitant downregulation of MDM2 at the protein level. Moreover, the oral administration of AD-1 doses from 10-40 mg/kg in mice resulted in the dose-dependent suppression of HepG2 and A549 xenograft tumor growth without adversely impacting body weight [13, 14]. However, they are not active at sufficiently low concentrations, and their lower selectivity and cell permeability have largely hindered their therapeutic applications. Our group sought to overcome these issues through the design and synthesis of a series of protopanoxadiol AD-1 derivatives such as introduction of side chains containing amino acid, benzoic acid, bromoacetyl bromide, and triphenylphosphine unit to obtain excellent efficacy [15-17].

The PROTAC (Proteolysis-Targeting Chimera) technology is a novel therapeutic platform that leverages the ubiquitin-proteasome system to facilitate targeted protein degradation. The PROTAC space is an area of active and growing drug discovery interest [18, 19], with two orally available small molecule PROTACs, ARV-110 (NCT03888612, Fig. 1) and ARV-471 (NCT04072952, Fig. 1), having entered into clinical trials in 2019 and achieved satisfactory safety and efficacy outcomes, encouraging further PROTAC-based drug design efforts [20]. At the simplest level, PROTACs are tripartite heterobifunctional compounds composed of an E3 ligand and a target protein ligand joined together by a chemical linker [21]. These molecules thereby recruit E3 ubiquitin ligases to ubiquitinate target proteins such that they subsequently undergo proteasomal degradation and can no longer function [22]. Unlike traditional small molecule drugs that inhibit individual signaling pathways for multifunctional proteins, PROTACs can induce target protein degradation and thereby impact the functions of that protein, disrupting feedback mechanisms that might be engaged and thus partially mitigating the onset of drug resistance [23, 24]. In contrast to high-affinity small molecule inhibitor drugs, chimeric PROTACs also exhibit less restrictive target protein affinity requirements given that even relatively weak binding can facilitate ubiquitination and proteasomal degradation [25]. These PROTACs can also play catalytical roles at subchemical doses, with dissociated PROTAC molecules iteratively functioning, thereby reducing the required concentrations for effective treatment and reducing dose-related toxicities. Given the marked advantages of

PROTACs, they are currently a focus of pronounced anti-tumor drug development [26]. Thus, we hypothesize that by linking PROTACs to natural compound AD-1 through alkyl chains of different lengths as chemical linker could target AD-1 to cancer cells and further improve its cytotoxicity.

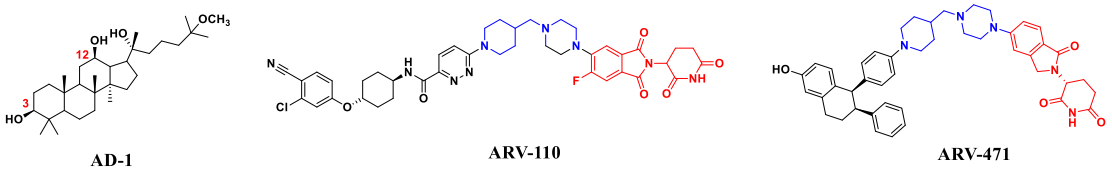


Fig. 1. The chemical structure of AD-1, ARV-110 and ARV-471.

In this study, we report the rational design and synthesis of a series of AD-1 derivatives based on PROTAC technology by linking AD-1 at the C-3 and C-12 positions with pomalidomide through linkers of different alkyl chain, with the aim of increasing the efficacy of the parent compound (Fig. 2). In addition, the underlying cytotoxic mechanisms of representative AD-1 PROTAC derivative **A05** were also elucidated. The anti-tumor effects of the optimal compound were also additionally assessed *in vivo* using a zebrafish xenograft model.

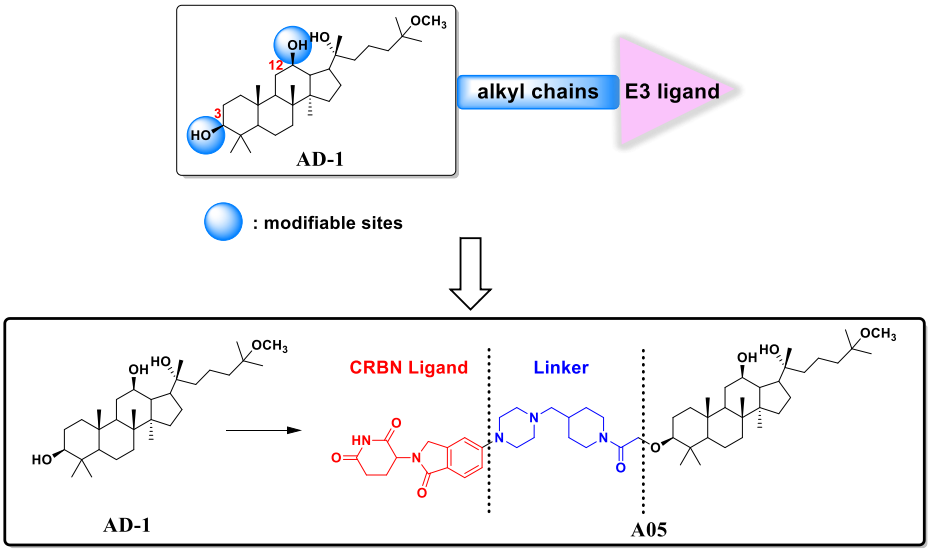


Fig. 2. The design strategy of AD-1 PROTAC derivatives.

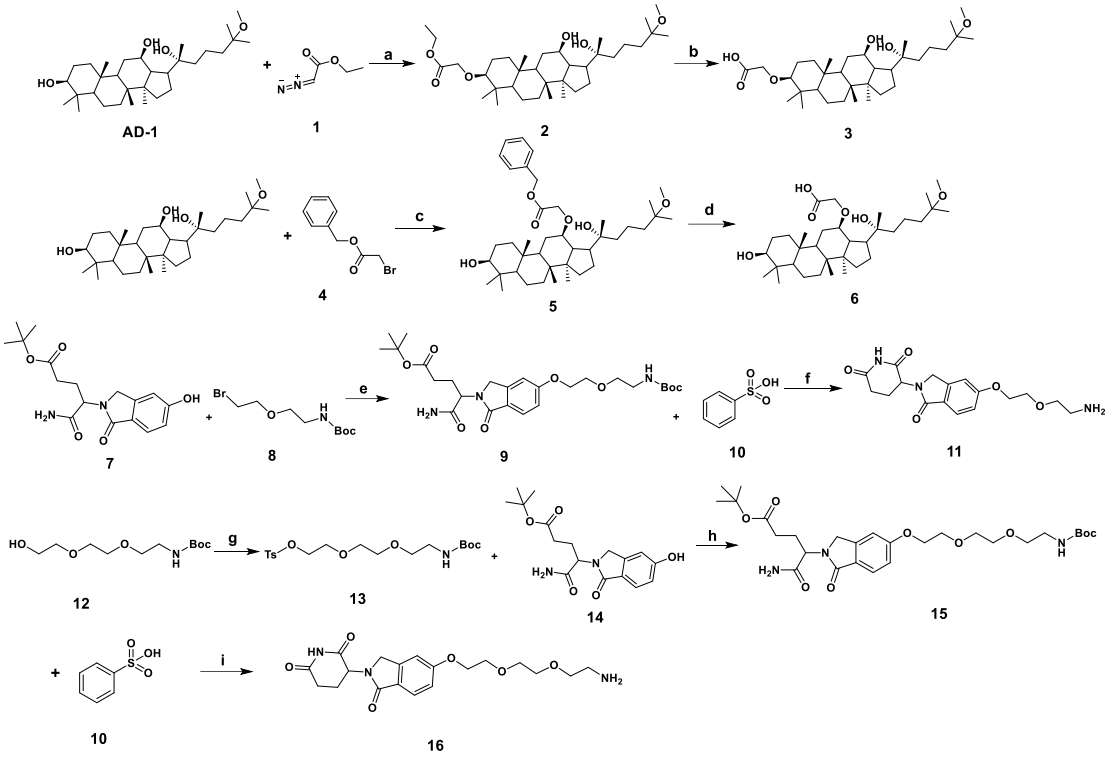
2. Results and Discussion

2.1. Design and chemistry of PROTACs

Balancing the appropriate composition and assembly of the three components of PROTACs is critical to their proper functioning. In an effort to develop PROTACs with only low levels of toxicity and superior binding affinity, the CRBN component of ARV-110/ARV-471 was used to explore the modification of linkage positions with the CRBN ligand. Appropriate linker selection is known to be critical to obtaining efficient and potent PROTACs. As such, the composition and length of the linker domain were systematically varied, and AD-1 served as the core payload for the designed PROTACs. As AD-1 contains just three hydroxyl groups amenable to traditional structural modification, these residues were explored as suitable sites for linker-mediated E3 ligase tethering. A series of PROTACs based on AD-1 derivatives were prepared by replacing these hydroxyl groups with oxyacetic acid, after which these derivatives were tethered to the CRBN ligand with a range of linkers, yielding compounds **A01-A10** (Scheme 1-4), as the reactivity of the C-3 hydroxyl group was significantly higher than that of the C-12 hydroxyl group. Structure-activity relationship (SAR) analysis of these different substitutions was performed by generating compounds **A11-A13** (Scheme 5).

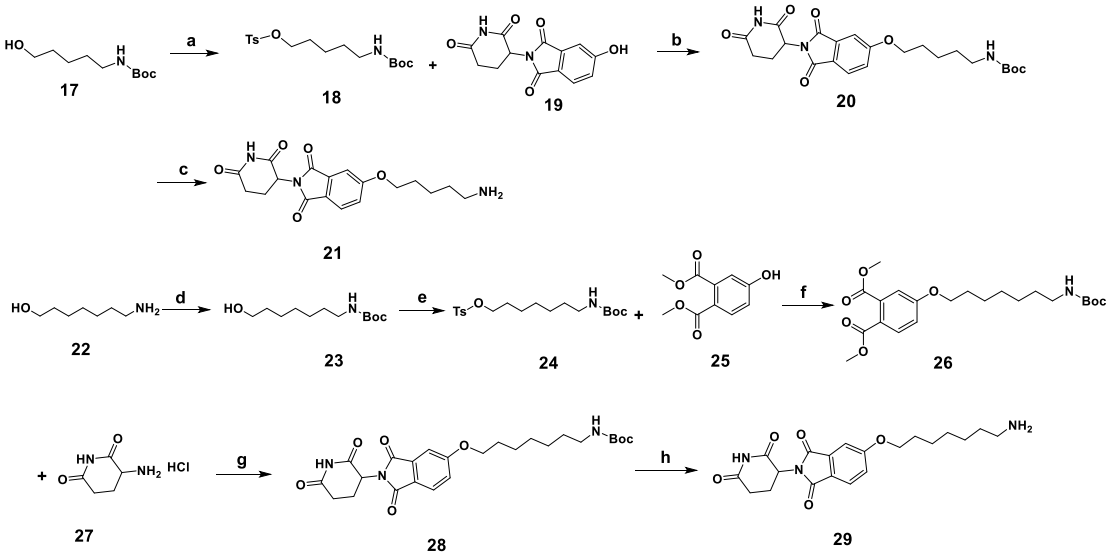
The synthetic strategy for compounds **A01**-A**13** is represented by the synthetic route for **A03**, which is summarized in Scheme 1. Briefly, intermediate **2** was obtained from ethyl 2-diazoacetate and AD-1. COSY, HMBC, and HSQC approaches were used to characterize the chemical structural characteristics of compound **2**. Next, lithium hydroxide was added to a mixture of **2** and tetrahydrofuran in methanol to yield compound **3**. Compound **A01** was synthesized from compound **3** and 3-[5-(5-aminopentoxy)-1-oxo-isoindolin-2-yl] piperidine-2,6-dione, with products then being purified via semi-preparative reverse phase HPLC. A similar approach was also used to synthesize compounds **A01-A13** using corresponding amines in place of the CRBN component.

Scheme 1. Synthesis strategies for the key intermediates **3**, **6, 11** and **16**.



Reagents and conditions: (a) $Rh(OAc)_2$, DCM, 25°C, 12 h; (b)MeOH, THF, H_2O, 25°C, 12 h; (c) NaH, DMF, 0-50°C, 4 h; (d) Pd/C, H_2, THF, MeOH, 30°C, 12 h; (e) K_2CO_3, DMF, 80°C, 2 h; (f) ACN, 80°C, 12 h; (g) TsCl, TEA, DCM, 25°C, 12 h; (h) K_2CO_3, DMF, 80°C, 2 h; (i) ACN, 80°C, 12 h.

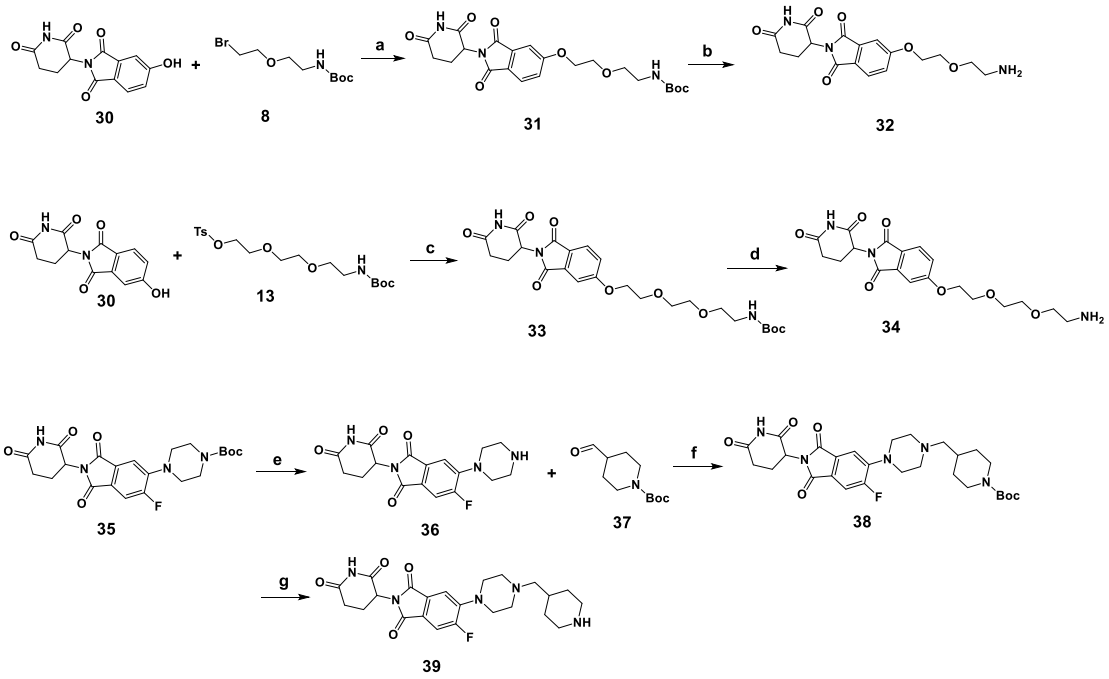
Scheme 2. Synthetic route for intermediates **21** and **29**.



Reagents and conditions: (a) TsCl, TEA, DCM, 25°C, 12 h; (b) K_2CO_3, DMF, 60°C, 12 h; (c) HCl, dioxane, DCM, 25°C, 2 h; (d) Boc_2O, TEA, DCM, 0-25°C, 2 h; (e) TsCl, TEA, DCM,

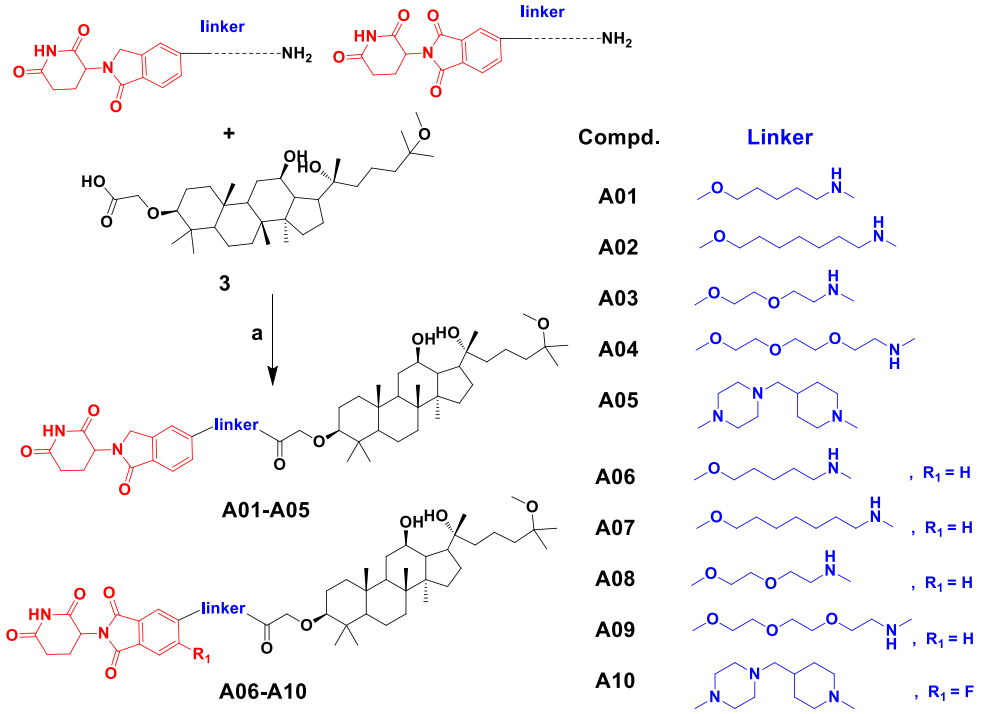
25°C, 12 h; (f) Cs_2CO_3, DMF, 80°C, 12 h; (g) LiI, Py, 130°C, 12 h; (h) HCl, dioxane, DCM, 25°C, 2 h.

Scheme 3. Synthetic route for intermediates **32**, **34**, and **39**.



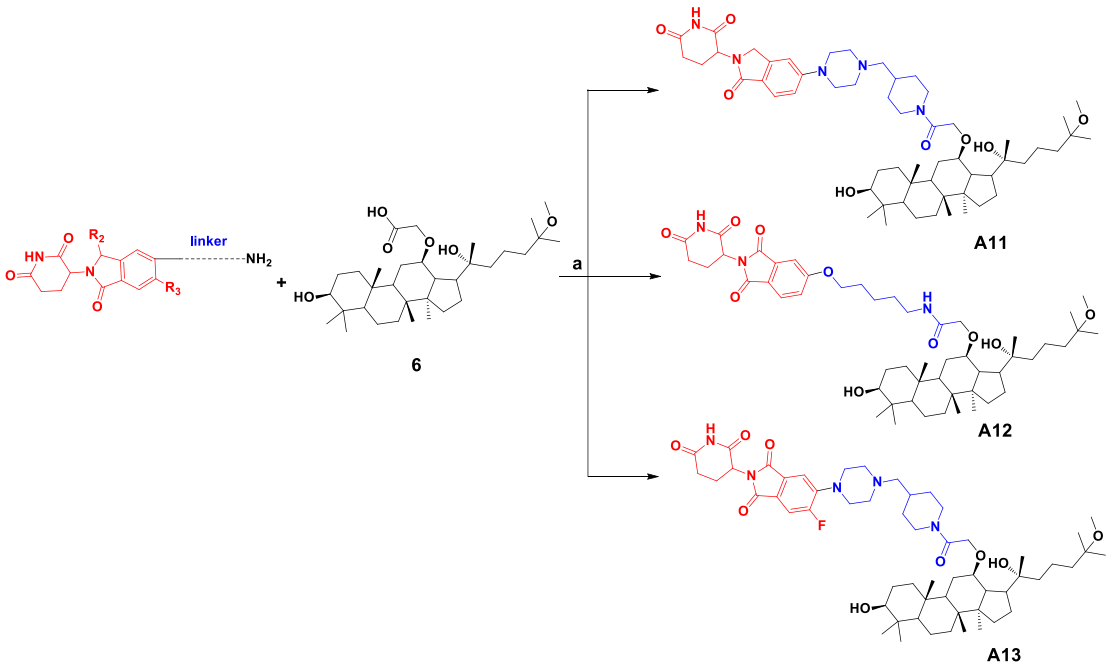
Reagents and conditions: (a) K_2CO_3, DMF, 60°C, 12 h; (b) HCl, dioxane, DCM, 25°C, 2 h; (c) K_2CO_3, DMF, 60°C, 12 h; (d) HCl, dioxane, DCM, 25°C, 2 h; (e) HCl, dioxane, 25°C, 2 h; (f) NaOAc, $NaBH(OAc)_3$, DCM, MeOH, 25°C, 1 h; (g) HCl, dioxane, DCM, 25°C, 2 h.

Scheme 4. Synthesis approach for compounds **A01-A10.**



Reagents and conditions: (a) EDCI, HOBt, DIPEA, DMF, 25°C, 12 h.

Scheme 5. Synthesis approach for compounds **A11-A13.**



Reagents and conditions: (a) EDCI, HOBt, DIPEA, DMF, 25°C, 12 h.

2.2. Biological characterization of representative PROTACs

*2.2.1 Compound **A05** markedly suppresses tumor cell proliferative activity*

Five model human cancer cell lines known to exhibit MDM2 overexpression were selected for use as models for the validation of the biological activity of the candidate AD-1-derived PROTACs (PANC-1, HepG-2, A549, MCF-7, and HeLa cells). These cells were treated for 48 h with a range of doses of compounds **A01**-**A13**, AD-1, or Mitomycin C (0, 5, 10, 20, 40 μM). SPSS 16.0 was then used to compute IC_{50} values for these different compounds, revealing that compounds **A02**, **A04**, **A05**, and **A10** exhibited IC_{50} values below 10 μM (Table 1). These results indicated that the combination of AD-1 with chelating linkers was associated with enhanced antitumor activity relative to the parent compound AD-1. All synthesized PROTACs exhibited moderate to robust antitumor activity against these five target cell lines, with IC_{50} values ranging from 2~10 μM. Among these PROTACs, compound **A05** exhibited the most potent antitumor activity with an IC_{50} of 2.16 μM against A549 cells, superior to that of AD-1 or Mitomycin C.

Table 1. IC_{50} values of compounds **A01~A13**, AD-1 and Mitomycin C in PANC-1, HepG-2, A549, MCF-7, HeLa.

Compd.	$IC_{50}(\mu M)^a$				
	PANC-1	HepG-2	A549	MCF-7	HeLa
A01	21.88 ± 2.13	6.91± 0.23	11.62 ± 0.99	7.95 ± 1.33	12.52 ± 1.88
A02	13.57 ± 1.21	5.06 ± 0.94	4.91 ± 1.29	9.23 ± 1.42	5.89 ± 0.35
A03	14.84 ± 0.89	10.13 ± 1.37	7.54 ± 3.01	43.81 ± 2.11	20.17 ± 1.55
A04	12.23 ± 0.82	7.13 ± 2.41	8.28 ± 0.73	8.16 ± 0.76	13.50 ± 0.67
A05	**8.49 ± 0.61**	**5.74 ± 0.92**	**2.16 ± 0.62**	**7.26 ± 0.66**	**8.80 ± 0.33**
A06	57.19 ± 3.65	7.28 ± 0.72	9.63 ± 0.07	7.463 ± 0.53	11.52 ± 0.88
A07	45.36 ± 3.11	9.84 ± 0.84	12.32 ± 0.87	9.19 ± 0.92	9.62 ± 0.65
A08	15.59 ± 1.36	47.37 ± 2.65	9.27 ± 0.56	10.35 ± 0.71	12.42 ± 1.12
A09	13.45 ± 1.15	25.61 ± 1.88	9.873 ± 1.19	10.43 ± 0.82	13.96 ± 0.68
A10	13.31 ± 1.09	6.87 ± 1.12	9.57 ± 0.64	9.23 ± 1.19	11.59 ± 0.96
A11	24.96 ± 1.66	5.164 ± 0.36	8.09 ± 1.09	7.66 ± 0.36	12.96 ± 0.69
A12	16.39 ± 1.13	76.80 ± 5.13	13.45 ± 2.06	12.38 ± 1.23	22.31 ± 1.32
A13	40.28 ± 2.11	26.95 ± 2.68	9.05 ± 2.94	12.39 ± 0.89	15.20 ± 1.09
AD-1	>100	7.43 ± 0.56	17.98 ± 0.36	25.40 ± 1.32	18.94 ± 2.31
Mitomycin C	23.65 ± 1.12	17.61 ± 0.66	18.96 ± 0.48	37.02 ± 1.68	16.22 ± 1.65

[a]Values are the mean of triplicate experiments.

According to preliminary SAR analyses, the overall activity of the synthesized compounds was superior to those of parental AD-1. In particular, the C-3 substituted derivatives were superior to C-12 substituted derivatives based on a comparison of compounds **A05** and **A11**. Linker length and type can also influence the physicochemical properties and biological activity of PROTACs. [27, 28] Additional studies of PROTAC linkers through the synthesis and incorporation of alkyl or alkyl diamines of varying lengths revealed that the length of nine atoms linker may be the most optimal, with a rigid linker being conducive to enhanced activity as observed for compound **A05**.

*2.2.2 Analysis of the effects of compound **A05** on MDM2-p53 interactions*

MDM2 is a critical oncogenic protein that negatively regulates p53 and can maintain appropriate p53 activity under physiological conditions [29, 30]. However, many tumors exhibit MDM2 overexpression, and p53 pathway activation through MDM2 inhibition has long been thought to represent a promising approach to treating cancer. MDM2 inhibitor-based therapies thus have the potential to restore the ability of p53 to suppress oncogenic processes through the disruption of MDM2-p53 interactions.

Accordingly, the ability of compound **A05** to interfere with interactions between p53 and MDM2 was assessed, revealing a marked drop in MDM2 protein levels following **A05** treatment with a corresponding increase in p53 protein levels in A549 cells. **A05** markedly suppressed MDM2 protein expression, with respective inhibition ratios of 33.78%, 49.53%, and 65.39% at concentrations of 1.11 μM, 3.33 μM, and 10 μM. At these same respective doses, p53 levels rose by 133.45%, 138.01%, and 147.15% (Fig. 3).

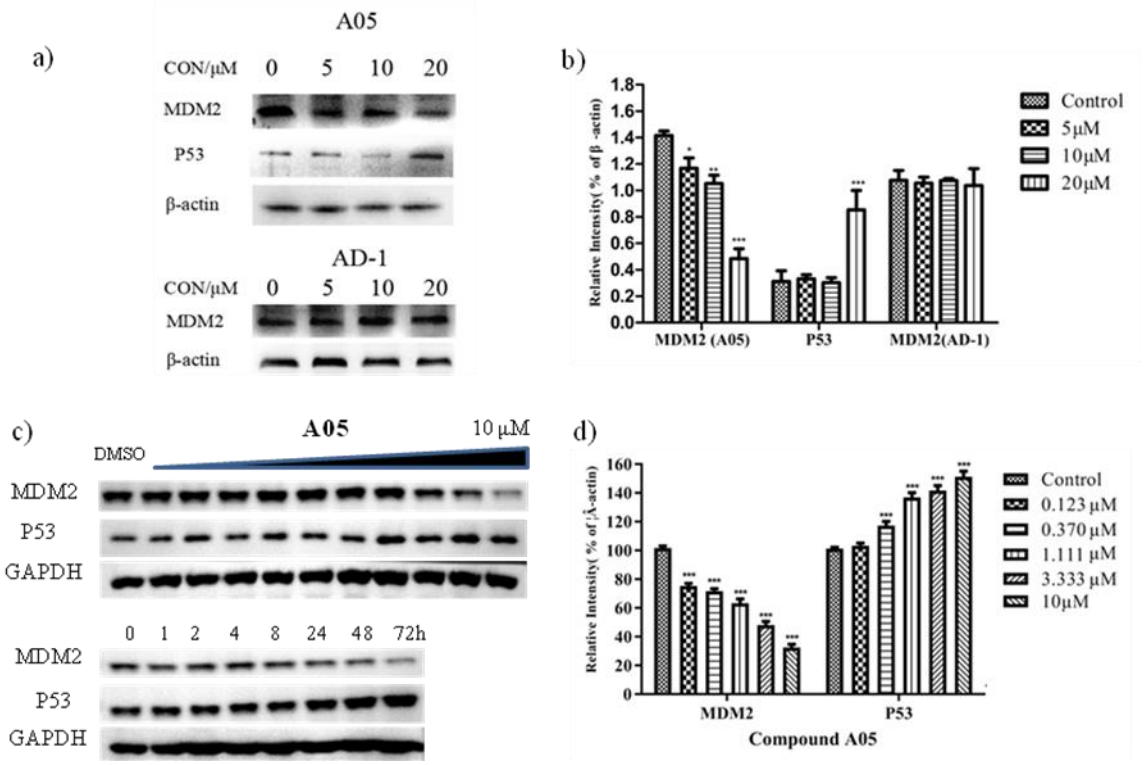


Fig. 3. a) Western blot analysis of MDM2 and p53 proteins expression with compound **A05** and **AD-1** treatment in A549 cells for 48 h. Data were expressed as means ± SDs of triplicate experiments performed independently. **P*< 0.05, ***P*< 0.01, ****P*< 0.001. b) **A05** obviously inhibited the MDM2 protein expression, the inhibition ratios were 17.52%, 25.66%, 65.80% at the concentration of 5, 10, 20 μM, respectively. c) Western blotting analysis of MDM2 and p53 protein expression with compound **A05** (0.00051, 0.00152, 0.00457, 0.0137, 0.041, 0.123, 0.37, 1.11, 3.33, 10 μM) treatment in A549 cells for 72 h. d) Data are expressed as means ± SDs from independent experiments performed in triplicate. **P*< 0.05, ***P*< 0.01, ****P*< 0.001.

*2.2.3 Compound **A05** promotes the apoptotic death of A549 cells via the mitochondrial pathway*

DAPI staining was used to monitor A549 cell nuclei in order to gauge the ability of compound **A05** to promote apoptotic tumor cell death. Marked chromatin condensation was observed in cells treated with compound **A05** but not in cells treated with AD-1 (Fig. 4), consistent with the induction of apoptosis. Moreover, an Annexin V-FITC staining approach confirmed that treatment with compound **A05** at

doses of 10 or 20 μM resulted in the pronounced induction of apoptotic death in A549 cells. Consistently, levels of cleaved PARP, cleaved caspase-3, and cleaved caspase-9 protein levels rose with increasing compound **A05** concentrations (Fig. 5). Together, these data thus confirmed that treatment with compound **A05** triggered apoptotic death in A549 cells.

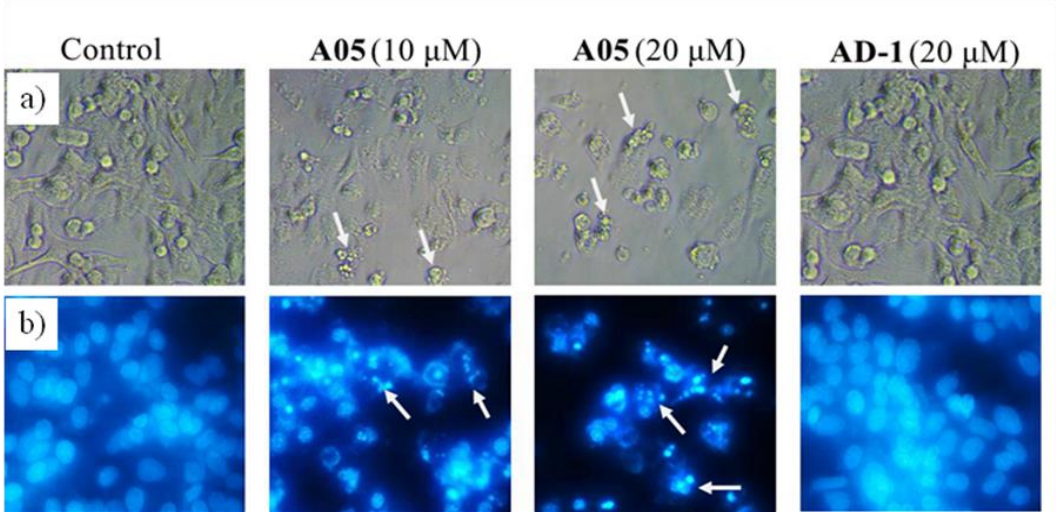


Fig. 4. a) Morphological observations of A549 cells treated with compound **A05** and AD-1. b) The effect of compound **A05** on the induction of apoptosis in A549 cells stained with DAPI.

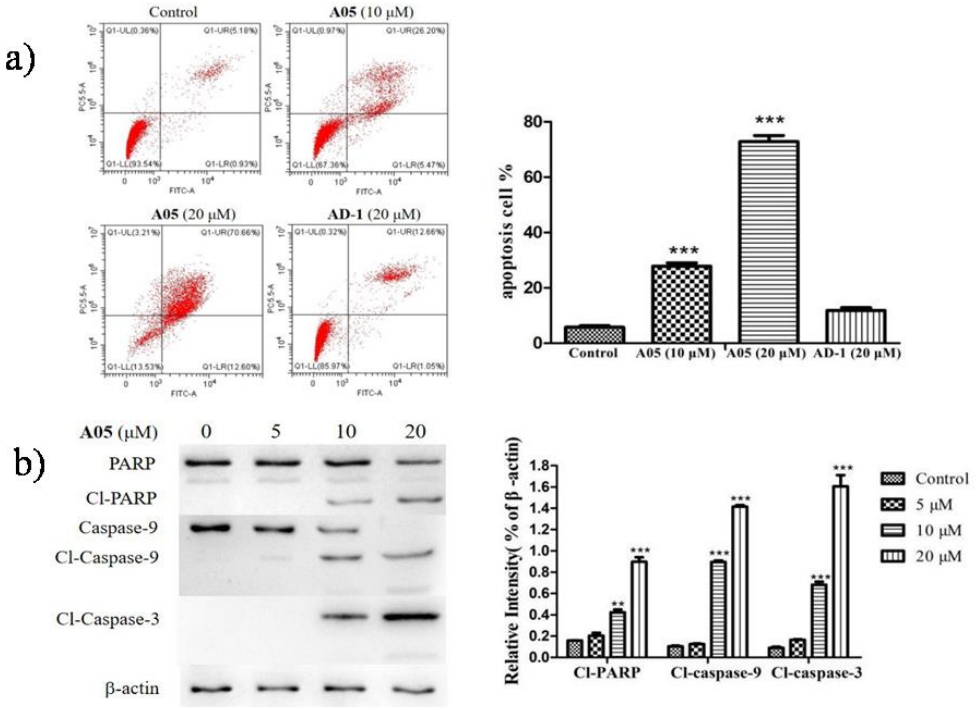


Fig. 5. a) The effects of compound **A05** on apoptotic induction in A549 cells as detected via flow cytometry. Data are expressed as means ± SDs from independent experiments performed in triplicate. ***$P < 0.001$ *vs*. control. b) The expression levels of apoptosis-related proteins in A549 cells treated with compound **A05**. Data are expressed as means ± SDs from independent experiments performed in triplicate. **$P < 0.01$, ***$P < 0.001$.

The mitochondrial pathway is one of the primary mechanisms of apoptotic cell death [31, 32], and both pro-apoptotic Bax and anti-apoptotic Bcl-2 regulate the integrity of the mitochondria [33]. In the context of apoptotic induction, Bax is transferred to the membranes of mitochondria from the cytoplasm, resulting in a loss of mitochondrial membrane potential and cytochrome C release [34]. To explore the antitumor activity of compound **A05,** shifts in mitochondrial apoptosis-related protein levels were next assessed by evaluating levels of Bax and cytochrome C in the mitochondrial and cytoplasmic compartments following **A05** treatment. Significant reductions in cytosolic Bax were observed in **A05**-treated cells whereas its levels were increased in the mitochondrial fraction, whereas the opposite finding was observed for cytochrome C (Fig. 6). These data thus confirmed the ability of compound **A05** to

induce apoptotic death via the mitochondrial pathway.

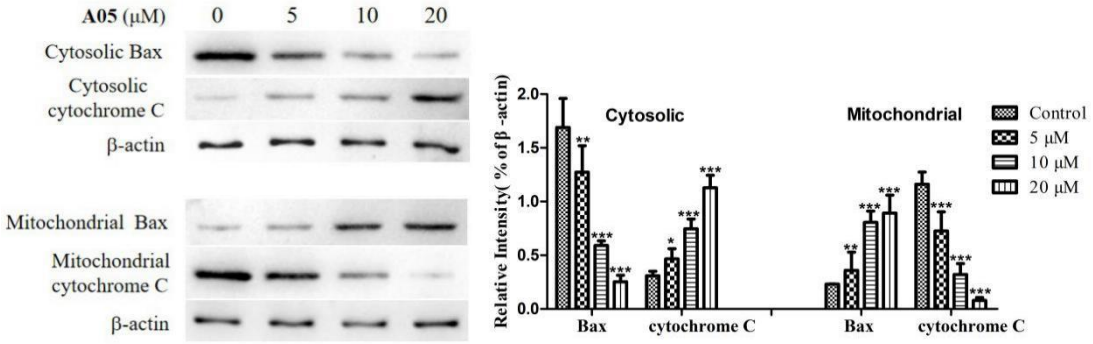


Fig. 6. Bax and cytochrome C protein levels in the cytoplasm and mitochondria following the **A05** treatment of A549 cells. Data are expressed as means ± SDs from independent experiments performed in triplicate. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

*2.2.4 Analyses of the in vivo antitumor activity of compound **A05** in zebrafish*

To validate the *in vivo* antitumor activity of compound **A05**, its ability to suppress A549 cell growth and metastasis in a well-established zebrafish model system that is widely used for drug screening was assessed. [35,36] After labeling with the fluorescent CM-DiI dye, A549 cells were implanted in zebrafish that were subsequently treated with a range of **A05** concentrations in an aqueous solution (0.78, 1.56, or 3.12 μM). Cisplatin (50 μM) was utilized as a positive control. The fluorescence intensity of A549 tumors in cisplatin-treated zebrafish was significantly reduced relative to the model group (335,657 pixels vs. 535,401 pixels). The A549 tumor xenograft fluorescence intensity values in zebrafish were 525,656, 485,896, and 402,713 pixels, respectively, when the animals were treated with **A05** concentrations of 0.78, 1.56, and 3.12 μM with the most robust antiproliferative activity thus being evident at a concentration of 3.12 μM (Fig. 7).

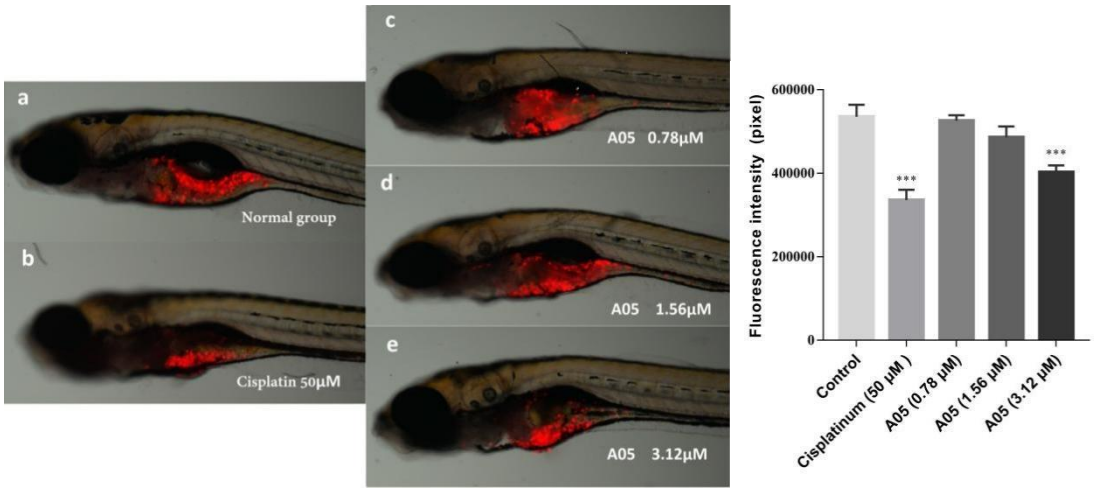


Fig. 7. The fluorescence intensity of A549 xenografts in zebrafish treated with **A05**. Images were taken via fluorescence microscope and analyzed using the NIS-Elements D 3.20 advanced image processing software.

A549 xenograft tumors treated with both cisplatin (50 μM) and compound **A05** (3.12 μM) exhibited significant reductions in cellular migration (Fig. 8). These results thus confirmed the ability of compound **A05** to suppress A549 xenograft tumor growth and metastasis in zebrafish.

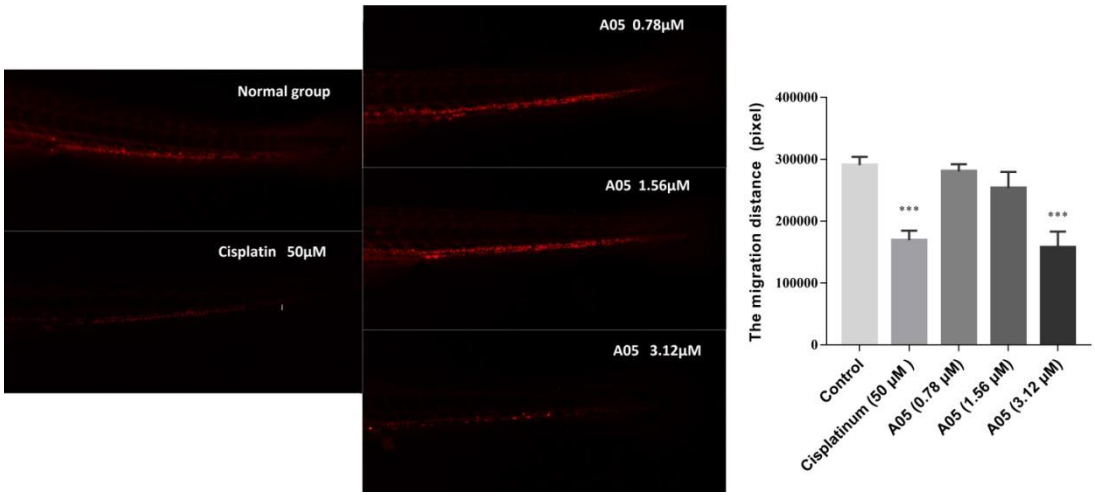


Fig. 8. A549 xenograft migration distance in zebrafish treated with **A05**. Images were taken via fluorescence microscopy and analyzed using the ImageJ advanced image processing software.

Wild-type AB zebrafish were additionally utilized as an animal model for minimum toxic concentration (MTC) testing. These animals were treated with a range

of **A05** concentrations (0.78, 1.56, 3.12, 6.25, 12.5, or 25 μM) in water, and **A05** toxicity was then assessed (Table 2). No precipitation, death, or toxicity phenotypes were observed at a concentration of 3.12 μM, suggesting that this was the MTC for compound **A05** in zebrafish.

Table 2. The relationship between concentration and lethality in wild-type AB zebrafish treated with **A05**.

Group (n=180)	Concentration (μM)	Number of deaths (tail)	mortality rate (%)	Toxic phenotype
Normal group*	-	0	0	Normal
A05	1.56	0	0	Normal
A05	3.12	0	0	Normal
A05	6.25	0	0	slightly precipitation
A05	12.5	0	0	obvious precipitation
A05	25.0	4	13	–

*****The number of zebrafish is thirty in each group.

3. Conclusion

In summary, by introducing different PROTAC substitutions at the C-3 and C-12 positions of natural protopanoxadiol, a series of novel ginsenoside AD-1 derivatives based PROTAC technology were designed and synthesized through a structure-based discovery approach. The majority of these new compounds were found to exhibit potent anti-tumor activities in five human cancer cell lines (PANC-1, HepG-2, A549, MCF-7, and HeLa). Among these PROTACs, the representative compound **A05** exhibited the most potent antitumor activity with an IC_{50} of 2.16 μM against A549 cells, superior to that of AD-1 or positive Mitomycin C. Furthermore, compound **A05** was found to repress MDM2 protein expression, in addition to disrupt interactions between p53 and MDM2. Mechanistic studies revealed that compound **A05** induced apoptotic death via the mitochondrial pathway. Importantly, the *in vivo* studies demonstrated that compound **A05** (at concentration of 3.12 μM) was able to

significantly suppress A549 xenograft tumor growth and cellular migration in zebrafish tumor model as compared to the control cisplatin (at concentration of 50 μM) treatment. Overall, these results thus provide a foundation for future efforts to develop novel PROTACs for the treatment of diverse cancers using natural ginsenosides.

4. Experimental

4.1 General Chemistry

AD-1 was obtained from Panax ginseng cultivated in China and the purity was more than 99.0% in HPLC analysis. All solvents and reagents used in this study were obtained from commercial sources and utilized without any additional purification. Reaction progress was monitored via TLC on pre-coated silica gel plates (silica gel 60 F254), with spots being detected via UV or other appropriate stains. A Bruker spectrometer was used to detect ^{1}H (400 MHz) spectra, with TMS serving as an internal standard. Coupling constants (*J*) are reported in Hz. ESI-MS was used to generate ordinary and high-resolution mass spectra. Semi-preparative reverse phase HPLC (column: Phenomenex luna C18 150 * 25 mm *10 μm) was used to purify synthesized compounds (silica gel, 200-300 mesh).

Synthesis of compound 2. To a mixture of AD-1 (2 g, 4.06 mmol, 1 eq) and diacetoxyrhodium (125 mg, 0.28 mmol, 0.07 eq) in dichloromethane (30 mL) was added a solution of ethyl 2-diazoacetate (1.16 g, 10.15 mmol, 1.1 mL, 2.5 eq) in dichloromethane (10 mL) dropwise. The mixture was stirred at 25°C for 12 h. Thin layer chromatography (petroleum ether: ethyl acetate = 2:1) showed the reactant (R_f = 0.10) was not consumed and one new major spot (R_f = 0.25) was detected. Water (50 mL) was poured into the mixture and stirred for 1 min. The aqueous phase was extracted with dichloromethane (50 mL × 2). The combined organic phase was washed with brine (50 mL × 2), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. The residue was purified by Flash silica gel column chromatography (ethyl acetate/ petroleum ether = 10%~40%). The product compound **2** (1.01 g, 1.75 mmol, 43% yield) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.23-4.18 (m, 2H), 4.16-4.05 (m, 2H), 3.65-3.55 (m, 1H), 3.18-3.16 (m, 3H),

2.90 (dd, *J* = 3.6, 11.6 Hz, 1H), 2.22-0.67 (m, 53H). MS(ESI) m/z 579.5 $[M + H]^+$.

Synthesis of compound 3. To a mixture of **2** (1.01 g, 1.74 mmol, 1 eq), water (10 mL) and tetrahydrofuran (15 mL) in methanol (20 mL) was added lithium hydroxide (209 mg, 8.72 mmol, 5 eq) in one portion at 25°C. The mixture was stirred at 25°C for 12 h. The mixture was concentrated and then poured into aqueous hydrochloric acid (1 M, 10 mL) and stirred for 1 min. The aqueous phase was extracted with dichloromethane (30 mL × 2). The combined organic phase was washed with brine (30 mL × 2), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. The product compound **3** (0.782 g, 1.42 mmol, 81% yield) was obtained as a white solid, which was directly used into the next step without further purification. MS(ESI) m/z 551.4 $[M + H]^+$.

Synthesis of compound A01. To a mixture of **3** (70 mg, 0.13 mmol, 1 eq) and diisopropylethylamine (66 mg, 0.51 mmol, 4 eq) in dimethylformamide (2 mL) was added 1-hydroxybenzotriazole (21 mg, 0.15 mmol, 1.2 eq) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (29 mg, 0.15 mmol, 1.2 eq) in one portion at 25°C. The mixture was stirred at 25°C for 5 min, then 3-[5-(5-aminopentoxy)-1-oxo-isoindolin-2-yl] piperidine-2,6-dione (53.38 mg, 0.14 mmol, 1.1 eq, hydrochloride) was added, the mixture was stirred at 25°C for 11 h and 55 min. 0.5 mL of acetonitrile was added and the solution was filtered. The residue was purified by Semi-preparative reverse phase HPLC (column: Phenomenex luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 63%-93%, 11 min). The product **A01** (14.8 mg, 0.02 mmol, 13% yield) was obtained as a white solid. ^{1}H NMR: (400 MHz, DMSO-d_6) δ 10.97 (s, 1H), 7.61 (d, *J* = 8.4 Hz, 1H), 7.37 (t, *J* = 6.4 Hz, 1H), 7.15 (d, *J* = 2.0 Hz, 1H), 7.03 (dd, *J* = 2.0, 8.4 Hz, 1H), 5.85 (s, 1H), 5.71 (s, 1H), 5.07 (dd, *J* = 5.2, 13.2 Hz, 1H), 4.42-4.22 (m, 2H), 4.06-4.00 (m, 2H), 3.99-3.90 (m, 2H), 3.76 (d, *J* = 14.4 Hz, 1H), 3.19-3.10 (m, 2H), 3.04 (s, 3H), 2.92-2.82 (m, 3H), 2.60 (d, *J* = 2.0 Hz, 2H), 2.43-2.36 (m, 1H), 2.04-1.96 (m, 1H), 1.90 (d, *J* = 6.8 Hz, 1H), 1.74 (d, *J* = 6.0 Hz, 3H), 1.60 (d, *J* = 10.4 Hz, 3H), 1.45 (s, 3H), 1.37 (s, 3H), 1.34-1.30 (m, 6H), 1.21 (s, 3H), 1.06 (s, 6H), 1.02 (s, 1H), 0.97 (s, 3H), 0.92 (s, 6H), 0.82 (s, 7H), 0.77 (s, 3H), 0.73 (s, 3H), 0.67 (s, 1H).

^{13}C NMR (150 MHz, DMSO-d_6) δ 172.9, 171.2, 169.3, 167.9, 161.8, 144.4, 124.3, 124.0, 115.4, 108.5, 87.1, 86.3, 73.9, 72.2, 69.7, 69.0, 67.9, 66.3, 55.6, 55.4, 53.4, 51.5, 49.5, 49.3, 48.4, 48.4, 48.0, 47.4, 42.6, 40.1, 38.5, 38.0, 36.5, 35.4, 34.4, 31.2, 30.6, 28.9, 28.2, 27.9, 26.5, 25.7, 24.9, 24.8, 22.9, 22.2, 17.7, 16.8, 16.3, 15.9, 15.4. MS (ESI) m/z: 878.5 $[M + H]^+$.

Synthesis of compound A02. Compound **A02** was synthesized by compound **3** and 3-[5-(7-aminoheptoxy)-1-oxo-isoindolin-2-yl] piperidine-2,6-dione from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 80%-100%, 10 min). Yellow solid (39.7 mg, 38% yield). ^{1}H NMR: (400 MHz, DMSO-d_6) δ 10.93 (s, 1H), 7.61 (d, J = 8.4 Hz, 1H), 7.29 (t, J = 5.6 Hz, 1H), 7.14 (s, 1H), 7.03 (dd, J = 1.6, 8.4 Hz, 1H), 5.89-5.72 (m, 1H), 5.71-5.53 (m, 1H), 5.06 (dd, J = 5.2, 13.2 Hz, 1H), 4.48-4.20 (m, 2H), 4.05 (t, J = 6.4 Hz, 2H), 3.97-3.70 (m, 2H), 3.39 (s, 1H), 3.17-3.08 (m, 2H), 3.05 (d, J = 2.0 Hz, 3H), 2.97-2.83 (m, 2H), 2.63-2.56 (m, 1H), 2.44-2.35 (m, 1H), 2.03-1.86 (m, 2H), 1.80-1.53 (m, 8H), 1.48 (s, 9H), 1.37 (s, 2H), 1.35-1.28 (m, 7H), 1.23 (d, J = 8.4 Hz, 3H), 1.19-1.09 (m, 2H), 1.06 (d, J = 2.0 Hz, 6H), 1.00 (d, J = 17.6 Hz, 3H), 0.92 (d, J = 8.8 Hz, 7H), 0.82 (s, 6H), 0.77 (s, 3H), 0.71 (d, J = 11.2 Hz, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.9, 171.2, 169.2, 167.9, 161.9, 144.4, 124.3, 124.0, 115.3, 108.6, 87.0, 73.9, 72.2, 69.8, 69.7, 69.0, 68.0, 55.5, 53.4, 51.5, 51.0, 50.9, 49.5, 49.3, 48.4, 48.4, 47.4, 47.0, 42.6, 40.4, 40.1, 38.5, 38.1, 38.0, 36.5, 35.4, 34.3, 31.2, 30.6, 29.1, 28.5, 28.4, 27.9, 26.3, 25.7, 25.4, 24.9, 22.5, 22.2, 17.7, 16.8, 15.9, 15.3. MS (ESI) m/z: 906.7 $[M + H]^+$.

Synthesis of compound 5. To a mixture of AD-1 (480 mg, 0.97 mmol, 1 eq) in tetrahydrofuran (10 mL) was added sodium hydride (78 mg, 1.95 mmol, 60% purity, 2 eq) batchwise at 0°C. And then benzyl 2-bromoacetate (335 mg, 1.46 mmol, 1.5 eq) was added. The mixture was stirred at 50°C for 4 hours. The residue was poured into aqueous sulfuric acid (1 N, 40 mL) and stirred for 1 min. The aqueous phase was extracted with ethyl acetate (30 mL × 2). The combined organic phase was washed

with brine (30 mL × 2), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. The residue was purified by Flash silica gel column chromatography (ethyl acetate/ petroleum ether = 0%~30%).The product **5** (240 mg, 0.37 mmol, 38% yield) was obtained as a colorless solid. ^{1}H NMR: (400 MHz, DMSO-d_6) δ 7.43-7.29 (m, 5H), 5.27-5.21 (m, 1H), 5.16-5.11 (m, 1H), 5.10-4.85 (m, 1H), 4.34-4.19 (m, 1H), 4.15-4.00 (m, 1H), 3.44 (qd, *J* = 5.2, 10.4 Hz, 1H), 3.27 - 3.15 (m, 4H), 2.24-2.06 (m, 1H), 1.98-1.82 (m, 3H), 1.58-1.52 (m, 3H), 1.49-1.41 (m, 6H), 1.39-1.24 (m, 6H), 1.22-1.09 (m, 11H), 1.05 (dd, *J* = 8.8, 11.6 Hz, 1H), 0.98 (s, 7H), 0.91-0.83 (m, 7H), 0.78 (s, 3H), 0.72 (d, *J* = 10.0 Hz, 1H). MS: (ESI) m/z: 641.5 $[M + H]^+$.

Synthesis of compound 6. To a solution of **5** (240 mg, 0.37 mmol, 1 eq) in tetrahydrofuran (10 mL) and methanol (10 mL) was added pallidum on activated carbonate (200 mg, 5% purity) under nitrogen atmosphere. The suspension was degassed under vacuum and purged with hydrogen several times. The mixture was stirred under hydrogen atmosphere (50 psi) at 30°C for 12 h. The reaction mixture was filtered and the filter was concentrated. The product **6** (206 mg, 0.37 mmol, 99% yield) was obtained as a white solid, which was directly used into the next step without further purification. MS: (ESI) m/z: 551.4 $[M + H]^+$.

Synthesis of compound 9. To a mixture of tert-butyl 5-amino-4-(5-hydroxy-1-oxo-isoindolin-2-yl)-5-oxo-pentanoate (200 mg, 0.60 mmol, 1 eq) and potassium carbonate (165 mg, 1.20 mmol, 2 eq) in dimethylformamide (3 mL) was added tert-butyl *N*-[2-(2-bromoethoxy)ethyl]carbamate (192 mg, 0.72 mmol, 1.2 eq) in one portion at 25 °C. The mixture was stirred at 80 °C for 2 hours. 40 mL of water was added into the mixture. The aqueous phase was extracted with ethyl acetate (30 mL × 3). The combined organic phase was washed with brine (30 mL × 3), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. The residue was purified by Flash silica gel chromatography (methanol/ dichloromethane = 0%~7%). The product **9** (311 mg, 0.55 mmol, 92% yield) was obtained as a light yellow oil. MS (ESI) *m/z*: 522.2$[M + H]^+$.

Synthesis of compound 11. To a mixture of **9** (311 mg, 0.55 mmol, 92% purity, 1 eq)

in acetonitrile (25 mL) was added benzenesulfonic acid (260 mg, 1.65 mmol, 3 eq) in one portion at 25°C. The mixture was stirred at 80°C for 12 h. The mixture was concentrated in vacuum. The residue was dissolved in 4 mL dimethylformamide and water (v:v = 1:3). The solution was purified by Semi-preparative reverse phase HPLC (column: Phenomenex Luna C18, 200*40 mm, 10 μm; mobile phase: [water (hydrochloric acid)-acetonitrile]; B%: 1%-24%, 10 min). The product **11** (277 mg, 0.55 mmol) was obtained as a colorless solid. MS (ESI) m/z: 348.1 $[M + H]^+$.

Synthesis of compound A03. Compound **A03** was synthesized by compound **3** and 3-[5-[2-[2-(benzenesulfonic acid amino) ethoxy] ethoxy]-1-oxo-isoindolin-2-yl] piperidine-2,6-dione from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 57%-87%, 10 min). White solid (27.7 mg, 27% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.95 (s, 1H), 8.46 (s, 1H), 7.62 (d, J = 8.4 Hz, 1H), 7.33 (t, J = 5.6 Hz, 1H), 7.16 (s, 1H), 7.05 (dd, J = 2.0, 8.4 Hz, 1H), 5.90-5.51 (m, 2H), 5.07 (dd, J = 4.8, 13.2 Hz, 1H), 4.43-4.22 (m, 2H), 4.20-4.13 (m, 2H), 3.94 (d, J = 14.8 Hz, 1H), 3.84-3.71 (m, 3H), 3.53 (t, J = 5.6 Hz, 2H), 3.05 (d, J = 2.4 Hz, 3H), 2.96-2.82 (m, 2H), 2.62-2.56 (m, 2H), 2.38 (dd, J = 4.4, 13.2 Hz, 1H), 2.03-1.94 (m, 1H), 1.92-1.85 (m, 1H), 1.77-1.64 (m, 3H), 1.63-1.55 (m, 2H), 1.49-1.29 (m, 12H), 1.25-1.13 (m, 4H), 1.06 (d, J = 2.4 Hz, 6H), 1.02 (s, 1H), 0.98 (s, 1H), 0.95-0.84 (m, 9H), 0.82-0.77 (m, 6H), 0.74-0.66 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.9, 171.1, 169.5, 167.9, 161.7, 144.4, 124.3, 124.2, 115.4, 108.7, 87.1, 73.9, 72.2, 72.1, 69.8, 69.7, 69.1, 68.8, 68.5, 67.7, 55.4, 53.4, 51.5, 51.0, 49.5, 49.3, 48.4, 48.4, 47.4, 47.0, 42.6, 40.4, 40.1, 38.4, 38.1, 37.9, 36.5, 34.3, 31.2, 30.6, 27.9, 26.5, 25.7, 24.9, 22.6, 17.7, 16.8, 16.2, 15.8, 15.3. MS (ESI) m/z: 880.6 $[M + H]^+$.

Synthesis of compound 13. To a mixture of tert-butyl *N*-[2-[2-(2-hydroxyethoxy)ethoxy]ethyl] carbamate (1 g, 4.01 mmol, 1 eq) and triethylamine (1.22 g, 12.03 mmol, 1.7 mL, 3 eq) in dichloromethane (20 mL) was added4-methylbenzenesulfonyl chloride (994 mg, 5.21 mmol, 1.3 eq) in one portion at 25°C. The mixture was stirred at 25°C for 12 h. Thin layer chromatography

(petroleum ether: ethyl acetate = 3:1) showed the reactant (R_f = 0.10) was not consumed and one new major spot (R_f = 0.30) was detected. Water (30 mL) was poured into the mixture and stirred for 1 min. The aqueous phase was extracted with dichloromethane (30 mL × 2). The combined organic phase was washed with brine (30 mL × 2), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. The residue was purified by Flash silica gel chromatography (ethyl acetate/petroleum ether = 0%~30%). The product **13** (1.18 g, 2.92 mmol, 73% yield) was obtained as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 8.0 Hz, 2H), 4.94 (s, 1H), 4.22-4.15 (m, 2H), 3.75-3.66 (m, 2H), 3.62-3.54 (m, 4H), 3.51 (t, J = 5.2 Hz, 2H), 3.30 (d, J = 4.8 Hz, 2H), 2.46 (s, 3H), 1.45 (s, 9H). MS (ESI) m/z: 426.1 $[M + Na]^+$.

Synthesis of compound 15. Compound **15** was synthesized by compound **13** and compound **14** from following the general procedure for preparing compound **9**. A light yellow oil (507 mg, 98% yield). MS (ESI) m/z: 566.3 $[M + H]^+$.

Synthesis of compound 16. Compound **16** was synthesized by compound **15** and compound **10** from following the general procedure for preparing compound **11**. A colorless solid.^{1}H NMR (400 MHz, DMSO-d_6) δ 10.95 (s, 1H), 7.84 (d, J = 0.8 Hz, 2H), 7.68-7.53 (m, 3H), 7.31 (d, J = 4.4 Hz, 3H), 7.18 (s, 1H), 7.06 (d, J = 8.0 Hz, 1H), 5.14-5.01 (m, 1H), 4.41-4.24 (m, 2H), 4.20 (s, 2H), 3.79 (s, 2H), 3.67-3.57 (m, 3H), 3.04-2.85 (m, 3H), 2.04-1.92 (m, 1H). MS (ESI) m/z: 392.2 $[M + H]^+$.

Synthesis of compound A04. Compound **A04** was synthesized by compound **3** and compound **16** from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex luna C18, 150*25 mm, *10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 54%-84%, 10 min). White solid (15.1 mg, 13% yield).^{1}H NMR (400 MHz, DMSO-d_6) δ 10.97 (s, 1H), 7.62 (d, J = 8.4 Hz, 1H), 7.30 (t, J = 5.6 Hz, 1H), 7.17 (d, J = 1.6 Hz, 1H), 7.06 (dd, J = 2.0, 8.4 Hz, 1H), 5.90-5.74 (m, 1H), 5.73-5.54 (m, 1H), 5.07 (dd, J = 5.2, 13.2 Hz, 1H), 4.43-4.22 (m, 2H), 4.20-4.12 (m, 2H), 3.95 (d, J = 14.8 Hz, 1H), 3.82-3.73 (m, 3H), 3.62-3.53 (m, 4H), 3.47-3.43 (m, 2H), 3.07-3.02 (m, 3H), 2.96-2.84 (m, 2H), 2.61 (s, 2H), 2.38 (dd, J = 4.0, 13.2 Hz,

1H), 2.03-1.94 (m, 1H), 1.92-1.85 (m, 1H), 1.76-1.66 (m, 3H), 1.63-1.54 (m, 2H), 1.49-1.39 (m, 5H), 1.38-1.27 (m, 8H), 1.25-1.12 (m, 4H), 1.07-1.05 (m, 6H), 1.03-0.96 (m, 4H), 0.91 (d, *J* = 11.6 Hz, 8H), 0.80 (d, *J* = 6.8 Hz, 6H), 0.75 (s, 2H), 0.72-0.67 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.9 171.1, 169.4, 167.9, 161.7, 144.4, 124.3, 124.2, 115.4, 108.6, 87.1, 73.9, 72.2, 72.1, 70.0, 69.8, 69.7, 69.6, 69.0, 68.8, 68.8, 67.7, 55.4, 53.4, 51.5, 51.0, 49.5, 49.3, 48.4, 48.4, 47.4, 47.0, 42.6, 40.4, 40.1, 38.4, 38.1, 37.9, 36.5, 34.3, 31.2, 30.6, 27.9, 26.5, 25.7, 24.9, 22.5, 17.7, 16.8, 16.3, 15.9, 15.3. MS (ESI) m/z: 924.4 $[M + H]^+$.

Synthesis of compound A05. Compound **A05** was synthesized by compound **3** and 3-[1-oxo-5-[4-(4-piperidylmethyl)piperazin-1-yl]isoindolin-2-yl]piperidine-2,6-dione from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex Luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 31%-61%, 10 min). White solid (58.0 mg, 48% yield). ^{1}H NMR: (400MHz, DMSO-d_6) δ 10.95 (s, 1H), 7.59-7.44 (m, 1H), 7.07 (s, 2H), 5.87-5.75 (m, 1H), 5.72-5.56 (m, 1H), 5.05 (dd, *J* = 5.2, 13.2 Hz, 1H), 4.35-4.12 (m, 4H), 4.06-3.84 (m, 2H), 3.28 (s, 4H), 3.04 (d, *J* = 2.4 Hz, 3H), 3.01-2.83 (m, 3H), 2.63-2.53 (m, 7H), 2.41-2.35 (m, 1H), 2.18 (d, *J* = 1.2 Hz, 2H), 1.98-1.84 (m, 2H), 1.83-1.64 (m, 6H), 1.62-1.55 (m, 1H), 1.50-1.26 (m, 12H), 1.25-1.18 (m, 2H), 1.15-1.11 (m, 1H), 1.06 (d, *J* = 2.0 Hz, 7H), 1.02 (s, 1H), 1.01-0.85 (m, 12H), 0.82 (s, 6H), 0.75-0.63 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.9, 171.3, 168.3, 167.3, 163.0, 144.0, 123.8, 114.7, 108.5, 86.3, 73.9, 72.1, 69.7, 68.8, 55.5, 53.4, 52.7, 51.4, 51.0, 50.9, 49.5, 49.3, 48.5, 48.4, 48.0, 47.4, 47.0, 44.7, 42.6, 41.0, 40.4, 40.1 38.6, 38.5, 38.2, 36.8, 36.5, 35.4, 34.4, 31.3, 31.1, 30.6, 30.0, 28.1, 26.5, 25.7, 24.9, 24.8, 22.6, 22.2, 17.7, 17.5, 17.0, 16.8, 16.5, 15.4. MS (ESI) m/z: 958.4 $[M + H]^+$.

Synthesis of compound 18. Compound **18** was synthesized by compound **17** from following the general procedure for preparing compound **13**. A colorless oil (1.47 g, 83% yield). ^{1}H NMR: (400MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.4 Hz, 2H), 7.36 (d, *J* = 8.0 Hz, 2H), 4.48 (s, 1H), 4.03 (t, *J* = 6.4 Hz, 2H), 3.07 (q, *J* = 6.0 Hz, 2H), 2.46 (s, 3H), 1.71-1.63 (m, 2H), 1.44 (s, 11H), 1.39-1.31 (m, 2H). MS (ESI) m/z: 380.1 $[M + Na]^+$.

Synthesis of compound 20. Compound **20** was synthesized by compound **18** and compound **19** from following the general procedure for preparing compound **9**. The product was further purified by Semi-preparative reverse phase HPLC (column: Phenomenex Luna C18, 200*40 mm, 10 μm; mobile phase: [water (formic acid) - acetonitrile]; B%: 38%-68%, 10 min). Saturated aqueous sodium bicarbonate was added to adjust the pH = 9. The aqueous phase was extracted with ethyl acetate (60 mL × 2). The combined organic phase was washed with brine (60 mL × 2), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. A colorless oil (0.28 g, 56% yield).

Synthesis of compound 21. To a mixture of **20** (0.28 g, 0.61 mmol, 1 eq) in dichloromethane (8 mL) was added hydrochloric acid (4 M in dioxane, 3 mL, 20 eq) in one portion at 25°C. The mixture was stirred at 25°C for 2 h. The mixture was concentrated in vacuum. The product **20** (0.269 g, crude, hydrochloride) was obtained as a white solid, which was directly used into the next step without further purification. MS (ESI) m/z: 360.1 $[M + H]^+$.

Synthesis of compound A06. Compound **A06** was synthesized by compound **3** and compound **21** from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex Luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 64%-94%, 10 min). White solid (19.1 mg, 17% yield). ^{1}H NMR:(400 MHz, DMSO-d_6) δ 11.11 (s, 1H), 7.83 (d, J = 8.4 Hz, 1H), 7.41 (d, J = 2.0 Hz, 1H), 7.39-7.31 (m, 2H), 5.84 (d, J = 2.4 Hz, 1H), 5.70 (s, 1H), 5.11 (dd, J = 5.2, 12.8 Hz, 1H), 4.16 (t, J = 6.4 Hz, 2H), 3.98-3.71 (m, 2H), 3.21-3.10 (m, 2H), 2.98-2.78 (m, 3H), 2.63-2.57 (m, 2H), 2.08-2.01 (m, 1H), 1.93-1.86 (m, 1H), 1.81-1.66 (m, 6H), 1.62-1.54 (m, 2H), 1.51-1.39 (m, 10H), 1.35-1.26 (m, 7H), 1.23-1.12 (m, 4H), 1.06 (s, 6H), 0.97 (s, 3H), 0.92 (d, J = 8.0 Hz, 7H), 0.82 (s, 6H), 0.77 (s, 3H), 0.75-0.66 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.8, 169.9, 169.3, 166.9, 166.8, 164.1, 133.9, 125.3, 122.9, 120.7, 108.9, 87.1, 73.9, 72.1, 70.0, 69.0, 68.7, 55.4, 51.0, 49.5, 49.3, 49.0, 48.4, 48.3, 48.0, 42.6, 40.1, 38.5, 38.1, 37.9, 36.5, 34.3, 31.1, 31.0, 30.6, 28.8, 28.0, 27.9, 25.7, 24.9, 24.8, 22.8, 22.3, 22.2, 22.1,

17.7, 17.0, 16.8, 16.3, 15.9, 15.3. MS (ESI) m/z: 892.3 $[M + H]^+$.

Synthesis of compound 23. To a mixture of 7-aminoheptan-1-ol (1 g, 7.62 mmol, 1 eq) in dichloromethane (30 mL) was added triethylamine (1.54 g, 15.24 mmol, 2 mL, 2 eq) and di-tert-butyl dicarbonate (2.49 g, 11.43 mmol, 1.5 eq) in one portion at 0°C. The mixture was stirred at 25°C for 2 h. Thin layer chromatography (petroleum ether : ethyl acetate = 1:1) showed the reactant (R_f = 0.75) was detected. The residue was poured into water (10 mL) and stirred for 1 min. The aqueous phase was extracted with dichloromethane (20 mL × 2). The combined organic phase was washed with brine (30 mL × 2), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. The residue was purified by Flash silica gel chromatography (ethyl acetate/petroleum ether = 0%~40%). The product **23** (1.62 g, 7.01 mmol, 92% yield) was obtained as a light colorless oil. ^{1}H NMR:(400 MHz, $CDCl_3$) δ 4.50 (d, J = 1.6 Hz, 1H), 3.65 (t, J = 6.4 Hz, 2H), 3.11 (s, 2H), 1.57 (td, J = 6.8, 14.0 Hz, 2H), 1.50-1.44 (m, 12H), 1.35 (s, 6H). MS:(ESI) m/z: 254.1 $[M + Na]^+$.

Synthesis of compound 24. Compound **24** was synthesized by compound **23** from following the general procedure for preparing compound **18**. A colorless oil (1.87 g, 67% yield). MS (ESI) m/z: 408.1 $[M + Na]^+$.

Synthesis of compound 26. To a mixture of dimethyl 4-hydroxybenzene-1,2-dicarboxylate (200 mg, 0.95 mmol, 1 eq) and **24** (367 mg, 0.95 mmol, 1 eq) in dimethylformamide (7 mL) was added cesium carbonate (620 mg, 1.90 mmol, 2 eq) in one portion at 25°C. The mixture was stirred at 80°C for 12 h. The mixture was poured into ice-water (w/w = 1/1) (10 mL) and stirred for 1 min. The aqueous phase was extracted with ethyl acetate (30 mL × 2). The combined organic phase was washed with brine (30 mL × 2), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. The product **26** (0.338 g, 0.78 mmol, 82% yield) was obtained as a colorless oil, which was directly used into the next step without further purification. MS: (ESI) m/z: 424.1 $[M + H]^+$.

Synthesis of compound 28. To a solution of **26** (338 mg, 0.78 mmol, 98% purity, 1 eq) in pyridine (5 mL) was added lithium iodide (1.05 g, 7.82 mmol, 0.3 mL, 10 eq) and 3-aminopiperidine-2,6-dione (257 mg, 1.56 mmol, 2 eq, hydrochloride). The

mixture was stirred at 130°C for 12 h. The mixture was concentrated in vacuum. Aqueous hydrochloric acid (1 N) was added to adjust the pH = 4. Water (50 mL) was poured into the mixture and stirred for 1 min. The aqueous phase was extracted with tetrahydrofuran (60 mL × 3). The combined organic phase was washed with brine (60 mL × 2), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. The oil was purified by Semi-preparative reverse phase HPLC (column: Phenomenex luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 5%-35%, 10 min). Saturated aqueous sodium bicarbonate was added to adjust the pH = 9. Water (50 mL) was poured into the mixture and stirred for 1 min. The aqueous phase was extracted with tetrahydrofuran (80 mL × 3). The combined organic phase was washed with brine (80 mL × 2), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum.The product **28** (94 mg, 0.19 mmol, 25% yield) was obtained as a colorless oil.

Synthesis of compound 29. Compound **29** was synthesized by compound **28** from following the general procedure for preparing compound **21**. White solid (80 mg, 98% yield, hydrochloride), which was directly used into the next step without further purification. MS:(ESI) *m/z*: 388.1 $[M + H]^+$.

Synthesis of compound A07. Compound **A07** was synthesized by compound **3** and compound **29** from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex Luna C18, 100*30 mm, 5 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 80%-100%, 8 min). White solid (16.3 mg, 18% yield). ^{1}H NMR: (400 MHz, DMSO-d_6) δ 11.09 (s, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.41 (d, J = 2.0 Hz, 1H), 7.37-7.27 (m, 2H), 5.89-5.73 (m, 1H), 5.72-5.55 (m, 1H), 5.34-5.08 (m, 1H), 4.16 (t, J = 6.4 Hz, 2H), 3.99-3.71 (m, 2H), 3.38 (s, 2H), 3.16-3.08 (m, 2H), 3.05 (d, J = 2.4 Hz, 3H), 2.92-2.82 (m, 2H), 2.61 (d, J = 2.4 Hz, 2H), 2.08-1.96 (m, 3H), 1.92-1.85 (m, 1H), 1.74 (dd, J = 8.0, 14.0 Hz, 4H), 1.65-1.55 (m, 3H), 1.48-1.39 (m, 10H), 1.35-1.30 (m, 6H), 1.24 (s, 4H), 1.06 (d, J = 2.4 Hz, 6H), 1.02 (s, 1H), 0.97 (s, 3H), 0.93 (s, 3H), 0.90 (d, J = 2.4 Hz, 4H), 0.82 (s, 7H), 0.77 (s, 2H), 0.73 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.8, 169.9, 169.2, 166.9, 166.8, 164.1, 134.0, 125.3,

122.9, 120.7, 108.8, 87.0, 73.9, 72.2, 69.8, 69.7, 69.0, 55.5, 51.0, 49.5, 49.3, 49.0, 48.4, 48.3, 48.0, 42.6, 40.1, 38.4, 38.1, 38.0, 36.5, 34.3, 31.3, 31.0, 30.6, 29.1, 28.8, 28.4, 27.9, 26.0, 25.3, 24.9, 24.8, 22.3, 22.2, 22.1, 17.7, 17.0, 16.8, 16.3, 15.9, 15.3, 14.0. MS: (ESI) m/z: 920.4 $[M + H]^+$.

Synthesis of compound 32. Compound **32** was synthesized by compound **31** from following the general procedure for preparing compound **21**. White solid (0.196 g, 89.7% yield), which was directly used into the next step without further purification.^{1}H NMR: (400 MHz, DMSO-d_6) δ 7.93-7.76 (m, 3H), 7.47 (d, J = 2.4 Hz, 1H), 7.38 (dd, J = 2.4, 8.4 Hz, 1H), 5.12 (dd, J = 5.6, 12.8 Hz, 1H), 4.43-4.29 (m, 2H), 3.84 (dd, J = 3.6, 5.2 Hz, 2H), 3.68 (t, J = 5.2 Hz, 2H), 3.01 (d, J = 4.4 Hz, 2H), 2.93-2.80 (m, 1H), 2.09-1.99 (m, 2H), 1.22-1.10 (m, 2H). MS: (ESI) m/z: 361.9 $[M + H]^+$.

Synthesis of compound A08. Compound **A08** was synthesized by compound **3** and compound **32** from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 58%-88%, 10 min). White solid (41.8 mg, 37% yield).^{1}H NMR (400 MHz, DMSO-d_6) δ 11.11 (s, 1H), 7.84 (d, J = 8.4 Hz, 1H), 7.43 (d, J = 2.0 Hz, 1H), 7.36 (dd, J = 2.0, 8.4 Hz, 1H), 7.32 (t, J = 6.0 Hz, 1H), 5.89-5.73 (m, 1H), 5.72-5.54 (m, 1H), 5.12 (dd, J = 5.2, 13.2 Hz, 1H), 4.33-4.25 (m, 2H), 3.99-3.74 (m, 4H), 3.53 (t, J = 6.0 Hz, 2H), 3.04 (d, J = 2.8 Hz, 3H), 2.96-2.82 (m, 2H), 2.63-2.57 (m, 2H), 2.09-2.00 (m, 1H), 1.94-1.84 (m, 1H), 1.77-1.65 (m, 3H), 1.63-1.56 (m, 2H), 1.46-1.27 (m, 13H), 1.25-1.11 (m, 4H), 1.06 (d, J = 2.8 Hz, 7H), 1.02 (s, 1H), 0.97 (s, 2H), 0.93-0.91 (m, 1H), 0.89 (s, 6H), 0.84-0.79 (m, 4H), 0.77 (s, 3H), 0.73-0.70 (m, 3H), 0.67 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.7, 169.9, 169.5, 166.8, 166.8, 163.9, 133.9, 125.3, 123.1, 120.8, 109.0, 87.1, 73.9, 72.2, 72.1, 69.7, 69.6, 69.0, 68.8, 68.5, 68.3, 55.4, 53.4, 51.0, 49.5, 49.3, 49.0, 48.4, 47.4, 47.0, 42.6, 40.4, 40.1, 38.4, 381, 37.9, 36.4, 34.3, 30.9, 30.6, 27.9, 26.5, 25.7, 24.9, 22.2, 17.7, 16.8, 16.2, 15.8, 15.3. MS: (ESI) m/z: 894.3 $[M + H]^+$.

Synthesis of compound 33. Compound **33** was synthesized by compound **30** and

compound **13** from following the general procedure for preparing compound **20**. A colorless oil (0.372 g, 58% yield). MS: (ESI) m/z: 528.1 $[M + Na]^+$.

Synthesis of compound 34. Compound **34** was synthesized by compound **33** from following the general procedure for preparing compound **21**. A light yellow oil (0.445 g, crude, hydrochloride), which was directly used into the next step without further purification. MS: (ESI) *m/z*: 406.0 $[M + H]^+$.

Synthesis of compound A09. Compound A09 was synthesized by compound **3** and compound **34** from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex Luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 60%-90%, 11 min). White solid (41.9 mg, 34% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.11 (s, 1H), 7.83 (d, J = 8.4 Hz, 1H), 7.45 (d, J = 2.0 Hz, 1H), 7.37 (dd, J = 2.0, 8.4 Hz, 1H), 7.29 (t, J = 5.6 Hz, 1H), 5.91-5.73 (m, 1H), 5.72-5.53 (m, 1H), 5.12 (dd, J = 5.6, 12.8 Hz, 1H), 4.34-4.23 (m, 2H), 3.95 (d, J = 14.8 Hz, 1H), 3.83-3.72 (m, 3H), 3.62-3.52 (m, 4H), 3.47-3.43 (m, 2H), 3.28 (d, J = 6.0 Hz, 2H), 3.04 (d, J = 2.8 Hz, 3H), 2.95-2.82 (m, 2H), 2.62-2.56 (m, 2H), 2.09-1.99 (m, 1H), 1.94-1.84 (m, 1H), 1.78-1.66 (m, 3H), 1.63-1.54 (m, 2H), 1.46-1.26 (m, 13H), 1.24-1.11 (m, 4H), 1.06 (d, J = 2.8 Hz, 6H), 1.02-0.97 (m, 3H), 0.90 (d, J = 11.6 Hz, 7H), 0.80 (d, J = 9.2 Hz, 6H), 0.74 (s, 3H), 0.69 (d, J = 11.2 Hz, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.7, 169.9, 169.4, 166.8, 166.8, 163.9, 133.9, 125.3, 123.1, 120.9, 108.9, 87.1, 73.9, 72.2, 72.1, 70.0, 69.8, 69.6, 69.6, 69.0, 68.8 68.7, 68.4, 55.4, 53.4, 51.0, 49.5, 49.3, 49.0, 48.4, 48.3, 47.4, 42.6, 40.4, 40.1, 38.4, 38.1, 37.9, 36.5, 34.3, 31.0, 30.6, 27.9, 26.5, 25.7, 24.9, 22.2, 17.7, 16.8, 16.3, 15.9, 15.3. MS: (ESI) m/z: 938.4 $[M + H]^+$.

Synthesis of compound 36. Compound **36** was synthesized by compound **35** from following the general procedure for preparing compound **21**. Yellow solid (515 mg, crude, hydrochloride), which was directly used into the next step without further purification. MS: (ESI) m/z: 361.0 $[M + H]^+$.

Synthesis of compound 38. To a mixture of **36** (515 mg, 1.30 mmol, 1 eq, hydrochloride) in dichloromethane (10 mL) and methanol (10 mL) was added sodium

acetate (426 mg, 5.19 mmol, 4 eq) in one portion at 25°C. The mixture was stirred at 25°C for 1 min. And then tert-butyl 4-formylpiperidine-1-carboxylate (277 mg, 1.30 mmol, 1 eq) was added in one portion at 25°C. The mixture was stirred at 25°C for 1 min. Then sodium triacetoxyborohydride (825 mg, 3.89 mmol, 3 eq) was added in one portion. The mixture was stirred at 25°C for 1 h. Saturated aqueous sodium bicarbonate was added to adjust the pH = 9. Water (30 mL) was poured into the mixture and stirred for 1 min. The aqueous phase was extracted with tetrahydrofuran (30 mL × 2). The combined organic phase was washed with brine (30 mL × 2), dried with anhydrous sodium sulfate, filtered and concentrated in vacuum. The residue was purified by flash silica gel column chromatography (ethyl acetate/petroleum ether = 10%-60%).The product **38** (469 mg, 0.84 mmol, 65% yield) was obtained as a yellow solid. MS: (ESI) m/z: 558.3 $[M + H]^+$.

Synthesis of compound 39. Compound **39** was synthesized by compound **38** from following the general procedure for preparing compound **21**. Light yellow solid (489 mg, crude, hydrochloride), which was directly used into the next step without further purification. MS: (ESI) m/z: 458.1 $[M + H]^+$.

Synthesis of compound A10. Compound **A10** was synthesized by compound **3** and compound **39** from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 30%-60%, 11 min). Yellow solid (32.6 mg, 26% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.11 (s, 1H), 7.73 (d, J = 11.6 Hz, 1H), 7.45 (d, J = 7.8 Hz, 1H), 5.88-5.74 (m, 1H), 5.73-5.57 (m, 1H), 5.10 (dd, J = 5.6, 12.8 Hz, 1H), 4.41-4.24 (m, 1H), 4.21-4.07 (m, 1H), 4.04-3.84 (m, 2H), 3.24 (s, 4H), 3.04 (d, J = 2.4 Hz, 3H), 3.02-2.83 (m, 3H), 2.63-2.54 (m, 8H), 2.19 (d, J = 6.4 Hz, 2H), 2.03 (td, J = 5.2, 10.4 Hz, 1H), 1.95-1.85 (m, 1H), 1.82-1.54 (m, 8H), 1.51-1.27 (m, 12H), 1.25-1.12 (m, 4H), 1.06 (d, J = 2.1 Hz, 7H), 1.03-0.96 (m, 4H), 0.96-0.89 (m, 8H), 0.82 (s, 6H), 0.73 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.8, 169.9, 167.3, 166.6, 166.2, 158.2, 156.5, 145.4, 128.8, 123.4, 108.5, 86.3, 73.9, 72.2, 72.1, 69.7, 68.8, 63.6, 55.5, 53.4, 52.9, 51.0, 49.6, 49.3, 48.4, 48.4, 48.0, 47.4, 44.8, 42.6, 41.1,

40.4, 40.1, 38.5, 38.2, 36.5, 36.4, 35.4, 34.4, 32.7, 31.1, 30.6, 30.1, 27.9, 26.5, 25.7, 24.9, 24.8, 22.2, 22.1, 17.7, 17.5, 17.0, 16.8, 16.5, 15.3. MS: (ESI) m/z: 990.5 $[M + H]^+$.

Synthesis of compound A11. Compound **A11** was synthesized by compound **6** and 3-[1-oxo-5-[4-(4-piperidylmethyl)piperazin-1-yl]isoindolin-2-yl]piperidine-2,6-dione from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 32%-62%, 8 min). White solid (69.5 mg, 61% yield). ^{1}H NMR (400M Hz, DMSO-d_6) δ 10.94 (s, 1H), 7.53 (d, J = 7.6 Hz, 1H), 7.08 (s, 2H), 5.05 (dd, J = 5.2, 13.2 Hz, 1H), 5.00-4.68 (m, 1H), 4.40-4.04 (m, 6H), 3.78-3.65 (m, 1H), 3.44-3.38 (m, 1H), 3.30-3.18 (m, 4H), 3.04 (s, 3H), 3.01-2.85 (m, 3H), 2.62-2.55 (m, 2H), 2.49-2.46 (m, 4H), 2.42-2.34 (m, 1H), 2.18 (d, J = 4.0 Hz, 1H), 2.07 (t, J = 4.4 Hz, 1H), 2.02-1.92 (m, 2H), 1.85-1.58 (m, 6H), 1.55-1.17 (m, 16H), 1.06 (s, 7H), 1.00-0.78 (m, 19H), 0.68 (s, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.9, 171.3, 168.3, 166.9, 163.0, 144.0, 123.8, 114.8, 108.5, 80.1, 79.8, 74.0, 71.8, 71.6, 66.0, 55.4, 51.5, 51.4, 49.8, 49.7, 49.4, 48.5, 48.4, 47.0, 46.5, 45.8, 42.8, 40.5, 40.2, 40.1, 38.6, 38.5, 36.8, 36.2, 34.3, 31.3, 30.7, 29.9, 28.1, 27.1, 26.9, 26.7, 26.3, 24.9, 24.9, 22.9, 22.8, 22.6, 17.9, 17.7, 17.2, 16.9, 16.9, 15.9, 15.8, 15.3. MS (ESI) m/z: 958.5 $[M + H]^+$.

Synthesis of compound A12. Compound **A12** was synthesized by compound **6** and 5-(5-aminopentoxy)-2-(2,6-dioxo-3-piperidyl)isoindoline- 1,3-dione from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex luna C18, 150*25 mm, 10 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 65%-95%, 8 min). Off-white solid (73 mg, 68% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.10 (s, 1H), 7.92-7.85 (m, 1H), 7.83 (d, J = 8.4 Hz, 1H), 7.41 (d, J = 2.0 Hz, 1H), 7.34 (dd, J = 2.0, 8.4 Hz, 1H), 6.93-6.60 (m, 1H), 5.11 (dd, J = 5.2, 12.8 Hz, 1H), 4.84-4.44 (m, 1H), 4.27 (d, J = 5.2 Hz, 1H), 4.16 (t, J = 6.4 Hz, 2H), 4.03-3.82 (m, 2H), 3.22-3.08 (m, 2H), 3.06-3.01 (m, 3H), 2.99-2.80 (m, 2H), 2.61 (d, J = 2.4 Hz, 2H), 2.09-2.01 (m, 1H), 1.99-1.89 (m, 2H), 1.86-1.70 (m, 4H), 1.59 (d, J = 13.2 Hz, 1H), 1.53-1.40 (m,

10H), 1.35 (s, 3H), 1.32-1.17 (m, 8H), 1.04 (s, 6H), 0.99 (s, 3H), 0.94 (s, 4H), 0.87 (s, 3H), 0.81 (d, *J* = 12.8 Hz, 6H), 0.72-0.62 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.8, 169.9, 168.8, 166.9, 166.8, 164.1, 139.2, 134.0, 128.0, 125.3, 120.7, 108.8, 80.7, 76.7, 73.9, 72.3, 72.1, 68.7, 67.5, 55.3, 51.6, 49.7, 49.4, 49.0, 48.4, 48.4, 46.2, 45.6, 40.1, 40.1, 38.6, 38.5, 38.0, 36.7, 34.4, 34.3, 31.0, 30.4, 28.9, 28.1, 25.7, 24.9, 22.7, 22.1, 21.0, 17.9, 17.1, 16.9, 16.0, 15.7, 15.3. MS(ESI) m/z: 892.6 $[M + H]^+$.

Synthesis of compound A13. Compound **A13** was synthesized by compound **6** and 2-(2,6-dioxo-3-piperidyl)-5-fluoro-6-[4-(4-piperidylmethyl)piperazin-1-yl]isoindoline -1,3-dione from following the general procedure for preparing compound **A01**. The product was purified by Semi-preparative reverse phase HPLC (column: Phenomenex Luna C18, 100*30 mm, 5 μm; mobile phase: [water (formic acid)-acetonitrile]; B%: 35%-65%, 8 min). Yellow solid (81.2 mg, 69% yield). ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 11.10 (s, 1H), 7.73 (d, *J* = 12.0 Hz, 1H), 7.45 (d, *J* = 6.4 Hz, 1H), 5.10 (dd, *J* = 5.2, 12.8 Hz, 1H), 5.01-4.63 (m, 1H), 4.42-3.98 (m, 4H), 3.80-3.64 (m, 1H), 3.48-3.38 (m, 1H), 3.25 (s, 4H), 3.07-3.03 (m, 3H), 3.02-2.83 (m, 3H), 2.63-2.52 (m, 6H), 2.19 (s, 2H), 2.10-1.92 (m, 3H), 1.85-1.59 (m, 6H), 1.53-1.18 (m, 16H), 1.06 (d, *J* = 3.2 Hz, 6H), 1.04-0.86 (m, 14H), 0.82 (d, *J* = 12.0 Hz, 6H), 0.72-0.64 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 172.7, 169.9, 166.9, 166.6, 166.2, 158.2, 156.5, 145.3, 128.8, 113.7, 111.9, 80.1, 76.7, 74.0, 71.8, 71.6, 66.5, 66.0, 63.6, 55.4, 53.3, 52.8, 51.5, 49.80,49.7, 49.4, 49.1, 48.4, 48.4, 46.5, 45.8, 42.8, 41.1, 40.6, 40.2, 40.1, 38.5, 36.8, 36.2, 34.3, 30.9, 30.7, 30.0, 28.1, 27.1, 26.9, 26.6, 24.9, 24.9, 22.2, 22.1, 17.9, 17.2, 15.9, 15.8, 15.3. MS(ESI) m/z: 990.5 $[M + H]^+$.

4.2 Cell culture

PANC-1, HepG-2, A549, MCF-7, and HeLa cells from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) were cultured in DMEM or RPMI-1640 containing 10% FBS and 1% penicillin/streptomycin in a 37°C 5% CO_2 incubator.

4.3 MTT assays

The cytotoxic effects of treatment with compounds **A01**-**A13**, AD-1, or Mitomycin C were examined via MTT assay. Briefly, tumor cells were added to 96-well plates

(5,000/well) for 24 h, after which they were treated for 48 h with a range of concentrations of these compounds (5, 10, 20, or 40 μM). Then MTT reagent (10 μL, 5 mg/mL) was added to each well. After a 4 h incubation, cell viability was measured via plate reader (Bio-Rad iMARK, USA).

4.4 Analyses of cellular morphology

A549 cells were added to 6-well plates (1×10^5/well) for 24 h, after which they were treated for 24 h with AD-1 or compound **A05**, with control cells instead being treated using 0.1% DMSO. After treatment, 4% formaldehyde was used to fix cells at 4°C for 1 h, followed by staining in the dark for 10 min with DAPI (5 μM) at 37°C. Cells were then imaged via fluorescence microscopy.

4.5 Analysis of apoptotic cell death

The apoptotic death of cells was analyzed using an Annexin V-FITC apoptosis assay. Briefly, A549 cells were added to 6-well plates (2×10^5/well) for 24 h, after which they were treated for 24 h with AD-1 or compound **A05** (0, 10, 20 μM). Apoptotic cells were then detected using a Cytomics FC500 Flow Cytometer (Beckman Coulter, USA).

4.6 Western immunoblotting

Cellular proteins were extracted using RIPA buffer, with cytosolic and mitochondrial protein fractions being separately isolated using a Mitochondria Isolation Kit. Lysates were centrifuged for 10 min at 750 xg at 4°C, after which supernatants were centrifuged for 15 min at 12,000 xg at 4°C to separate the cytosolic (supernatant) and mitochondrial (pellet) fractions. A BCA assay was used to detect protein concentrations in these samples, followed by the separation of 35μg protein per lane via 10% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes that were subsequently blocked using 5% non-fat milk for 1 h followed by probing with 1:1000 dilutions of primary antibodies specific for Cl-Caspase-9, Cl-Caspase-3, Cl-PRAP, Bax, cytochrome C, MDM2, P53, or β-actin (Cell Signaling Technology, Inc.). Blots were then rinsed in triplicate using TBST, and 1:5000 dilutions of anti-mouse or anti-rabbit IgG were used to probe blots for 1 h at room temperature. A chemiluminescence system (Eastman Kodak Company, USA) was

then used to detect protein bands with X-ray film. Data are means ± SD from triplicate experiments, and were compared via two-way ANOVAs with post hoc Student-Newman-Keuls (SNK) multiple comparison tests. $P < 0.05$ was the significance threshold.

4.7 Zebrafish xenograft model systems

Zebrafish were housed in a pathogen-free laboratory and all experimental protocols and facilities received AAALAC accreditation (Certification number: 001458), license number: SYXK (Zhejiang) 2022-0004. Zebrafish were raised in water at 28°C (water quality: 200 mg of instant sea salt was added per 1 L of reverse osmosis water; conductivity: 450-550 μS/cm; pH: 6.5-8.5; hardness: 50-100 mg/L $CaCO_3$).

Human A549 cells were labeled with CM-DiI and transplanted via microinjection into the yolk sac of wild-type AB zebrafish at 2 days post-fertilization (dpf). A total of 200 cells were transferred per zebrafish to establish a human lung cancer xenograft model, and fish were then incubated at 35°C for 3 dpf. Then, zebrafish with transplanted tumors were selected via microscopy and transferred at random into a 6-well plate (30/well). A range of compound **A05** doses in an aqueous solution was then added to each well, and fish were incubated for 48 h at 35℃, with cisplatin serving as a positive control. In total, 10 zebrafish per group were then selected for fluorescence imaging via microscopy, with data being collected in the NIS-Elements D 3.20 advanced image processing software. Fluorescence intensity values for tumors were measured to assess the suppression of tumor growth. The Image J advanced image processing software was also used to measure A549 cell migration as a means of assessing the anti-metastatic effects of compound **A05** treatment.

SPSS 26.0 was used to analyze data, which are reported as means ± SEM. $P < 0.05$ was the significance threshold.

4.8 Minimal toxic concentration (MTC) analyses

All zebrafish breeding and rearing were performed as described above. To assess MTC values, wild-type AB zebrafish were selected at random 3 dpf in 6-well plates. In total, 30 zebrafish were treated with aqueous solutions. A normal control group was also established. Each well could hold 3 mL of water. Fish were incubated for 48 h at

35°C, after which they were visually examined and the number of deaths was quantified.

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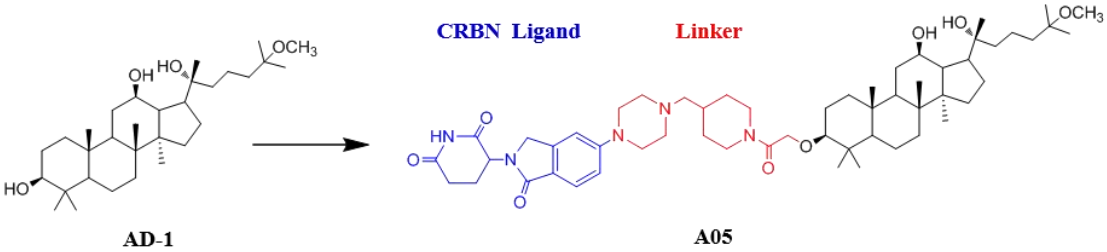
Graphical Abstract



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