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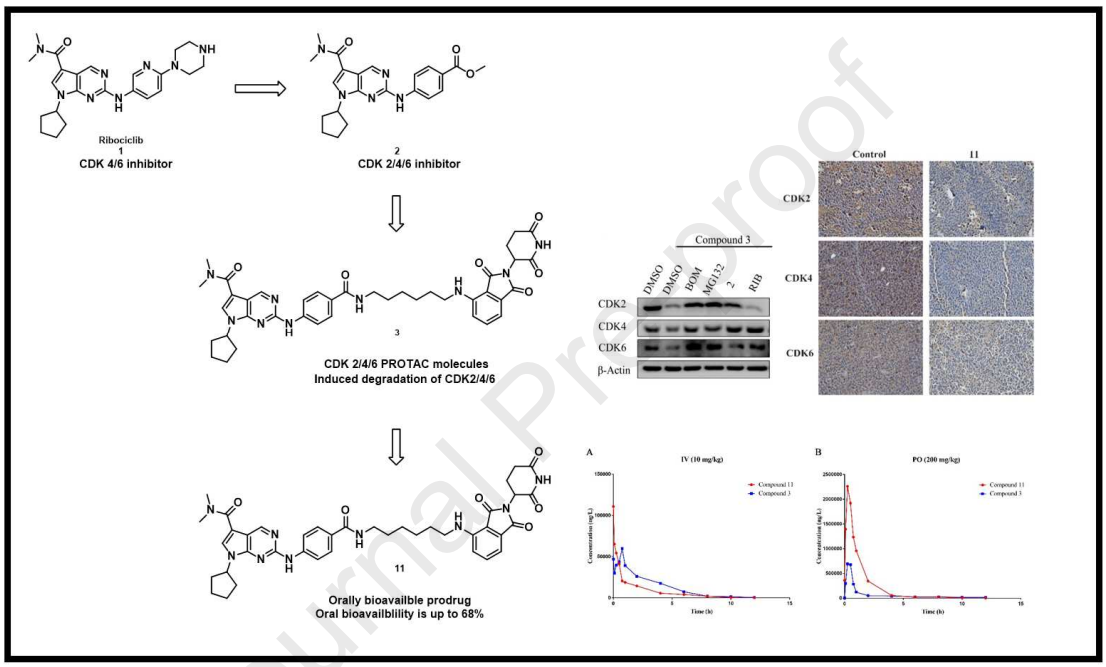


First Orally Bioavailable Prodrug of Proteolysis Targeting Chimera (PROTAC) Degrades Cyclin-Dependent Kinases 2/4/6 *in vivo*

Mingming Wei, [‡, a] Rui Zhao, [‡, a] Yuting Cao, [‡, a] Yujiao Wei, [‡, a] Ming Li, [‡, b] Zhiqiang Dong, [‡, a] Yulin Liu, [‡, a] Hao Ruan, [a] Ying Li, [a] Sheng Cao, [a] Zhiwen Tang, [a] Yuanyuan Zhou, [a] Wei Song, [a] Yubo Wang, [a] Jiefu Wang, [*, c] Guang Yang, [*, a] Cheng Yang [*, a]

a The State Key Laboratory of Medicinal Chemical Biology, College of Pharmacy, Nankai University, Tianjin, 300071, P.R. China.

b Cangzhou Institutes for Food and Drug Control, Cangzhou, 061000, P.R. China.

c Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Tianjin's Clinical Research Center for Cancer, Key Laboratory of Cancer Prevention and Therapy, Tianjin, 300060, P.R. China.

ABSTRACT

A growing number of reports suggested that the inhibitor targeting cyclin-dependent kinases (CDK) 2/4/6 can act as a more feasible chemotherapy strategy. In the present paper, a novel PROTAC molecule was developed based on the structure of Ribociclib's derivative. In malignant melanoma cells, the degrader can not only degrade CDK 2/4/6 simultaneously and effectively, but also remarkably induce cell cycle reset and apoptosis of melanoma cells. Moreover, PROTAC molecules with CRBN ligands always have poor oral bioavailability. We developed the orally bioavailable prodrug for the first time. It would provide general solution for oral administration of the PROTAC molecules, derived from CRBN ligands, for animal test conveniently.

Cyclin-dependent kinases (CDKs) are critical cellular enzymes in regulating eukaryotic cell division and cell proliferation. [1] CDKs catalytic units are activated by regulatory subunits, i.e., cyclins. At least 16 mammalian cyclins have been identified, among which Cyclin B/CDK1, cyclin A/CDK2, cyclin E/CDK2, cyclin D/CDK4 and cyclin D/CDK6 act as vital regulators of cell cycle progression. [2] Additional functions of cyclin/CDKs (e.g., transcription regulation, DNA repair, differentiation and apoptosis) have been reported as well. [3-5]

Enhanced activity or temporally abnormal activation of CDKs leads to the development of many types of tumors. Accordingly, cyclin-dependent kinases inhibitors have been suggested to be powerful in the treatment of cancer. [6] To be specific, CDK4/6 inhibitors have exhibited noticeably high clinical efficacy in breast and other cancers as single agents or combined with other therapeutics. [7] For instance, Palbociclib and Ribociclib have been approved by FDA for the treatment of HR-positive and HER2-negative advanced or metastatic breast cancer in combination with aromatase inhibitors. Nevertheless, with the development of primary or acquired drug resistance, CDK4/6 inhibitors are also limited over time.

Recent results revealed that over-expression of CDK2 and its complex with cyclins is highly correlated with abnormal regulation of cell-cycle. [8] The cyclin E/CDK2 complex is critical to regulate the G1/S transition, histone biosynthesis and centrosomes duplication. Through the progressive phosphorylation of Rb by cyclin D/CDK4/6 and cyclin E/CDK2, the G1 transcription factor, E2F, is released, which can significantly promote S-phase entry. [9] Activation of cyclin A/CDK2 during early S-phase also facilitates DNA replication and inactivation of E2F to

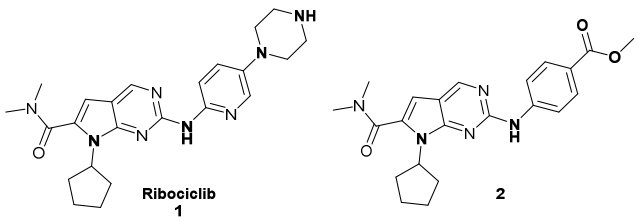
complete S-phase. [9] Cyclin E refers to the regulatory cyclin with CDK2. Recent studies have also reported that cyclin E/CDK2 inhibition restore sensitivity to tamoxifen or CDK4/6 inhibitors in breast cancer cells. [10, 11] Amplification or over-expression of cyclin E/CDK2 has long been related to poor outcomes in breast cancer [12-14] and other types of cancers. [15-18]

To the best of our knowledge, there has been no approved drug targeting CDK2 thus far. [19] However, two small molecules, Dinaciclib (MK-7965) and Seliciclib (CYC202), inhibiting CDK1/2/5/9 and CDK2/7/9, respectively, are being clinically developed for the treatment of advanced tumors as single reagents and in conjunction with chemotherapies. [19] Very recently, a novel molecule labeled as PF069736, a CDK2/4/6 inhibitor, has been recruited for phase I study now. In brief, it has been indicated that combination therapies targeting CDK2 are arousing huge attention by both medicinal and pharmaceutical areas. At this stage, a novel chemotherapeutical strategy was proposed by chemical knock-down of CDK2/4/6 simultaneously for tumor therapies.

The technique of Proteolysis-targeting chimeras (PROTACs) was first reported by Crews. [20] In practice, PROTAC molecules are hetero-bifunctional small molecules conjugated by a proper chemical linker. They can drag the target proteins close to an E3 ligase, thus causing consequent degradation of the target proteins by proteasome. Thus far, PROTAC molecules have become a valuable tool for the chemical knockdown of specific proteins for the treatment of tumor. Numerous inhibitors of target proteins (e.g., FLT3, [21] AR, [22] MDM2, [23] CDK6, [24] CDK9, [25] BRDs, [26] BET, [27] ALK, [28] as well as PARP-1, [29]) have been developed into PROTAC molecules.

Nevertheless, most of the previously reported PROTAC molecules only degrades single one target. There have been few publications on a PROTAC molecule, which can mitigate proteasomal down-regulation of multiple proteins simultaneously. In this paper, a novel compound was synthesized and screened by linking a derivative of Ribociclib and Pomalidomide. This PROTAC molecule can degrade CDK 2/4/6 in various cancer cells. It also down-regulated the phosphorylation of Rb, exhibiting potent anti-proliferation of malignant melanomas both *in vitro* and *in vivo*.

The structure of Ribociclib **1** was taken as the CDKs binding moiety for its high binding potency for CDK 4/6. [30] However, Ribociclib has no effect on inhibiting CDK2. Great efforts have been made to synthesize and screen appropriate analogue (data are not shown in this paper). Fortunately, core structure **2,** delivered from the straightforward structural modification of Ribociclib, exhibited enhanced potency for CDK2. The results of kinase activity assays suggested that compound **2**, provided significant improvement its binding capability to CDK 2, though its affinity to CDK 4/6 slightly decreased. We therefore hypothesized that an extended linker attached as amides will exert minimal effects on binding to CDK 2/4/6.



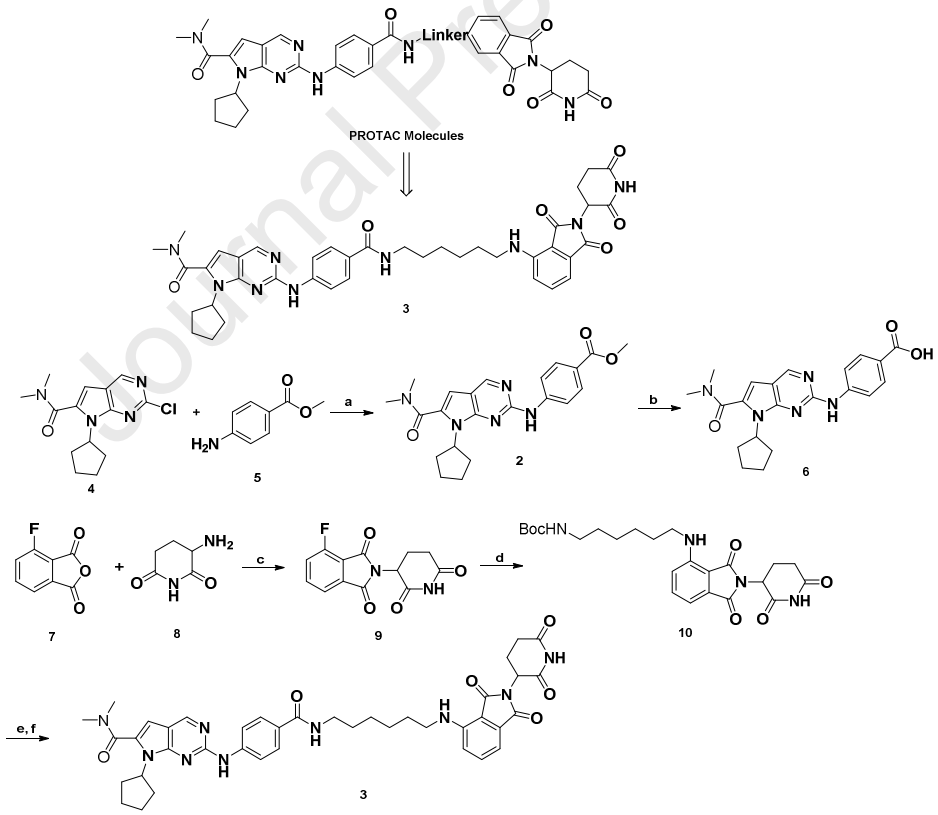
Scheme 1. Structure of Ribociclib 1 and its derivative 2

Table 1. Kinase activity of Ribociclib **1** and analogue **2**

	IC_{50} value (nM)		
Compounds	Cyclin E/CDK2	Cyclin D/CDK4	Cyclin D/CDK6
1	>20000	29	73
2	390	112	101

In accordance with the principle of designing PROTAC molecules, 23 compounds were synthesized (for details, please refer to the ESI files). After carefully screened, compound **3** was proven as the most efficient one to degradation of CDK 2/4/6 in various cancer cells, exhibiting remarkable inhibiting effect of tumor growth. Control assays clearly demonstrated that Ribociclib, thalidomide or their combination at equimolar could not induce the cleavage of CDK 2/4/6 in cancer cells or induce the apoptosis of cancer cells.

The forwards synthesis of compound **3** was straightforward and efficient. One hand, the main core **2** was obtained from commercially available materials **4** and **5** under Buchwald coupling conditions. Hydrolysis of the methyl ester delivered carboxylic acid **6** as coupling precursor. On the other hand, 2-fluoro-thalidomide **9**, prepared from the commercially available starting materials **7** and **8**, reacted with N-Boc-hexane-1,6-diamine under basic conditions to delivery amide **10**. Removal Boc protecting group by TFA, and subsequential amide coupling with intermediate **6** furnished the compound **3** required in moderate yield in gram-scale.



Reaction reagents and conditions: a $Pd(OAc)_2$, BINAP, Cs_2CO_3, dioxane, reflux, 79%; b LiOH, THF/H_2O, rt, 98%; c AcONa, HOAc, reflux, 80%; d N-Boc-hexane-1,6-diamine, DIPEA, DMF, 80 ^oC, 55%; e TFA, CH_2Cl_2, 0 ^oC to rt; f compound **6**, EDCI, HOBt, DIPEA, MeCN, rt, 43% over 2 steps.

Scheme 2. Design of PROTAC molecule and Synthesis of Compound **3**

For all PROTACs, the effect on the cell viability in a panel of cancer cell lines was examined

by standard MTT assay. Ribociclib (RIB), Pomalidomide (POM) and their mixture acted as reference control. Compound **3** exhibited prominent anti-proliferative activity against a broad spectrum of human cancer cell lines (Table 1, ESI), in particular for Melanomas (Table 2). IC_{50} of B16F10 and A375 was ascertained as 0.09761±0.021 and 0.1659±0.150, respectively.

Table 2. Cytotoxic Activity of Compound **3** Against Melanoma Cells

Compounds	IC_{50} value (μM)	
	B16F10	A375
3	0.09761±0.021	0.1659±0.150
RIB	>10	>10
POM	>10	>10
RIB+POM (1:1 ratio)	>10	>10

IC_{50} > 10 μmol/L indicated no significant inhibition; IC_{50} was determined from three independent experiments. MTT assay was employed to ascertain the cytotoxic effect of compound **3** after 72 h incubation.

Subsequently, compound **3** was tested for CDK2/4/6 degradation in melanoma cells (Figure 1). It was reported that the compound **3** induced significant intracellular cleavage of all CDK 2, CDK 4 and CDK 6 proteins in both concentration-dependent and time-dependent manners (Figure 1A and 1B). It also effectively mitigated downstream p-Rb signaling pathway activation of Melanoma Cells (Figure 1A and 1B). To verify whether the cleavage effect of CDK2/4/6 by the treatment of compound **3** was PROTAC-mediated degradation, the B16F10 cells were first pre-treated by Pomalidomide, MG132 as well as compound **2** (Figure 1C). Degrading effect of CDK2, 4 and 6 was totally impeded. Note that pre-treatment with Ribociclib, CDK4/6 degradation was dramatically inhibited, while CDK2 was down-regulated as normal. These results were consistent with our original finding and design.

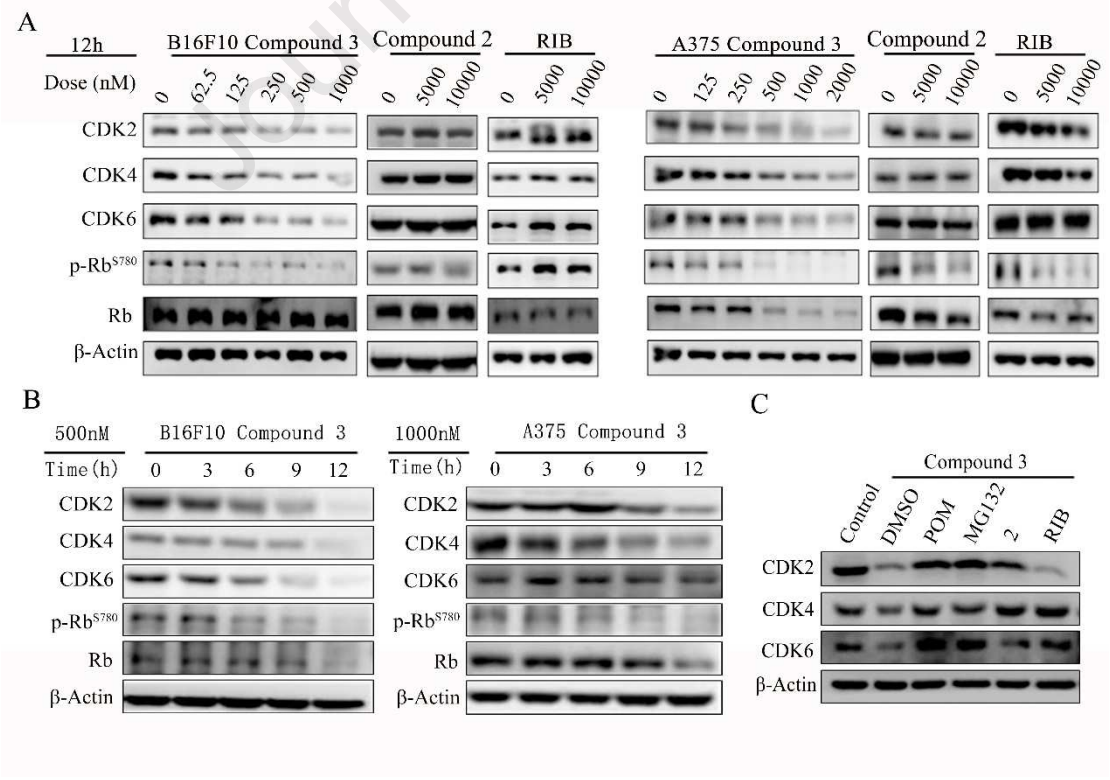


Figure 1. Compounds **3** Induced CDK2/4/6 Protein Degradation and Inhibited the Downstream

Rb Signaling Was Mitigated by Hijacking the E3 Ubiquitin Ligase CRBN. (A) Compounds **3** significantly down-regulated CDK2/4/6 protein levels and inhibited the Rb downstream signaling in a concentration-dependent manner. B16F10 and A375 cells were treated with DMSO or serial dilutions of compounds **3** for 12 h. (B) Compounds **3** down-regulated CDK2/4/6 proteins and inhibited the p-Rb downstream signaling in a time-dependent manner. B16F10 andA375 cells were treated with DMSO or compound **3** for the indicated length of time. (C) Compounds **3** induced CDK2/4/6 protein degradation was proteasomal degradation. Before being treated with the 100 nM compounds **3** for 6 h, cells were pre-treated with DMSO, Pomalidomide (10 μM), MG132 (20 μM) for 2 h. The CDK2/4/6 protein were determined by western blot.

To biologically assess the anti-proliferative activities of compound **3** against two melanoma cancer cells (A375 and B16F10), colony formation assay was performed (Figure 2). Compound **3** exerted a remarkable inhibiting effect on colony formation in A375 and B16F10, two malignant melanoma cancer cells, in low nano molar concentration.

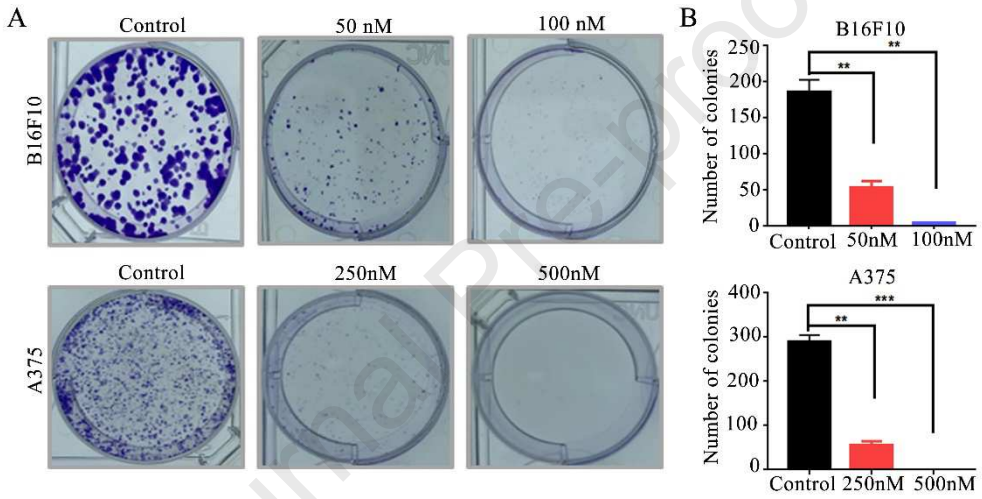


Figure 2. Inhibiting Effects of Compound **3** on Colony Formation of Malignant Melanoma Cancer Cells. Colony formation assay was performed to ascertain the proliferation capacity of A375 and B16F10 cellstreated with compound **3**. (A) Representative image of long-term growth assay of A375 and B16F10 cells following continuous compound **3** for 7 days. (B) Quantification of clonogenic growth illustrated in A. Student's T-test; mean ± SD, n = 3. **p<0.01;*** p<0.001.

Since cell apoptosis was distinctly observed under a microscope when cancer cells were treated with compound **3**, both A375 and B16F10 cells were treated with vehicle alone as control and with **3** at various concentrations for 48 h; then, they were stained with FITC-Annexin V and propidium iodide (PI). The percentages of apoptotic A375 and B16F10 cells were measured by flow cytometry. Figure 3A and 3B suggest that compound **3** significantly induced apoptosis of A375 and B16F10 cells in a dose-dependent manner.

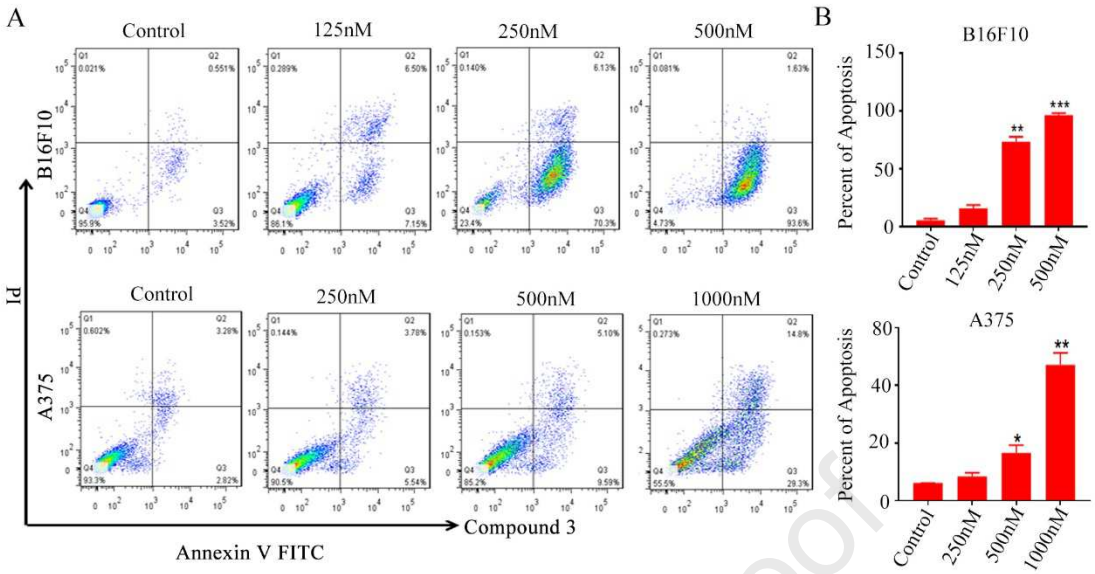


Figure 3. Compound **3** Induced of apoptosis in malignant melanoma cancer cells. (A) Flow cytometry analysis of apoptotic A375 and B16F10 cells induced by compound **3** at various concentrations. (B) Quantitative analysis of apoptosis illustrated in A. Student's T-test; mean ± SD, n= 3. * $P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

To identify the potential mechanism of apoptosis induced by compound **3**, several cell death-related proteins (e.g., PARP, Caspase-3, Bax, Bcl-2 and P53) were ascertained by western blot in A375 and B16F10 cells. Figure 4 suggests that cleaved Casepase-3 and cleaved PARP, two critical hallmarks of apoptosis, were noticeably up-regulated by the treatment with compound **3** in a dose-dependent manner. In the meantime, compound **3** also significantly induced up-regulation of the pro-protein level of P-53 and Bax. In contrast, the anti-apoptotic protein level of Bcl-2 was down-regulated following the treatment with compound **3**. On the whole, compound **3** induced Caspase-dependent apoptosis via the common P53/Bcl-2/Bax apoptotic pathway in melanoma cells.

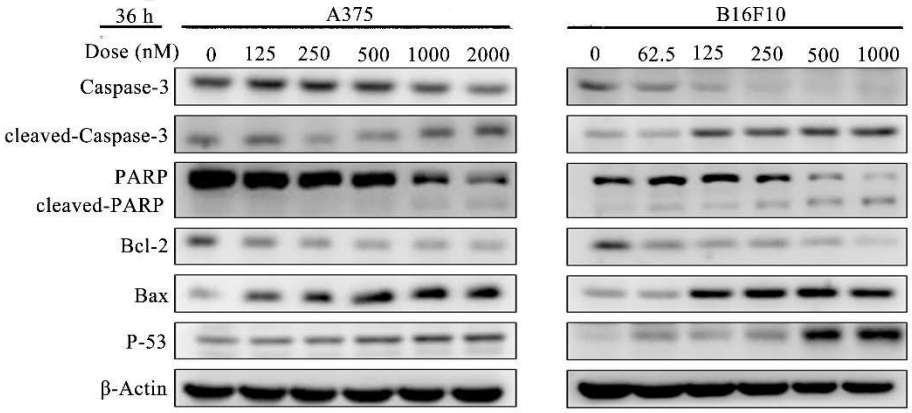


Figure 4. Apoptosis Induced by Treatment of Compound **3** through Caspase-dependent Manner. A375 and B16F10 cells were incubated with compound **3** for 48 h, cleaved-Caspase-3, cleaved-PARP, total expression of P53 and Bcl-2 members (Bcl-2 and Bax) were assessed by Western blot. β-Actin was used as an internal control.

To verify whether cytotoxic activity of compound **3** results from cell cycle arrest which

induced via degradation of CDK2/4/6, the cell cycle kinetics in human cancer cell lines, including A375 and B16F10 cells, were then investigated by treating those cancer cell lines with compound **3** (Figure 5). Tumor cells were incubated with complete medium supplemented by compound **3** and DMSO at various concentrations for 48 h; then they underwent flow cytometry to ascertain the proportion of cells in some phase of cell cycle. As shown in Fig.5, tumor cells were treated with DMSO normally distributed in each phase of cell cycle. In contrast, treatment of compound **3** increased accumulation of A375 cell population in G0/G1 pahse, but G2/M phase for B16F10 cell. These results indicated that compound **3** could induce cell cycle arrest via the degradation of cell cycle-related proteins CDK2/4/6 in tumor cells.

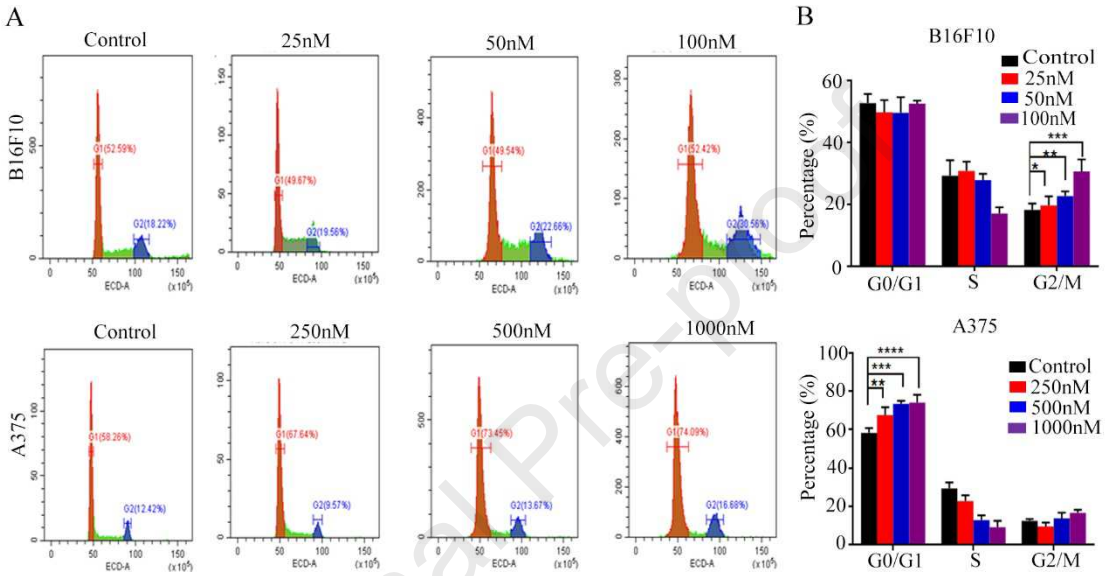
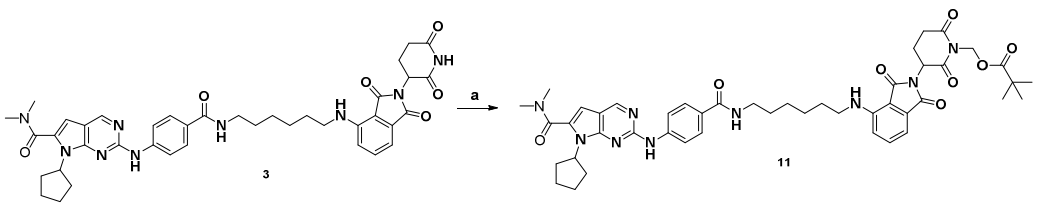


Figure 5. Effect of Compound **3** on Cell Cycle Progression of Tumor Cells. (A) Flow cytometry of the DNA content of A375 and B16F10 cells following staining with propidium iodide. (B) The bar graph illustrates the proportions of tumor cells in G0/G1, S and G2/M phase, respectively. Student's T-test; mean ± SD, n= 3. * P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

However, similar as most reported PROTAC molecules with CRBN ligands, the degrader **3** had alomst no oral bioavailblity (<1%) proparbly due to its amphotericity. Therefore, our hypothesized solution was to prepare a prodrug by adding a lipophilic group at active site of CRBN ligands (Scheme 3). It might provide convenient oral administration of the PROTAC molecule in animal test. Chemically, modification of the prodrug was very straightforward. As shown in scheme 3, a one-step reaction with chloromethyl pivalate under mild conditions converted degrader **3** into prodrug **11** in gram-scale. Based on the results of preliminary PK study (For more details, please see ESI), the orally bioavaiblility of compound **11** is up to 68%. Compound **3** was exposed to plasm of rats with a useful concentration within 4-8 hours. To our best of konwledge, this is the first time to report the successful development of orally bioavailble prodrug of PROTAC molecules based on the CRBN ligands.



Reaction reagents and conditions: a Chloromethyl pivalate, Cs_2CO_3, DMF, TBAI, rt, 65%.

Scheme 3. Synthesis of Prodrug **11**

Plasma concentration−time curves for compound **11** after a single dose in rats were shown in Figure **6**. Based on the results, after PO administration (PO, Figure 6B) of the compound **11**, which could steadily convert into active ingredient **3** in rat plasma, its blood concentration rapidly reached peak, and then remarkably declined in 5 hours. The pharmacokinetic of compound **11** was also investigated by tail intravenous injection (IV, Figure 6A). The pharmacokinetic parameters determined with a non-compartmental model were shown in table 2.

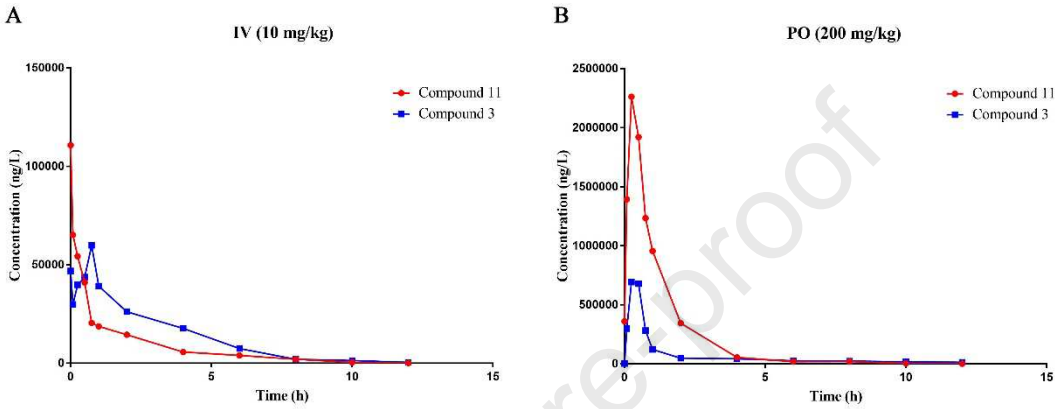


Figure 6. PK study of compound **11** in rats. (A) Plasma concentration−time curve of Compounds **11** and **3** in SD rats after a single IV administration of drug (10 mg/Kg, n = 3); (B) Plasma concentration−time curve of Compound **11** and **3** in SD rats after a single PO administration of drug (200 mg/Kg, n = 3);

Table 2. Preliminary Pharmacokinetic (PK) Parameters of Compound **11** and **3**

Compound	PO (200 mg/Kg)			
	C_{max}/ng.mL^{-1}	T_{max}/h	AUC_{0-12}/ng.mL^{-1}	F/%
11	692268	0.25	822803.9667	NA
3	2261897	0.25	2810197.617	NA
Compound	IV (10 mg/Kg)			
	C_{max}/ng.mL^{-1}	T_{max}/h	AUC_{0-12}/ng.mL^{-1}	F/%
11	59851	0.75	160704.5	68%
3	110809	NA	95665.81	NA

With the encouraging PK parameters of prodrug **11** in hand, its anticancer activity *in vivo* was assessed for its suppression of B16F10 tumor growth. As shown in Figure 7, tumor sizes were significantly suppressed in mice treated with compound **11**. There was no noticeable difference in body weight between the two groups. These results revealed that oral administration of compound **11** can distinctly inhibit B16F10 tumor growth and mediate degradation of CDK2/4/6 *in vivo*.

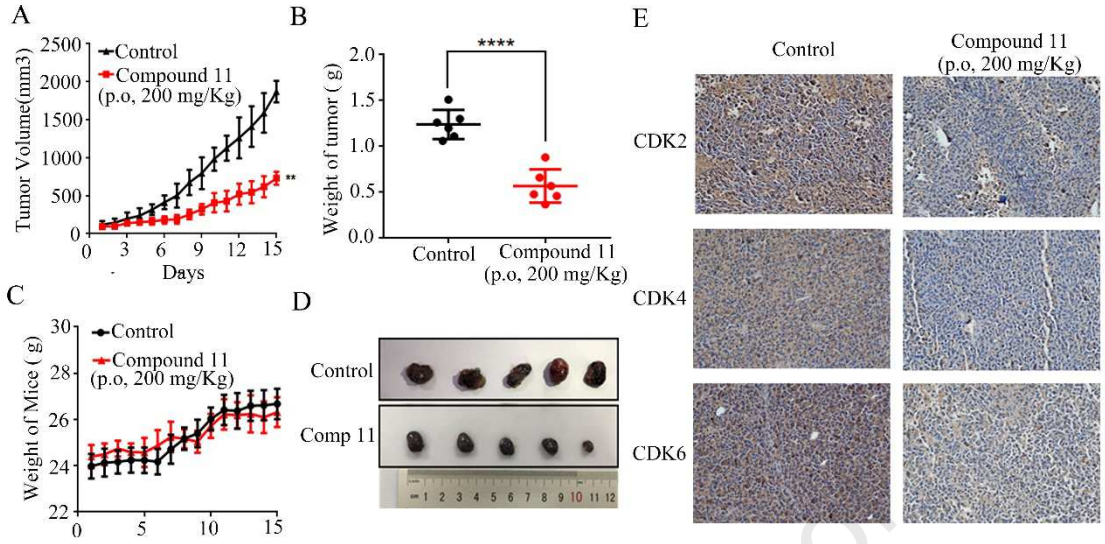


Figure 7. Effect of Compound **11** on B16F10 Tumor Growth Delay and Regression *in vivo* through oral administration. C57BL/6J mice intravenously injected with B16F10 cells. (A) Variations in the tumor volume of B16F10 xenografts. (B) Tumors were weighted on the15th day. (C) Body weights of animals. (D) The representative images of tumors dissected from vehicle-and compound **3** and **11**-treated mice are presented. Student's T-test;; mean ± SD, n = 6, **P < 0.001. (E) Tumor tissue lesions (n = 5) from B16F10 xenograft model were stained with monoclonal antibody against CDK2/4/6 and then photographed (100X).

In conclusion, a novel PROTAC degrader, compound **3**, based on a Ribociclib derivative and CRBN ligand has been developed. The small molecule degraded CDK 2/4/6 as well as their complex in malignant melanomas simultaneously and effectively. This compound could also rapidly reset the cell cycles and induce cell apoptosis in various cancer cells, in particular for melanomas. The mechanism should be interpreted that deficiency CDK 2/4/6 might lead to synthetic lethal effects to malignant melanomas in the presence of compound **3**. These results suggested that the combination of CDK 2/4/6 would become promising kinase targets for treatment of solid tumors. Furthermore, an orally bioavailable prodrug **11** was developed for oral drug delivery in animal test with high bioavailblity, for the first time. It might also provide a general solution for oral administration of the PROTAC molecules, which were derived from CRBN ligands.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the website.

The synthetic procedure and copies of $^{1}H/^{13}C$ NMR Spectrum of all compounds and intermediates. Biological evaluation of all selected compounds (PDF)

AUTHOR INFORMATION

Corresponding Author

* jwang05@tmu.edu.cn

* Guang.yang@nankai.edu.cn

* Cheng.yang@nankai.edu.cn

Author Contributions

§ These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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Highlights

Compound 3 is an efficient PROTAC molecule which can degrade CDK 2/4/6 simultaneously and effectively.

Compound 3 Remarkably inhibit the growth of melanoma cells.

The first orally bioavailable prodrug was developed for the PROTAC molecules with CRBN ligands.

The oral bioavailability of compound 11 is up to 68%.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: