Design and synthesis of NAD(P)H: Quinone oxidoreductase (NQO1)-activated prodrugs of 23-hydroxybetulinic acid with enhanced antitumor properties

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Abstract

A series of NQO1 selectively activated prodrugs were designed and synthesized by introducing indolequinone moiety to the C-3, C-23 or C-28 position of 23-hydroxybetulinic acid (23-HBA) and its analogues. Among them, the representative compound **32j** exhibited significant antiproliferative activities against NQO1-overexpressing HT-29 cells and A549 cells, with IC_{50} values of 1.87 and 2.36 μM, respectively, which were 20~30-fold more potent than those of parent compound 23-HBA. More importantly, it was demonstrated in the *in vivo* antitumor experiment that **32j** effectively suppressed the tumor volume and largely reduced tumor weight by 72.69% with no apparent toxicity, which was more potent than the positive control 5-fluorouracil. This is the first breakthrough in the improvement of *in vivo* antitumor activities of 23-HBA derivatives. The further molecular mechanism study revealed that **32j** blocked cell cycle arrest at G2/M phase, induced cell apoptosis, depolarized mitochondria and elevated the intracellular ROS levels in a dose-dependent manner. Western blot analysis indicated that **32j** induced cell apoptosis by interfering with the expression of apoptosis-related proteins. These findings suggest that compound **32j** could be considered as a potent antitumor prodrug candidate which deserves to be further investigated for personalized cancer therapy.

Keywords: NQO1, Prodrug, 23-Hydroxybetulinic acid, Indolequinone, Antitumor

1. Introduction

Although chemotherapy has long been recognized as one of the major strategies for cancer treatment, most of chemotherapeutic antitumor drugs are limited by their lack of specificity towards cancer cells, which results in drastic side effects and poor prognosis for cancer patients. The prodrug strategy has earned widespread attention due to their prominent dominance in improving selectivity and efficacy of targeting cancer cells [1]. The rationale prodrug design mainly relies on the unique biochemical alterations in cancer cells, such as some elevated enzymes in tumor tissues [2-4]. Among them, NAD(P)H: quinone oxidoreductase-1 (NQO1) is a cytosolic flavoenzyme that is overexpressed in multiple tumors (lung cancer, liver cancer and

colon cancer etc.) compared with normal tissue [5-9]. With the reduced pyridine nucleotide NADH or NADPH as a cofactor, NQO1 can catalyze the two-electron reduction of indolequinone-based prodrug with a potential leaving group at the indole 3-position (**1**, Figure 1) to dihydroxyindole (**2**), which is unstable to trigger expulsion of the drug owing to the markedly increased electron-density at the indole nitrogen [10-13]. As a result, the released drug and alkenyliminium electrophile **3** lead to apoptosis of NQO1-overexpressing cancer cells synergistically [14-17]. Thus, NQO1-activated indolequinone prodrugs with functionalization at the indole 3-position have emerged as an attractive strategy for antitumor prodrug design [13].

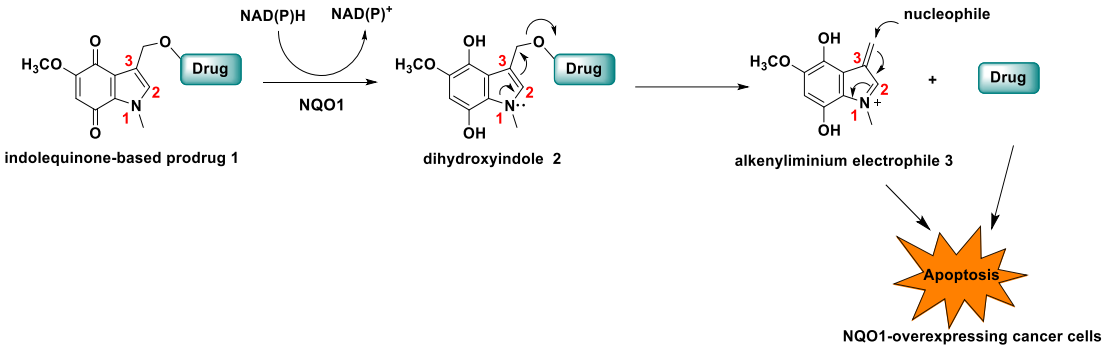
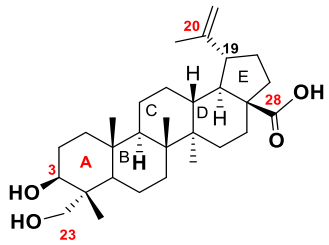


Figure 1. Schematic illustration for activation of NQO1-targeted indolequinone prodrugs.

23-Hydroxybetulinic acid (23-HBA, **4**, Figure 2), a natural lupane-type pentacyclic triterpene isolated from the root of the traditional Chinese herb *Pulsatilla chinensis* [18], has attracted substantial interest from medicinal chemists due to its effective and extensive antineoplastic activities [19]. Previous investigations have found that 23-HBA exhibited cytotoxicity against various of cancer cells through mechanisms like increasing the formation of intracellular ROS, reducing the mitochondrial membrane potential (MMP), inhibiting the telomerase activity, the down-regulation of the anti-apoptotic proteins and up-regulation of the pro-apoptotic proteins [20-24]. What's more, 23-HBA possessed synergistic effects on the cytotoxicity of clinically used drugs such as doxorubicin and paclitaxel *in vitro* and *in vivo* [25]. To improve the antitumor activity of 23-HBA, some studies on the structural modifications of 23-HBA have been performed by our group and others. Most of the C-23 and C-28 modified derivatives showed satisfactory activities, and an array of heterocyclic ring-fused derivatives on ring A of 23-HBA with excellent antitumor activities have also been obtained [26-31].

However, the moderate capacity of 23-HBA to target tumor cells has limited its clinical development. As a potential antitumor lead, it maybe the effective strategy by designing of NQO1 selectively activated prodrugs of 23-HBA to improve its selectivity and efficacy.



23-hydroxybetulinic acid (23-HBA), 4

Figure 2. Chemical structure of 23-hydroxybetulinic acid.

Inspired by the prodrug strategy for tumor site-specific activation through the NQO1 and the promising antitumor activities of the natural 23-HBA, a series of NQO1-activated prodrugs of 23-HBA were further designed and synthesized by introduction of indolequinone nucleus at C-3, C-23 or C-28 position of the parent compound and its analogues. Through bio-reductive transformation, 23-HBA or its derivative was expected to released from the prodrug in tumor tissue, thereby enhancing the specificity and reducing side effects (Figure 3). In this study, a total of eighteen synthesized target compounds were assayed for antiproliferative activities against a panel of cancer cell lines. Based on the outcomes of the potency and selectivity, the representative compound **32j** was selected for subsequent mechanism studies and *in vivo* antitumor study. To our knowledge, it is the first breakthrough in the improvement of *in vivo* antitumor activities in 23-HBA structural modification studies to date. Thus, compound **32j**, as a NQO1-activatable prodrug of 23-HBA, deserves to be further investigated for the selective anticancer therapy.

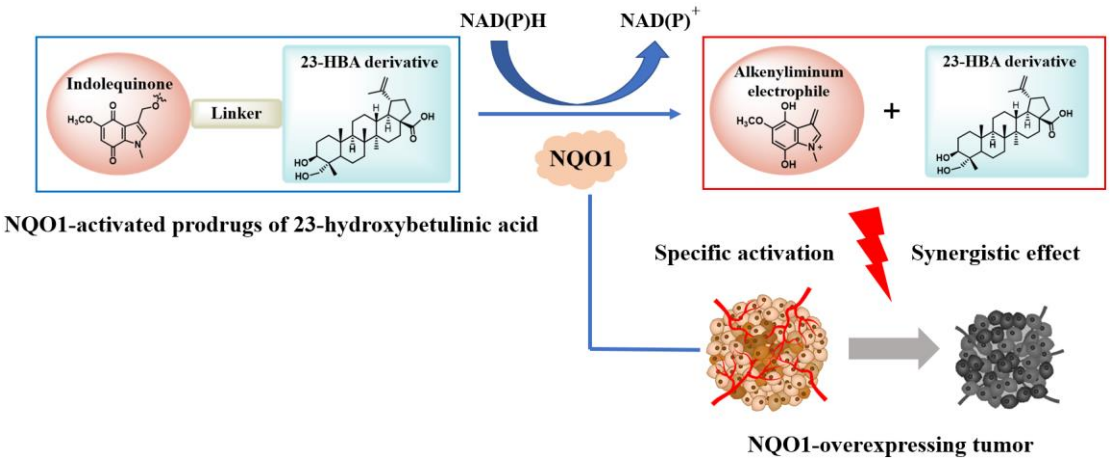
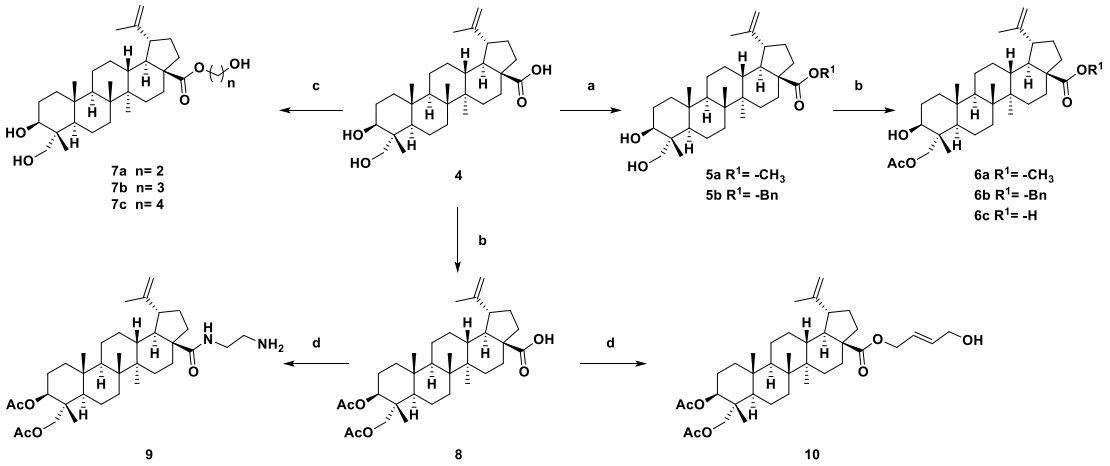


Figure 3. The rational design of NQO1-activatable prodrugs of 23-HBA.

2. Results and discussion

2.1. *Synthesis of NQO1-targeted 23-HBA prodrugs*

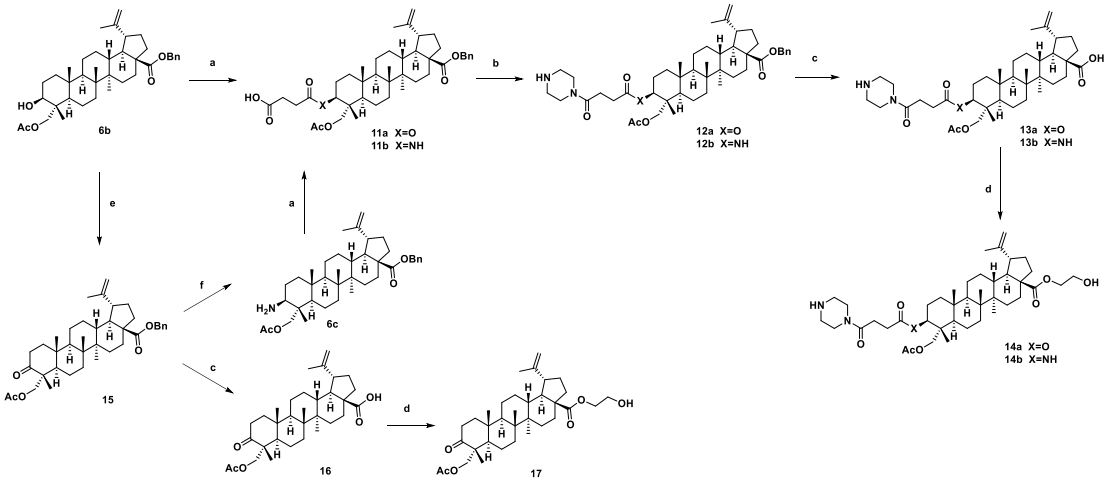
The synthetic route of key intermediates **5a-b**, **6a-c**, **7a-c** and **8-10** was outlined in Scheme 1. 23-HBA was isolated from the roots of *Pulsatilla chinensis* (Bge) Regel, and characterized by ^{1}H NMR, ^{13}C NMR and HR-MS [18]. It was treated with CH_3I or BnBr in DMF to yield 28-methyl-23-hydroxybetulinic ester **5a** or 28-benzyl-23-hydroxybetulinic ester **5b**. The C-23 hydroxyl group was protected using acetic anhydride in the presence of DMAP to give **6a-c**. Compounds **7a-c** were synthesized by the reaction of HBA with corresponding bromoalcohols. 3, 23-*O*-diacetyl compound **8** afforded **9** and **10** upon reactions with corresponding ethane diamine and 2-butene-1, 4-diol.



Scheme 1. Reagents and conditions: (a) BnBr/CH_3I, K_2CO_3, DMF, rt, 1-1.5 h, 86%-90%; (b) Ac_2O,

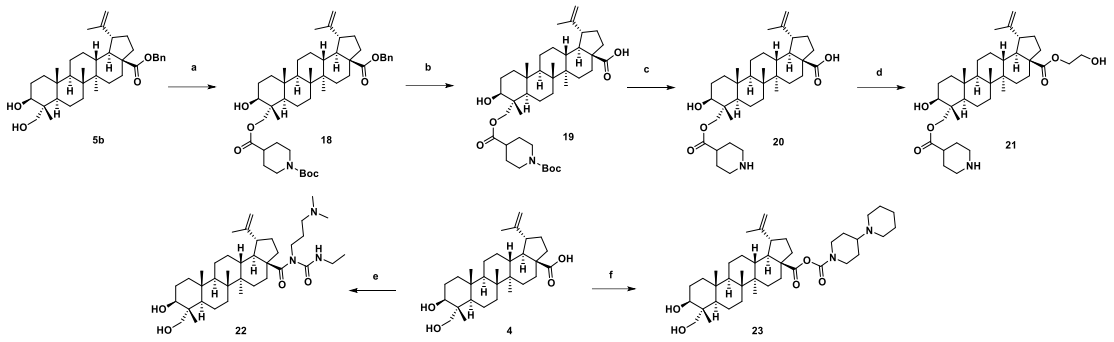
DMAP, DCM, rt, 1 h, 47%-76%; (c) 2-Bromoethanol/3-Bromo-1-propanol/4-Bromobutan-1-ol, K_2CO_3, DMF, rt, 1-1.5 h, 58%-66%. (d) Ethylenediamine or 2-butene-1,4-diol, EDCI, DMAP, DCM, 43%-49%.

As described in Scheme 2, compound **15** was afforded by oxidation with compound **6b** as the starting material. The intermediate **6c** was prepared by treatment of 3-oxo derivative **15** through Borch reduction [32, 33]. Compound **12a-b** were afforded by classical esterification and acylation. Subsequent debenzylation of these intermediates afforded compounds **13a-b** and **16** with Pd/C as a catalyst under atmospheric pressure of hydrogen. Finally, **13a-b** and **16** upon reaction with 2-bromoethanol led to compounds **14a-b** and **17** in the yields of 60-70%.



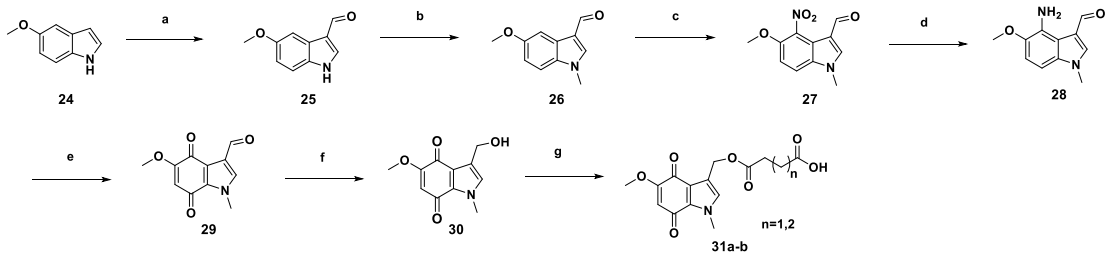
Scheme 2. Reagents and conditions: (a) Succinic anhydride, DMAP, DCM, rt, 3-5 h, 74%; (b) Piperazidine, EDCI, DMAP, DCM, rt, 1.5-2 h, 51%; (c) H_2, 10% Pd/C, THF, 0.5 h, 80-85%; (d) 2-Bromoethanol, K_2CO_3, DMF, rt, 1-1.5 h, 60%-70%; (e) Pyridinium chlorochromate (PCC), DCM, 3 h, 95%; (f) i) $NH_2OH{\cdot}HCl$, CH_3COONa, CH_3OH, rt, 12 h; ii) $NaBH_3CN$, $TiCl_3$, CH_3COONH_4, CH_3OH, 0 ^oC-rt, 3 h, 59.4%.

Compound **19** was prepared by esterification and debenzylation using the same methods mentioned above, and subsequent remove of protecting group with trifluoroacetic acid yielded compound **20**. Treatment of **20** with 2-bromoethanol gave compound **21** (Scheme 3). Reaction of C-28 carboxyl with EDCI in CH_2Cl_2 gave intermediate **22** in yield of 87%. 4-Piperidinopiperidine was converted to acyl chloride intermediate using triphosgene, which was further reacted with **4** to provide the derivative **23** in 86% yield (Scheme 3).



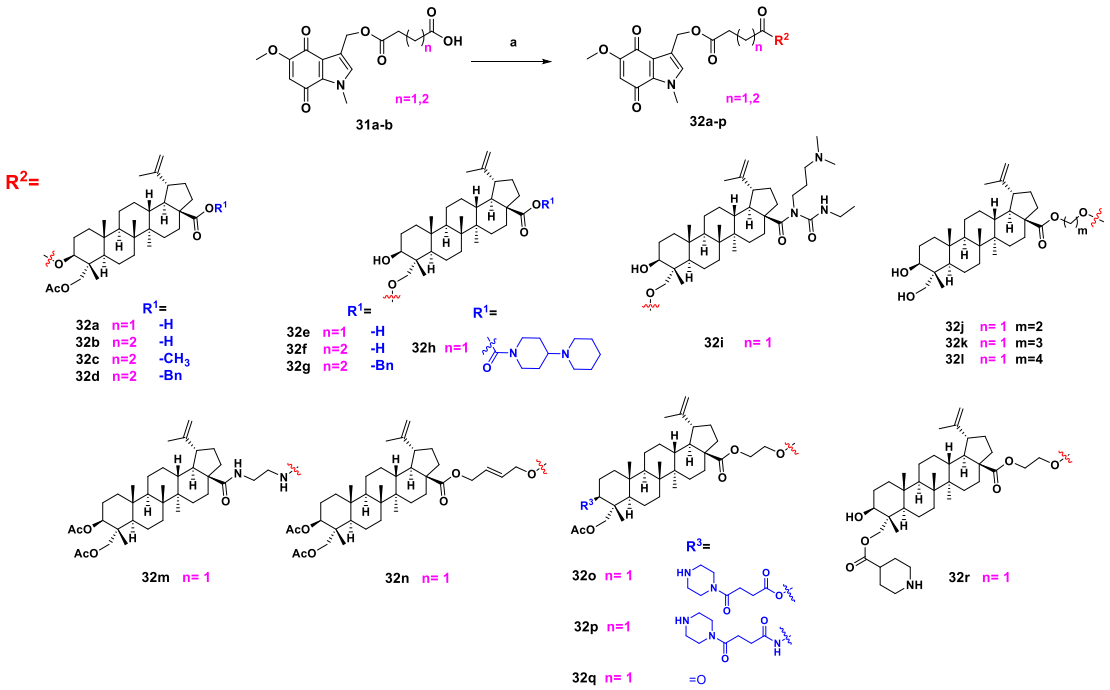
Scheme 3. Reagents and conditions: (a) 1-Boc-piperidine-4-carboxylic acid, DCC, DMAP, pyridine, rt, 8 h, 84%; (b) H_2, 10% Pd/C, THF, rt, 85%; (c) CF_3COOH, DCM, 0 °C-rt, 2 h, 51%; (d) 2-Bromoethanol, K_2CO_3, DMF, rt, 1-1.5 h, 38%; (e) EDCI, DCM, rt, 87%; (f) i) 4-Piperidinopiperidine, triphosgene, DCM, 0 °C; ii) Et_3N, DCM, rt, 86%.

Indolequinone skeleton was synthesized according to the route depicted in Scheme 4 [34]. Briefly, formylation of 5-methoxyindole **24** produced **25** in almost quantitative yield. The methylation of **25** using methyl iodide in the presence of sodium hydride gave **26** in 95% yield. Compound **27** was synthesized by nitration of **26** using conc. HNO_3 in acetic acid. The amine **28** was prepared by treatment of the intermediate **27** with Sn/HCl. Compound **29** was synthesized by oxidation using Fremy's salt, which was subsequently reduced using $NaBH_4$ to afford **30** in 70% yield. In order to install 23-HBA or its derivatives at the 3-position of indolequinone, we converted the hydroxyl group of **30** into the carboxyl function to give compounds **31a-b** using succinic anhydride or glutaric anhydride. The target compounds **32a-p** were obtained by coupling **31a-b** with 23-HBA and its analogues through the condensation reaction in the presence of EDCI and DMAP (Scheme 5).



Scheme 4. Reagents and conditions: (a) $POCl_3$, DMF, 0 °C to rt, 2 h, 90%; (b) NaH, MeI, DMF, rt, 30 min, 95%; (c) HNO_3, AcOH, 10-15 °C, 73%; (d) Sn, HCl, MeOH, rt, 57%; (e) Fremy's salt, acetone, PBS pH = 6.4, 3 h, 45%; (f) $NaBH_4$, MeOH, 10 min, 70%; (g) Anhydride, DMAP, DCM,

rt, 12-24 h, 55% - 80%.



Scheme 5. Reagents and conditions: (a) EDCI, DMAP, rt, 0.5-24 h, 31%-75%.

2.2. Effect of prodrugs on cancer cell growth

In order to analyze the cytotoxicity of these targeted compounds, compounds **32a-r** with indolequinone nucleus were preliminarily screened for their antiproliferative activities against human liver cancer cells HepG2, human melanoma A375 and human lung cancer cells A549 by the MTT assay (Figure 4, detailed data see Supplementary Table S1). The substituted position of indolequinone on the 23-HBA had little effect on the antitumor activities. When the C-3 position of 23-HBA was substituted with indolequinone, substitution of the C-28 carboxyl group led to a significant decrease of activity (**32a-b** *vs* **32c-d**). Most of 23-HBA derivatives with the indolequinone at C-23 position (**32e-i**) possessed moderate to good inhibition rates at a concentration of 10 μM. When the indolequinone was attached to the C-28 position of 23-HBA, increasing the chain length of the linker had negative effects on the antiproliferative activities (**32j** vs **32k-l**). It is interesting to note that the different modifications at the C-3 position led to a consistent increase in the antitumor activities (**32o-q**).

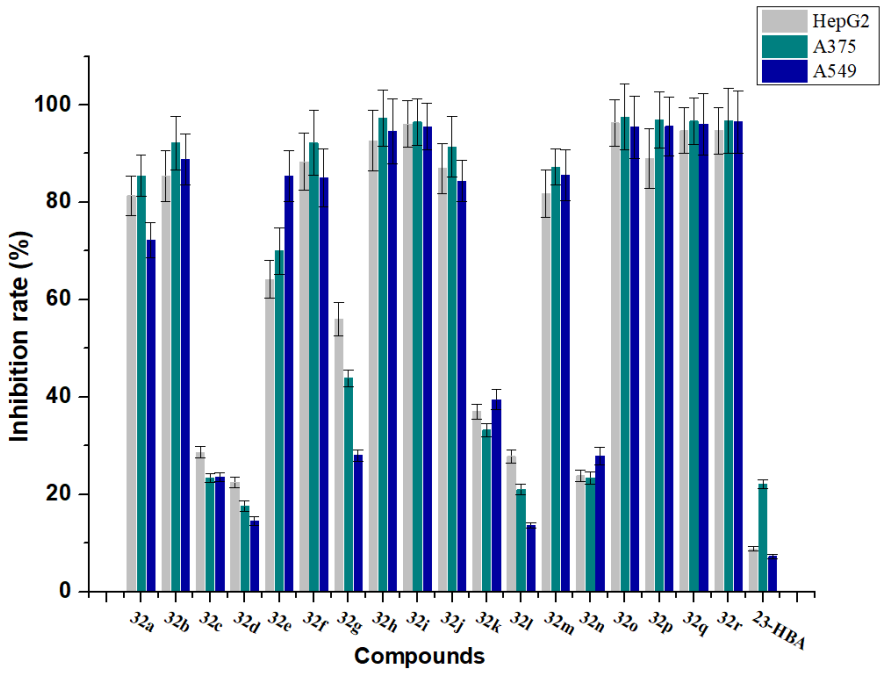


Figure 4. Histograms display the inhibitory rates of compounds **32a-r** against HepG2, A375 and A549 cells at 10 μM.

Based on preliminary assay data, the IC_{50} values of eleven selected compounds (**32a-b**, **32f**, **32h-j**, **32m** and **32o-r**) were further evaluated against three cancer cell lines: human lung carcinoma cells (A549), human colon carcinoma cells (HT-29), human lung adenosquamous carcinoma cells (H596) and one normal cell line human fetal lung fibroblast-1 (HFL-1) with 23-HBA and doxorubicin as the positive controls. In which, A549 and HT-29 cells have a higher expression level of NQO1, while H596 cells have a relative low expression level of NQO1. The cytotoxicity data of the representative compounds were listed in Table 1, and the results showed that most target compounds exhibited more potent antiproliferative activities than 23-HBA. The difference was most notable in NQO1-rich HT-29 cells, where compounds **32f**, **32h**, **32j** and **32m** were about 28-fold more potent than parent 23-HBA, with the IC_{50} values ranging from 1.73 to 1.92 μM. Among these four compounds, selectivity ratios defined as IC_{50}(H596)/IC_{50}(HT-29) and IC_{50}(HFL-1)/IC_{50}(HT-29) were generally > 3, except for compound **32f**, suggesting that NQO1-defficient H596 cell and normal cell HFL-1 were markedly less sensitive to these compounds. To further confirm whether the anticancer

activities of **32h**, **32j** and **32m** were related to their interaction with NQO1, we further measured their antiproliferative activities in the presence of dicoumarol (DIC), an effective NQO1 inhibitor that suppresses the catalytic efficiency by interacting with the NAD(P)H binding sites on oxidized enzyme. As shown in Table 2, DIC co-incubation reduced HT-29 cell sensitivity to compounds **32h**, **32j** and **32m**, and elevated the IC_{50} values to 4.1-, 4.9- and 4.2-fold, respectively. Based on these results, we speculated that the antiproliferative activities of these compounds may be influenced at least in part through their NQO1-dependent bioreductive activation. Given the potent antiproliferative activity and selectivity, compound **32j** was finally selected as the representative compound for further anticancer mechanism studies.

Table 1. Anti-proliferative activity of the representative compounds against human cancer and normal cells.

Compd.	IC_{50} values (μM) [a]				SI [b]	SI [c]
	A549 (NQO1+)	HT-29(NQO1+)	H596(NQO1-)	HFL-1		
32a	4.52 ± 0.24	3.69 ± 0.15	5.74 ± 0.34	7.25 ± 0.38	1.56	1.97
32b	4.03 ± 0.32	3.58 ± 0.13	4.58 ± 0.23	4.09 ± 0.22	1.27	1.36
32f	2.52 ± 0.12	1.92 ± 0.09	4.71 ± 0.36	3.23 ± 0.14	2.46	1.69
32h	2.66 ± 0.14	1.73 ± 0.09	6.41 ± 0.35	6.56 ± 0.43	3.71	3.79
32i	2.32 ± 0.11	2.28 ± 0.09	2.78 ± 0.13	4.22 ± 0.28	1.23	1.85
32j	2.36 ± 0.12	1.87 ± 0.11	7.78 ± 0.43	6.45 ± 0.24	4.16	3.44
32m	3.10 ± 0.21	1.92 ± 0.12	7.89 ± 0.52	5.78 ± 0.33	4.10	3.01
32o	5.45 ± 0.34	3.29 ± 0.20	6.39 ± 0.44	6.79 ± 0.30	1.99	2.12
32p	5.17 ± 0.57	3.63 ± 0.25	6.44 ± 0.46	7.11 ± 0.53	1.77	1.96
32q	3.04 ± 0.37	3.35 ± 0.11	5.64 ± 0.33	6.53 ± 0.42	1.68	1.95
32r	5.41 ± 0.34	4.30 ± 0.22	7.34 ± 0.37	6.83 ± 0.44	1.70	1.59
23-HBA	51.44 ± 3.57	54.81 ± 4.63	58.12 ± 5.15	75.00 ± 6.82	1.06	1.36
ADM [d]	1.15 ± 0.07	1.13 ± 0.06	1.52 ± 0.09	- [e]	1.74	- [e]

[a] Each data represents mean ± S.D. from three different experiments performed in triplicate.

[b] Selectivity：IC_{50} HFL-1 cells / IC_{50} HT-29 cells.

[c] Selectivity：IC_{50} H596 cells / IC_{50} HT-29 cells.

[d] ADM: Doxorubicin.

[e] Not determined.

Table 2. Evaluation of NQO1-dependent cytotoxicity of tested compounds.

Compd.	IC_{50} (μM) [a]		
	HT-29	H596	HT-29 + DIC [b]
32h	1.73 ± 0.09	6.41 ± 0.35	7.11 ± 0.52
32j	1.87 ± 0.11	7.78 ± 0.42	9.22 ± 0.32
32m	1.92 ± 0.12	7.89 ± 0.52	8.02 ± 0.66

[a] Each data represents mean ± S.D. from three different experiments performed in triplicate.

[b] Cells were pretreated with 10 mM DIC for 30 min.

2.3. *Compound **32j** was a good substrate of NQO1*

To further demonstrate that our synthesized compounds were the good substrates of NOQ1, HPLC analysis was used to monitor the decomposition of **32j** in the presence or absence of NQO1. As mentioned above, the drug at the 3-position of the indolequinone nuclear was released in a reverse-Michael-like process after reduction with NQO1. As shown in Figure 5, nearly 50% of **32j** was decomposed in the presence of NQO1 within 6 h. After 60 h, **32j** was almost consumed. However, about 80% of **32j** remained stable in pH 7.4 buffer without NQO1 even after 60 h. These results suggested that compound **32j** was a good substrate of NQO1.

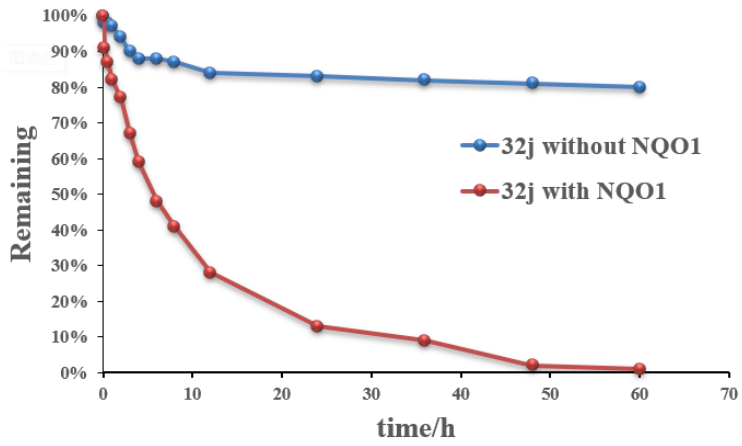


Figure 5. Decomposition profiles of **32j** in the presence and absence of NQO1 were determined by HPLC analysis.

*2.4 Cell apoptosis analysis of compound **32j***

To clarify whether the loss of cancer cell viability promoted by compound **32j** is associated with apoptosis, an Annexin V-FITC/propidium iodide (PI) co-staining assay was performed. Compound **32j** induced HT-29 cells apoptosis in a dose-dependent manner (Figure 6). Treatment of HT-29 cells with **32j** at 1, 2, and 4 μM for 48 h resulted in 22.35, 39.26 and 54.86% apoptotic cells (early and late-stage apoptosis), as compared with 3.45% in the untreated vehicle control group, indicating that compound **32j** effectively induced cell apoptosis in HT-29 cells.

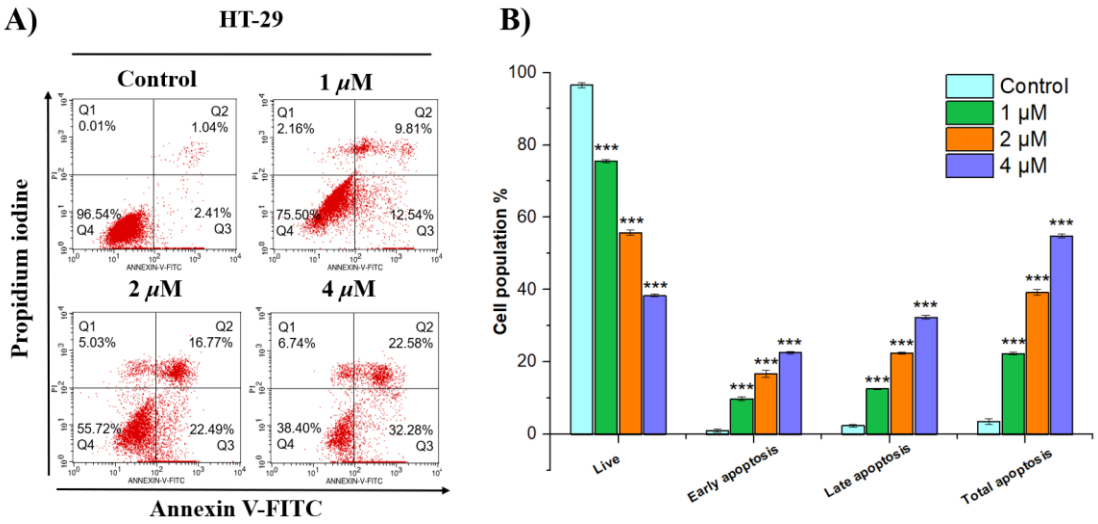


Figure 6. Compound **32j** induced apoptosis in HT-29 cells. (A) HT-29 cells were incubated with varying concentrations of **32j** (0, 1, 2 and 4 μM). After 48 h of incubation, cells were collected and stained with Annexin V-FITC/PI, followed by flow cytometric analysis. (B) Histograms display the

percentage of cell distribution. ***$p < 0.001$ *vs.* control group.

*2.5 Cell cycle analysis of compound **32j***

Since most antitumor agents can exert cell cycle arrest effects and ultimately cause cell death, the most active compound **32j** was examined for its effect on cell cycle progression of HT-29 cells using propidium iodide (PI) staining by flow cytometry. As shown in Figure 7, compound **32j** influenced cell cycle progression at micromolar concentrations and caused blockage of the cell cycle at the G2/M phase. When HT-29 cells were treated with increasing concentrations of **32j** (1, 2 and 4 μM), the percentage of cells at the G2/M phase increased from 20.54% to 32.73%. The above results suggested that compound **32j** may inhibited the growth of cancer cells *via* G2/M-phase arrest.

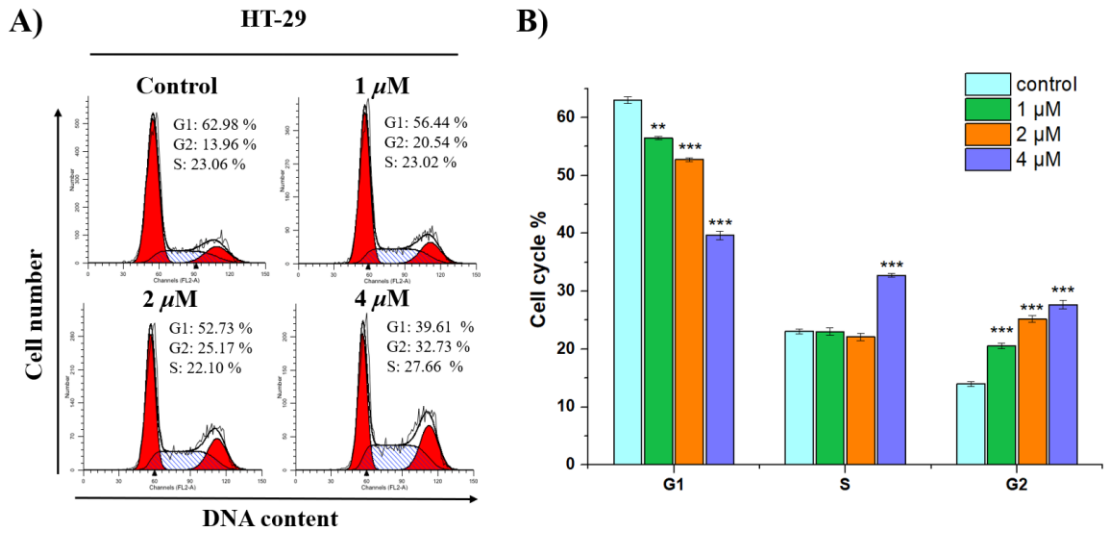


Figure 7. Compound **32j** induced G2/M arrest in HT-29 cancer cells. (A) HT-29 cells were incubated with DMSO and varying concentrations of **32j** (1, 2 and 4 μM) for 48 h. (B) Histograms display the percentage of cell cycle distribution. **p < 0.01, ***$p < 0.001$ *vs.* control group.

2.6 *Compound **32j** induced the generation of intracellular ROS*

ROS plays an important role in the induction of physiological and pathological apoptosis, which is highly associated with the NQO1-directed antitumor agents. In order to monitor intracellular ROS levels in the presence and absence of **32j**, ROS production induced by **32j** in HT-29 cells was detected by the fluorescent probe 2', 7'-

dichlorofluorescein diacetate (DCF-DA). As illustrated in Figure 8, **32j** induced intracellular ROS generation in a dose-dependent manner. These findings suggested that compound **32j** led to an increasing oxidative stress in HT-29 cells, thus resulting in significant cytotoxicity to tumor cells.

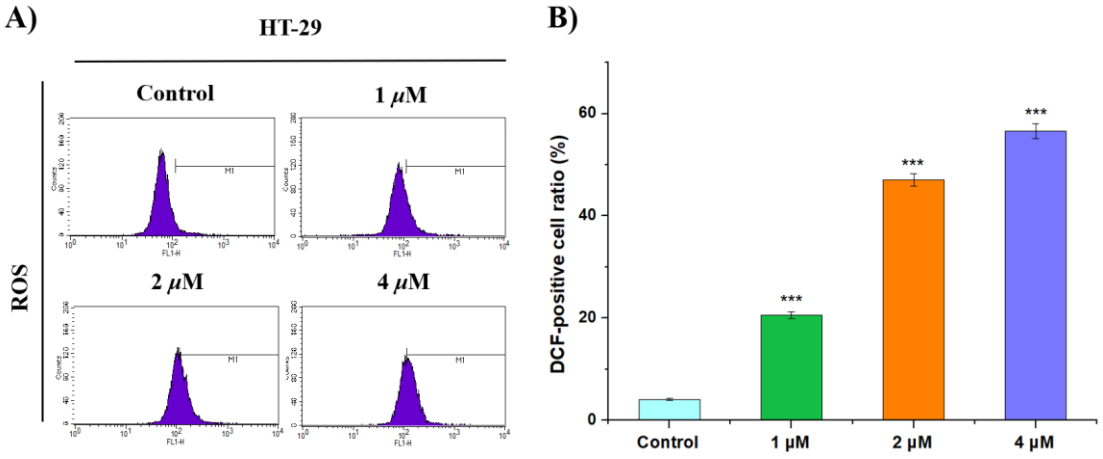


Figure 8. Effect of **32j** on ROS generation in HT-29 cells. A) Generation of ROS was measured using the ROS-detecting fluorescent dye DCF-DA in combination with FACScan flow cytometry; B) Histogram displays the DCF-positive cell radio in different concentrations (0, 1, 2 and 4 μM) of **32j**. ***$p < 0.001$ *vs.* control group.

*2.7 Compound **32j** reduced mitochondrial membrane potential*

It has been reported that mitochondria play an important role in regulating cellular functions, and mitochondrial dysfunction is involved in many pathological processes. To explore whether **32j** could induce mitochondrial dysfunction, mitochondrial membrane potential (MMP) assay by JC-1 staining of mitochondria in HT-29 was performed. As illustrated in Figure 9, with the concentration of **32j** increasing from 1 to 4 μM, the green fluorescence intensity (JC-1 monomers, low MMPs) correspondingly increased from 20.83% to 44.48%, suggesting that **32j** caused MMP collapse of HT-29 cells in a dose-dependent manner, which eventually triggered apoptotic cell death.

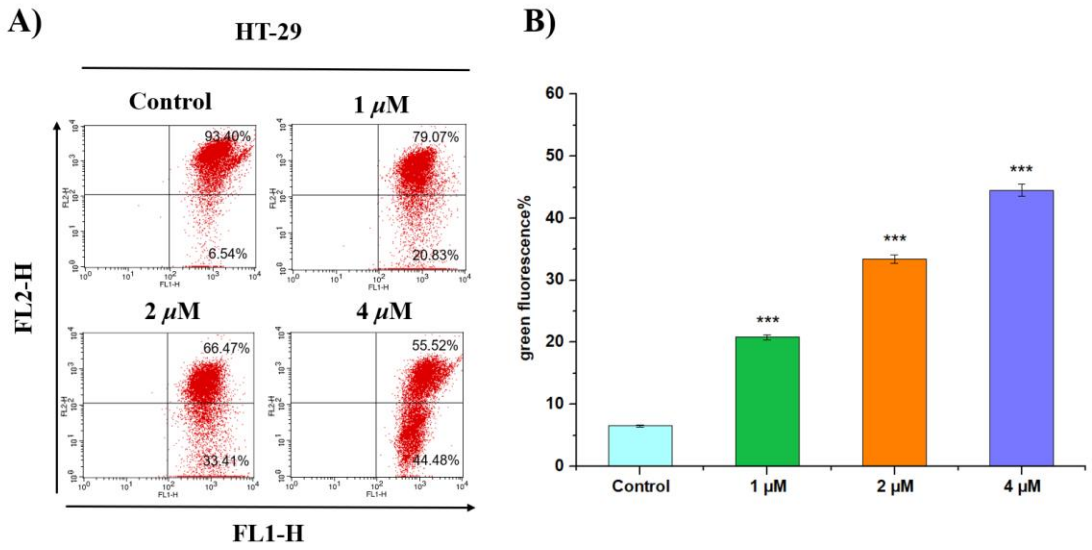


Figure 9. Effects of **32j** on the MMP of HT-29 cells. (A) After incubation with different concentrations (0, 1, 2 and 4 μM) of **32j** in HT-29 cells for 48 h prior to staining with JC-1 dye, the number of cells with collapsed MMPs was determined by flow cytometry analysis; (B) Histograms display the intracellular ROS contents in the absence or presence of **32j**. ***$p < 0.001$ *vs.* control group.

*2.8 Compound **32j** regulates apoptotic related proteins*

The above results showed that **32j**-induced apoptosis was closely related to the mitochondrial pathway. The Bcl-2 family members, including proapoptotic (e.g., Bax, Bad) and antiapoptotic proteins (e.g., Bcl-2, Bcl-xl), have been identified as essential proteins in controlling the mitochondrial pathway [35]. Bax acts on the mitochondria to increase the permeability of mitochondria, leading to the release of certain cellular components, such as Cytochrome C (Cyto C). Cyto C normally forms a caspase activation complex by combining with other molecules and plays a key role in the caspase-dependent apoptosis pathway [36]. Caspase 9 is one of the most important caspases, which cleaves many important cellular substrates. To explore the signaling mechanism of **32j**-induced apoptosis, the expression levels of Bax, Bcl-xL, Cyto C and caspase 9 were determined by Western blot analysis. As shown in Figure 10, the expression levels of Bax, Cyto C and caspase 3 in HT-29 cells were significantly increased in a dose-dependent manner after treatment with compound **32j** (1, 2 and 4

μM). Meanwhile, treatments with compound **32j** also dramatically down-regulated the expression of the anti-apoptotic protein Bcl-xl in a dose-dependent manner. These results suggested that compound **32j** induced cell apoptosis by interfering with the expression of apoptosis-related proteins.

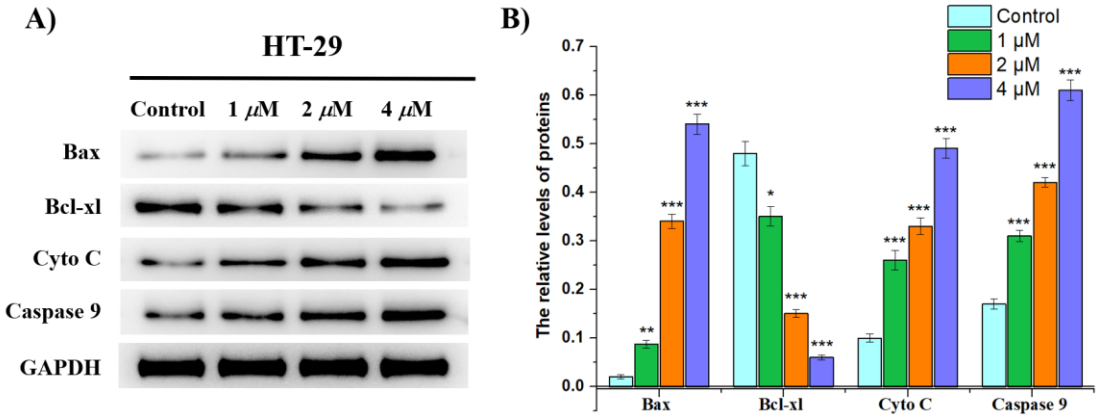


Figure 10. HT-29 cells were incubated with various concentrations of **32j** (0, 1, 2 and 4 μM) for 48 h. (A) The expression of Bax, Bcl-xl, Cyto C, Caspase 9 were determined by Western blotting using specific antibodies. GAPDH was used as internal control. (B) Histograms display the density ratios of Bax, Bcl-xl, Cyto C, and Caspase 9 to GAPDH. Data are represented as mean ± SD of three independent experiments. **p < 0.01, ***p < 0.001 *vs.* control group.

*2.9 In vivo antitumor activity of compound **32j***

In order to evaluate the *in vivo* anti-tumor activity of compound **32j**, colon cancer xenograft mouse model was established by subcutaneous inoculation of NQO1-overexpressing HT-29 cells into the right flank of mice. The tumor size and the body weights of mice were monitored and recorded every 2 days. As shown in Figure 11, compound **32j** reduced tumor weights by 72.69% at a dose of 30 mg/kg per day (i.p.), which was more potent than the positive controls 23-HBA and 5-fluorouracil (inhibitory rates of 56.81% at a dose of 30 mg/kg per day and 65.07% at a dose of 30 mg/kg per 2 days, respectively). Meanwhile, **32j** did not obviously affect the body weight even at a dose of 30 mg/kg, while treatment with 5-fluorouracil at a dose of 30 mg/kg led to a slight decrease of body weight. Collectively, compound **32j** displayed remarkable *in vivo* antitumor activity and safety, which presumably could be ascribed to

successful approaches by applying the NQO1-activated prodrugs. In terms of efficacy and safety, compound **32j** is worthy of further investigation for the treatment of cancers.

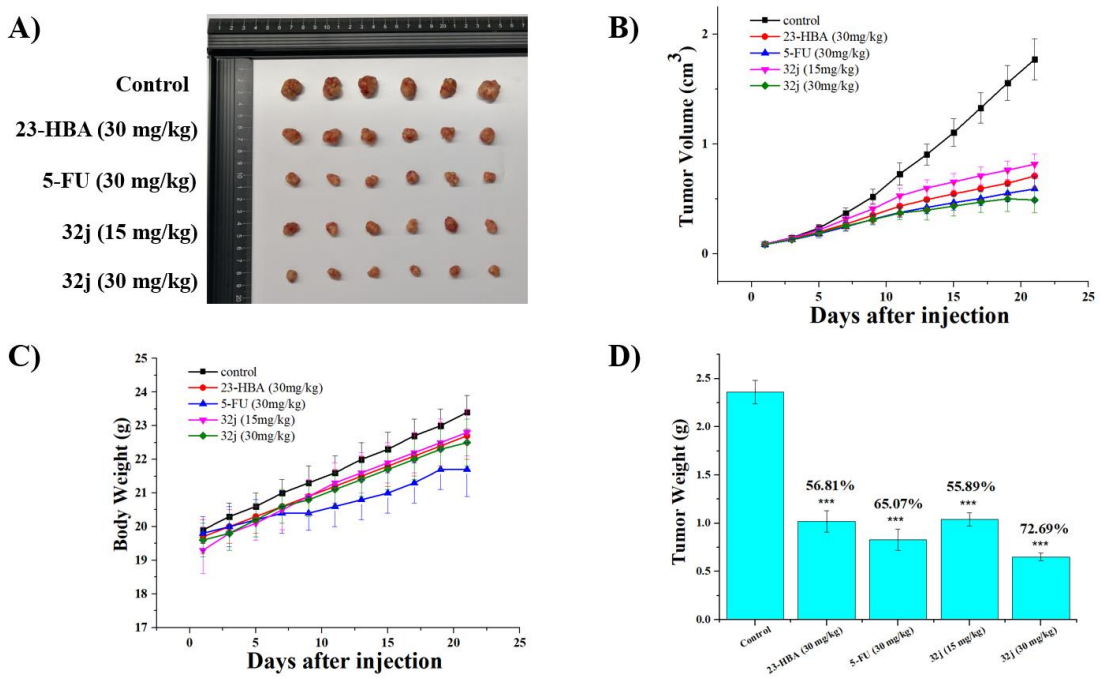


Figure 11. Compound **32j** inhibited colon tumor growth i*n vivo*. After administered with vehicle, 23-HBA (30 mg/kg per day), 5-fluorouracil (5-FU, 30 mg/kg per 2 days), **32j** (15 mg/kg per day) and **32j** (30 mg/kg per day) by intraperitoneal injection for three weeks, the mice were sacrificed, and the tumors were weighted. A) The images of tumors from mice at 21 days after initiation of treatment. B) Tumor volume changes of mice during treatment. C) Body weight changes of mice during treatment. D) The weight of the excised tumors of each group. ***P < 0.001 *vs* control group.

3. *Conclusion*

In summary, a series of NQO1-activated prodrugs of 23-HBA were designed and synthesized by introducing indolequinone nucleus to the C-3, C-23 or C-28 position of 23-HBA and its analogues. Antiproliferative screening of these target compounds validated that the representative compound **32j** showed satisfactory inhibitory activities against NQO1-overexpressing A549 and HT-29 cells (IC_{50} = 2.36 and 1.87 μM). Further mechanism study revealed that compound **32j** significantly increased cell apoptosis, caused cancer cells arrest at the G2/M phase, depolarized MMP and induced the generation of ROS. Moreover, western blot analysis indicated that compound **32j**

down-regulated the expression of anti-apoptotic protein Bcl-xl, up-regulated the expression of pro-apoptotic protein Bax, and activated cytochrome C and caspase 3 to cause cell apoptosis. More importantly, the results of *in vivo* antitumor experiment showed that **32j** effectively suppressed the xenografts tumor of NQO1-overexpressing HT-29 cells by 72.69% without apparent toxicity, suggesting compound **32j** deserves to be further investigated as a potent antitumor prodrug candidate for personalized cancer therapy.

4. Experimental section

4.1. Chemistry

4.1.1. General

Most chemicals and solvents were purchased from commercial sources. Further purification and drying by standard methods were employed when necessary. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AV-300 spectrometer in the indicated solvents (TMS as internal standard): the values of the chemical shifts are expressed in d values (ppm) and the coupling constants (*J*) in Hz. EI-MS spectra were recorded on an Agilent1100-LC-MSD-Trap/SL spectrometer and High-resolution mass spectra (HRMS) were recorded using an Agilent QTOF 6520.

*4.1.2 Methyl 3, 23-dihydroxy-lup-20(29)-en-28-oate (**5a**)*

K_2CO_3 (500 mg, 3.61 mmol) and iodomethane (0.079 mL, 1.27 mmol) were added to a solution of compound **4** (500 mg, 1.06 mmol) in DMF (10 mL) successively. The mixture was stirred for 5 h at room temperature and then poured into water (15 mL) and extracted with ethyl acetate (30 mL × 3). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether/ethyl acetate = 4:1) to afford compound **5a** as a white solid (447 mg, 86.9%), ESI-MS m/z: 487.4 $[M + H]^+$.

Compound **5b, 7a-c** were obtained by using the similar synthetic procedure of compound **5a**.

*4.1.3 Methyl 3-hydroxy-23-acetoxy-lup-20(29)-en-28-oate (**6a**)*

Compound **5a** (1 g, 1.78 mmol) was dissolved in dichloromethane (20 mL), after

which acetic anhydride (0.2 mL, 2.13 mmol) and 4-dimethylaminopyridine (44mg, 0.36 mmol) were added. The reaction mixture was stirred at room temperature for 0.5 h. After reaction completion, the mixture was diluted with water and extracted with ethyl acetate. The combined organic extracts were washed with water and brine, dried over Na_2SO_4, followed by concentration in vacuo to give the crude product. The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate = 10:1) to afford compound **6a** as a white solid (370mg, 76.1%), ESI-MS *m/z*: 529.4 $[M + H]^+$.

Compounds **6b-c** was obtained by using the similar synthetic procedure of compound **5a**.

*4.1.4 3, 23-diacetyl-lup-20(29)-en-28-oic acid (**8**)*

Compound **4** (1 g, 1.78 mmol) was dissolved in dichloromethane (20 mL), after which acetic anhydride (0.2 mL, 2.13 mmol) and 4-dimethylaminopyridine (44mg, 0.36 mmol) were added. The reaction mixture was stirred at room temperature for 0.5 h. After reaction completion, the mixture was diluted with water and extracted with ethyl acetate. The combined organic extracts were washed with water and brine, dried over Na_2SO_4, followed by concentration in vacuo to give the crude product. The product was purified by chromatography on silica gel (petroleum ether/ethyl acetate = 10:1) to afford compound **8** as a white solid (370mg, 76.1%), ESI-MS *m/z*: 557.4 $[M + H]^+$.

*4.1.5 N-[3, 23-dihydroxy-lup-20(29)-en-28-oyl]-1, 2-diaminoethane (**9**)*

Ethanediamine (14.4 μL, 0.22 mmol), EDCI (51.7 mg, 0.27 mmol) and DMAP (32.9 mg, 0.27 mmol) were added to a solution of compound **8** (100 mg, 0.18 mmol) in dichloromethane (4 mL). The solution was stirred at room temperature for 16 h, and then extracted with ethyl acetate (30 mL × 3). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo to give the crude product. The product was used for the next step without further purification. (53 mg, 49.3%), ESI-MS *m/z*: 599.4 $[M + H]^+$..

Compounds **10** was obtained by using the similar synthetic procedure of compound **9**.

*4.1.6 Ethanol 3-(4-oxo-4-(piperazin-1-yl)butanoate)-23-acetoxy-lup-20(29)-en-28-oate (**14a**)*

Succinic anhydride (414.3 mg, 4.14 mmol) and DMAP (303.5 mg, 2.48 mmol) were added to a solution of compound **6b** (500 mg, 0.83 mmol) in dichloromethane (20 mL). The mixture was refluxed for 5 h. After reaction completion, the reaction mixture was extracted with dichloromethane (80 mL × 3). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (dichloromethane / methanol = 40:1) to afford the intermediate **11a** as a white solid (455 mg, 74%). Subsequently, piperazine (24.46 mg, 0.142 mmol), EDCI (27.23 mg, 0.142 mmol) and DMAP (17.35 mg, 0.142 mmol) were added to a solution of compound **11a** (50 mg, 0.071 mmol) in dichloromethane (4 mL). The mixture was stirred for 2 h at room temperature. After reaction completion, the reaction mixture was extracted with ethyl acetate (20 mL × 3). The organic layer was washed with 1 N HCl solution, saturated sodium bicarbonate solution, water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether / ethyl acetate = 3:1) to afford the intermediate **12a** (40 mg, 64.6%). Pd/C (5 mg) was added to a solution of compound **12a** (50 mg, 0.65 mmol) in THF (5 mL). The mixture was stirred under atmospheric pressure of hydrogen for 0.5 h at room temperature. After reaction completion, the filtrate was obtained by filtration and concentrated in vacuo to obtain compound **13a**. K_2CO_3 (36 mg, 0.26 mmol) and 2-bromoethanol (6.4 μL, 0.088 mmol) were added to a solution of compound **13** (50 mg, 0.073 mmol) in DMF (5 mL) successively. The mixture was stirred for 1 h at room temperature and then poured into water (15 mL) and extracted with ethyl acetate (30 mL × 3). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether/ethyl acetate = 3:1) to afford compound **14a** as a white solid (37 mg, 69.5%), ESI-MS *m/z*: 727.4 $[M + H]^+$.

*4.1.7 Ethanol 3-(4-oxo-4-(piperazin-1-yl) butanamide)-23-acetoxy-lup-20(29)-en-28-oate (**14b**)*

Pyridinium chlorochromate (354.70 mg, 1.65 mmol) was added to a solution of compound **6b** (500.00 mg, 0.83 mmol) in dry dichloromethane (30 mL). The mixture

was stirred at room temperature for 3 h, then filtrated over Celite and the filtrate was concentrated in vacuo. The residue was chromatographed on silica gel (petroleum ether / ethyl acetate = 20:1) to give the product **15** as a white solid. Hydroxylamine hydrochloride (27.7 mg, 0.4 mmol) and sodium acetate (32.7 mg, 0.4 mmol) were added to a solution of compound **15** (120.0 mg, 0.2 mmol) in methanol (5 mL). The mixture was stirred overnight at room temperature. After reaction completion, the reaction mixture was concentrated in vacuo and extracted with ethyl acetate (20 mL × 3). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether / ethyl acetate = 10:1) to afford hydroxime intermediate as a white solid. Sodium cyanoborohydride (113.90 mg, 1.81 mmol) was added in portions to a solution of hydroxime intermediate (112.00 mg, 0.18 mmol) and ammonium acetate (139.70 mg, 1.81 mmol) in methanol (10 mL) at room temperature. Then, titanium trichloride hydrochloric acid solution (0.81 μL, 0.91 mmol, 15 - 20% in HCl) was added slowly into the reaction under ice bath. The mixture was stirred for 3h at room temperature. After reaction completion, the pH of the mixture was adjusted to 9 with 2N NaOH solution. The reaction mixture was extracted with dichloromethane (20 mL × 3). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether / ethyl acetate = 4:1) to afford compound **6c**. Then, compound **14b** was obtained by using the similar synthetic procedure of compound **14a**, ESI-MS *m/z*: 726.5 $[M + H]^+$.

*4.1.8 Ethanol 3-oxo-23-acetoxy-lup-20(29)-en-28-oate (**17**)*

The titled compound **17** was obtained following the procedure described for **14a,** ESI-MS *m/z*: 557.4 $[M + H]^+$.

*4.1.9 3-hydroxy-23-O-(4-piperidinylformyl)-lup-20(29)-en-28-oic acid (**21**)*

DCC (104 mg, 0.5 mmol) and DMAP (60 mg, 0.5 mmol) were added to a solution of 1-Boc-piperidine-4-carboxylic acid (114 mg, 0.5 mmol) in dry dichloromethane (10 mL). The mixture was stirred at room temperature for 1 h. Then, to the solution of mixture was added compound **5b** (281 mg, 0.5 mmol). The mixture was stirred at room

temperature for 12 h. After reaction completion, the reaction mixture was concentrated in vacuo and extracted with ethyl acetate (20 mL × 3). The organic layer was washed with 1N HCl, water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether / ethyl acetate = 8:1) to afford compound **18** as a white solid. Pd/C (25 mg) was added to a solution of compound **18** (120 mg, 0.16 mmol) in THF (10 mL). The mixture was stirred under atmospheric pressure of hydrogen for 0.5 h at room temperature. After reaction completion, the filtrate was obtained by filtration and concentrated in vacuo to obtain compound **19**. Trifluoroacetic acid (1.5 mL) was added to a solution of compound **19** (50 mg, 0.073 mmol) in dichloromethane (3 mL). The mixture was stirred for 30 min at room temperature. After reaction completion, the reaction mixture was concentrated in vacuo and extracted with ethyl acetate (20 mL × 3). The organic layer was washed with saturated sodium bicarbonate solution, water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (dichloromethane / methanol = 20:1) to afford compound **20** as a white solid (22 mg, 51%). K_2CO_3 (42 mg, 0.30 mmol) and 2-bromoethanol (7.3 μL, 0.103 mmol) were added to a solution of compound **20** (50 mg, 0.086 mmol) in DMF (5 mL) successively. The mixture was stirred for 1 h at room temperature and then poured into water (15 mL) and extracted with ethyl acetate (30 mL × 3). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether/ethyl acetate = 3:1) to afford compound **21** as a white solid (21 mg, 38%), ESI-MS *m/z*: 628.4 $[M + H]^+$.

*4.1.10 [N-(3-(dimethylamino) propyl)-N-(ethylcarbamoyl)] 3,23-dihydroxy-lup-20(29)-en-28-amide (**22**)*

EDCI (60.8 mg, 0.32 mmol) and DMAP (38.8 mg, 0.32 mmol) were added to a solution of 23-HBA (100 mg, 0.21 mmol) in dry dichloromethane (10 mL). The mixture was stirred at room temperature for 1.5 h. After reaction completion, the reaction mixture was concentrated in vacuo and extracted with ethyl acetate (20 mL × 3). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate,

and concentrated in vacuo. The residue was purified by chromatography on silica gel (dichloromethane / methanol = 30:1) to afford compound **22** (112 mg, 87.3%) as a white solid, ESI-MS *m/z*: 650.5$[M + Na]^+$.

*4.1.11 1', 4-bipiperidine-1-carbonyl 3, 23-dihydroxy-lup-20(29)-en-28-oate (**23**)*

Et_3N (0.1 mL, 0.71 mmol) and 4-piperidinopiperidine (500 mg, 2.97 mmol) were added to a solution of triphosgene (800 mg, 2.69 mmol) in dry dichloromethane (10 mL) under ice bath. The mixture was stirred at room temperature for 8 h. After reaction completion, the reaction mixture was concentrated in vacuo to give the acid chloride intermediate. Subsequently, the above acid chloride intermediate was added to a solution of 23-HBA (100 mg, 0.21 mmol) in dry pyridine (10 mL) under ice bath. The mixture was stirred at room temperature for 2 h. After reaction completion, the pH of the mixture was adjusted to 4-5 with 2N HCl solution. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (dichloromethane / methanol = 100:1) to afford compound **23** (122 mg, 86.5%) as a white solid, ESI-MS *m/z*: 667.5$[M + H]^+$.

Indolequinone compounds were synthesized according to previously reported procedures [4, 34], which were performed as follows:

*4.1.12 5-Methoxy-1H-indole-3-carbaldehyde (**25**)*

DMF (3.0 mL, 38.9 mmol) and $POCl_3$ (0.85 mL, 9.28 mmol) were stirred in an ice bath for 20 min until the yellow Vilsmeier compound formed. This Vilsmeier compound was then added to a solution of commercially available **24** (1.04 g, 6.45 mmol) in DMF (5 mL) and the reaction was stirred for 30 min at 0 °C. NaOH (2 M, 50 mL) was added and the solution was stirred for 10 min. Then the solution was extracted with dichloromethane (100 mL × 3), dried with Na_2SO_4 and evaporated. The obtained yellow solid 1.20 g (90%) was used without purification, ESI-MS *m/z*: 176.1 $[M + H]^+$.

*4.1.13 5-Methoxy-1-methyl-1H-indole-3-carbaldehyde (**26**)*

5-Methoxy-indole **25** (1.78 g, 9.40 mmol) was added gradually and under dry argon to a suspension of NaH (0.564 g of a 60% dispersion, 14.11 mmol) in dry DMF

(4 mL). The suspension was stirred at room temperature for 10 min and cooled to 0 °C, and MeI (0.81 g, 5.71 mmol) was added over 5 min. The solution was then heated at 40 °C for 30 min, cooled, poured into cold water. The mixture was extracted with ethyl acetate (150 mL × 3), dried and evaporated. The residue was used without purification (1.03 g, 95%), ESI-MS *m/z*: 190.1 $[M + H]^+$.

*4.1.14 5-Methoxy-1-methyl-4-nitro-1H-indole-3-carbaldehyde (**27**)*

Compound **26** (1.02 g, 5.39 mmol) was dissolved in acetic acid (88.7 mL), and a mixture of concentrated HNO_3 (3.2 mL in 18 mL acetic acid) was added dropwise at 0 ^oC over 1 h. After addition, the mixture was stirred at room temperature for 2 h, added to crushed ice (75 g), filtered, washed with H_2O and dried to give **27** (0.90 g, 70%) as a pale-yellow solid, ESI-MS *m/z*: 235.0 $[M + H]^+$.

*4.1.15 4-Amino-5-methoxy-1-methyl-1H-indole-3-carbaldehyde (**28**)*

To a suspension of **27** (1.2 g, 5.12 mmol) in EtOH (60 mL) was added tin (3.65 g, 30.73 mmol) followed by HCl (3.0 M, 60 mL). The mixture was stirred at 80 °C for 20 min. The solution was added to saturated $NaHCO_3$ (aqueous, 300 mL) and extracted with dichloromethane (200mL × 3). The organic layer was separated and evaporated, the yellow oil was purified on silica, eluting with ethyl acetate/hexane (1:1, R_f = 0.15) to give **28** (0.60 g, 57%) as a pale-yellow solid. ^1H NMR (300 MHz, Chloroform-*d*) δ 9.57 (s, 1H), 7.51 (s, 1H), 6.92 (d, J = 8.6 Hz, 1H), 6.51 (d, J = 8.6 Hz, 1H), 5.79 (s, 2H), 3.87 (s, 3H), 3.74 (s, 3H); ESI-MS *m/z*: 205.1 $[M + H]^+$.

*4.1.16 3-Formyl-5-methoxy-1-methylindole-4,7-dione (**29**)*

Fremy's salt (1.2 g, 4.4 mmol) in a solution of NaH_2PO_4/Na_2HPO_4 buffer (18.9 mL, 0.3 M, pH 6.4) was added to a solution of compound **28** (0.18 g, 0.9 mmol) in Me_2CO (25 mL). The mixture was stirred for 2 h, excess Me_2CO was removed and the product was purified on silica, eluting with dichloromethane/methanol (300:1, R_f=0.15) to give **29** as a yellow solid (0.09 g, 45%). ^1H NMR (300 MHz, DMSO-d_6) δ 10.15 (s, 1 H), 7.94 (s, 1 H), 5.96 (s, 1 H), 3.98 (s, 3 H), 3.85 (s, 3 H); ESI-MS *m/z*: 220.1 $[M + H]^+$.

*4.1.17 3-(Hydroxymethyl)-5-methoxy-1-methylindole-4,7-dione (**30**)*

$NaBH_4$ (0.21g, 5.47 mmol) was added to a solution of compound **29** (0.2 g, 0.91 mmol) in anhydrous MeOH (10 mL) and THF (10 mL). The solution was stirred for 10

min at 0 °C and then evaporated in vacuo to give a solid which was diluted with dichloromethane (20 mL), washed with H_2O (15 mL) and saturated NaCl (15 mL), and condensed to an orange solid. The residue was purified on silica, eluting with ethyl acetate/hexane (1:1, R_f=0.21) to afford **30** (0.14 g, 71%). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.72 (s, 1H), 5.69 (s, 1H), 4.65 (s, 2H), 3.94 (s, 3H), 3.85 (s, 4H); MS (ESI) *m/z*: 222.1 $[M+H]^+$.

*4.1.18 4-((5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol-3-yl) methoxy)-4-oxobutanoic acid (**31a**).*

Succinic anhydride (73 mg, 0.63 mmol) and DMAP (77 mg, 0.63 mmol) were added to a solution of compound **30** (140 mg, 0.63 mmol) in dichloromethane (10 mL), and the mixture was stirred at room temperature for 38 h. The solution was added to H_2O (100 mL) and extracted with DCM (30 mL × 3). The organic layer was separated and evaporated, then purified on silica, eluting with dichloromethane/methanol (100:1, R_f=0.15) to give **31a** (0.12g, 64%) as an orange solid. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 10.8 (s, 1 H), 6.41 (s, 1 H), 4.92 (s, 1 H), 4.64 (s, 2 H), 3.91 (s, 3 H), 3.83 (s, 3H), 2.31 (m, 2 H), 2.18 (m, 2 H); MS (ESI) *m/z*: 322.1 $[M+H]^+$.

*4.1.19 5-((5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol-3-yl) methoxy)-5-oxopentanoic acid (**31b**).*

Compound **31b** was obtained by using the similar synthetic procedure of compound **31a** as an orange solid (0.10 g, 61%). ^{1}H NMR (300 MHz, Chloroform-*d*): δ (ppm) 10.9 (s, 1H), 6.33 (s, 1H), 4.99 (s, 1H), 4.65 (s, 2H), 3.87 (s, 3H), 3.77 (s, 3H), 2.31 (m, 2H), 2.29 (m, 2H), 2.15 (m, 2H); MS (ESI) *m/z*: 336.1 $[M+H]^+$.

*4.1.20 Benzyl 3-O-[(5-Methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol)-3-methoxyacyl)-propionyloxy]-23-acetoxy-lup-20(29)-en-28-oate (**32a**)*

Compound **31a** (39.8 mg, 0.13 mmol), EDCI (24 mg, 0.13 mmol) and DMAP (17 mg, 0.13 mmol) were added to a solution of compound **6c** (50 mg, 0.083 mmol) in dry dichloromethane (4 mL). The mixture was stirred at room temperature for 5 h. After reaction completion, the reaction mixture was extracted with ethyl acetate (20 mL × 3).

The organic layer was washed with 1 N HCl solution, saturated sodium bicarbonate solution, water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (dichloromethane / methanol = 20:1) to afford compound **32a** (23 mg, 34.0%) as a yellow solid. $[\alpha]^{20}_{D}$ = 16.856 (c 0.070 g/100 mL, MeOH); mp 113-115 °C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.85 (s, 1H), 5.67 (s, 1H), 5.43 – 5.32 (m, 1H), 5.29 (s, 1H), 4.74 (s, 1H), 4.62 (s, 1H), 4.22 (t, *J* = 6.7 Hz, 2H), 4.16 (s, 1H), 4.09 (t, *J* = 6.7 Hz, 4H), 3.94 (s, 2H), 3.82 (s, 2H), 3.66 (d, *J* = 11.6 Hz, 1H), 3.37 (d, *J* = 13.0 Hz, 1H), 3.01 (s, 1H), 2.87 (s, 2H), 2.79 (d, *J* = 15.5 Hz, 2H), 2.63 (s, 2H), 2.27 – 2.18 (m, 3H), 2.09 (d, *J* = 6.0 Hz, 3H), 2.06 (s, 2H), 1.69 (s, 3H), 1.65 – 1.62 (m, 2H), 1.60 (s, 2H), 1.42 (d, *J* = 1.7 Hz, 1H), 1.40 (s, 2H), 1.37 (s, 2H), 1.35 (s, 3H), 0.98 (d, *J* = 3.2 Hz, 3H), 0.92 (s, 3H), 0.88 (s, 3H), 0.77 (d, *J* = 9.3 Hz, 4H); ^{13}C NMR (125 MHz, Chloroform-*d*) δ 177.4, 173.5, 172.1, 170.9, 169.8, 160.4, 136.6, 130.1, 129.9, 121.5, 109.9, 106.8, 103.6, 73.2, 66.2, 64.9, 58.1, 56.5, 49.2, 46.9, 46.6, 43.3, 42.4, 40.8, 38.2, 36.9, 36.3, 34.0, 31.9, 30.5, 30.4, 29.7, 29.3, 27.2, 25.5, 22.7, 20.9, 19.1, 16.6, 16.0, 14.6, 14.1, 13.6, 12.9; HR-MS (ESI) *m/z*: calculated for $C_{47}H_{63}NO_{11}Na$ $[M + Na]^{+}$: 840.4293, found: 840.4297.

*4.1.21 3-O-[(5-Methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol)-3-methoxyacyl)-butyryl]-23-acetoxy-lup-20(29)-en-28-oic acid (**32b**)*

Following the procedure described for preparation of compound **32a**, compound **32b** was prepared from **6c** and **31b** as a yellow solid with 30.5% yield. $[\alpha]^{20}_{D}$ = 9.499 (c 0.060 g/100 mL, MeOH); mp 125-127 °C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.85 (d, *J* = 2.6 Hz, 1H), 5.68 (s, 1H), 5.29 (s, 1H), 5.27 (s, 1H), 4.78 (d, *J* = 5.7 Hz, 1H), 4.74 (s, 1H), 4.61 (s, 1H), 3.94 (s, 3H), 3.82 (s, 4H), 3.68 (t, *J* = 10.7 Hz, 1H), 3.00 (d, *J* = 10.7 Hz, 1H), 2.39 (tt, *J* = 12.7, 7.2 Hz, 5H), 2.29 – 2.13 (m, 2H), 2.07 (s, 2H), 2.02 (s, 3H), 1.98 – 1.90 (m, 3H), 1.69 (s, 3H), 1.65 (s, 2H), 1.59 (d, *J* = 11.8 Hz, 2H), 1.42 (s, 3H), 1.35 (s, 3H), 1.25 (s, 3H), 1.14 (d, *J* = 7.6 Hz, 3H), 0.97 (d, *J* = 4.1 Hz, 3H), 0.93 (s, 3H), 0.86 (d, *J* = 7.3 Hz, 4H), 0.83 – 0.72 (m, 4H); ^{13}C NMR (75 MHz, Chloroform-*d*) δ 182.2, 179.1, 177.4, 172.8, 172.5, 170.7, 160.3, 150.4, 129.9, 129.5, 121.4, 119.8, 109.8, 106.8, 74.4, 65.3, 58.2, 58.0, 56.6, 56.4, 50.5, 49.2, 48.0, 47.0, 42.4,

40.7, 38.4, 38.0, 37.0, 36.4, 33.9, 33.6, 33.2, 32.1, 30.5, 29.7, 26.9, 25.4, 23.2, 21.3, 20.9, 20.2, 19.4, 17.9, 16.6, 16.0, 14.6, 13.0; HR-MS (ESI) *m/z*: calculated for $C_{48}H_{65}NO_{11}Na$ $[M + Na]^+$: 854.4450, found: 854.4458.

*4.1.22 Methyl 3-O-[(5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol)-3-methoxyacyl)-butyryl]-23-acetoxy-lup-20(29)-en-28-oate (**32c**)*

Following the procedure described for preparation of compound **32a**, compound **32c** was prepared from **6a** and **31b** as a yellow solid with 71.3% yield. $[\alpha]^{20}_D$ = 37.031 (c 0.060 g/100 mL, MeOH); mp 107-109 °C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.84 (s, 1 H), 5.67 (s, 1 H), 5.27 (s, 2 H), 4.78 (d, *J* = 5.0 Hz, 1 H), 4.74 (s, 1 H), 4.60 (s, 1 H), 3.94 (s, 3 H), 3.84 – 3.80 (m, 5 H), 3.68 – 3.64 (m, 4 H), 3.07 – 2.92 (m, 1 H), 2.41 – 2.32 (m, 5 H), 2.25 – 2.17 (m, 2 H), 2.06 (s, 3 H), 1.98 – 1.86 (m, 5 H), 1.69 (s, 5 H), 1.34 (s, 3 H), 0.96 (s, 3 H), 0.91 (s, 3 H), 0.87 (s, 3 H), 0.79 (s, 3 H); ^{13}C NMR (75 MHz, Chloroform-*d*) δ 176.6, 172.8, 172.4, 171.0, 160.4, 150.5, 129.9, 129.6, 121.4, 119.7, 109.7, 106.8, 74.5, 65.3, 58.0, 56.6, 51.3, 50.6, 49.4, 48.0, 47.0, 42.4, 40.7, 38.2, 38.0, 37.0, 36.3, 33.9, 33.6, 33.2, 32.1, 29.6, 23.2, 21.0, 20.2, 19.4, 17.9, 16.6, 15.9, 14.6, 13.0; HR-MS (ESI) *m/z*: calculated for $C_{49}H_{68}NO_{11}$ $[M + H]^+$: 846.4787, found: 846.4791.

*4.1.23 Benzyl 3-O-[(5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol)-3-methoxyacyl)-butyryl]-23-hydroxy-lup-20(29)-en-28-oate (**32d**)*

Following the procedure described for preparation of compound **32a**, compound **32d** was prepared from **6b** and **31b** as a yellow solid with 76.8% yield. $[\alpha]^{20}_D$ = 20.732 (c 0.060 g/100 mL, MeOH); mp 120-122 °C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.37 – 7.35 (m, 5 H), 6.84 (s, 1 H), 5.67 (s, 1 H), 5.28 (s, 2 H), 5.17 – 5.06 (m, 2 H), 4.73 (s, 1 H), 4.60 (s, 1 H), 3.93 (s, 3 H), 3.81 (s, 4 H), 3.66 (d, *J* = 11.6 Hz, 1 H), 3.02 (td, *J* = 10.9, 4.4 Hz, 1 H), 2.64 – 2.61 (q, *J* = 4.8, 3.9 Hz, 4 H), 2.30 – 2.14 (m, 2 H), 2.04 (s, 3 H), 1.68 (s, 3 H), 0.94 (s, 3 H), 0.84 (s, 3 H), 0.78 (s, 3 H), 0.75 (s, 3 H); ^{13}C NMR (75 MHz, Chloroform-*d*) δ 177.4, 175.8, 171.7, 171.0, 160.4, 150.5, 136.5, 129.7, 128.5, 128.3, 128.1, 119.6, 106.8, 74.9, 66.6, 65.8, 65.3, 58.3, 56.6, 49.4, 48.0, 47.0, 42.4, 40.7, 38.2, 38.0, 37.0, 36.3, 31.5, 30.6, 30.2, 29.5, 29.2, 24.1, 21.0, 19.4, 17.9, 16.6, 15.8,

14.6, 13.0; HR-MS (ESI) *m/z*: calculated for $C_{55}H_{72}NO_{11}$ $[M + H]^+$: 922.5100, found: 922.5105.

*4.1.24 3-Hydroxy-23-O-[(5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol)-3-methoxyacyl)-propionyloxy]-lup-20(29)-en-28-oic acid (**32e**).*

Following the procedure described for preparation of compound **32a**, compound **32e** was prepared from **4** and **31a** as a yellow solid with 35.2% yield. $[\alpha]^{20}_D$ = 29.489 (c 0.055 g/100 mL, MeOH); mp 126-128 °C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.83 (s, 1 H), 5.68 (s, 1 H), 5.31 (s, 2 H), 4.74 (s, 1 H), 4.61 (s, 1 H), 4.16 (dd, *J* = 20.7, 9.8 Hz, 1 H), 3.94 (s, 3 H), 3.82 (s, 4 H), 3.41 (s, 2 H), 2.99 (s, 2 H), 2.68 (s, 4 H), 2.23 (s, 3 H), 1.69 (s, 3 H), 0.97 (s, 3 H), 0.93 (s, 3 H), 0.86 (s, 3 H), 0.74 (s, 3 H); ^{13}C NMR (125 MHz, Chloroform-*d*) δ 180.0, 179.0, 177.4, 172.7, 172.0, 160.4, 150.4, 130.0, 129.5, 121.4, 119.6, 109.7, 106.8, 72.2, 67.1, 58.4, 56.6, 56.3, 50.6, 49.9, 49.3, 48.2, 46.9, 42.4, 42.2, 41.9, 40.7, 38.4, 37.1, 36.3, 34.0, 32.2, 30.6, 29.7, 29.2, 26.3, 25.5, 20.9, 19.4, 18.2, 16.6, 16.0, 14.7, 11.8, 11.3. HR-MS (ESI) *m/z*: calculated for $C_{45}H_{61}NO_{10}Na$ $[M + Na]^+$: 798.4188, found: 7798.4192.

*4.1.25 3-Hydroxy-23-O-[(5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol)-3-methoxyacyl)-butyryl]-lup-20(29)-en-28-oic acid (**32f**).*

Following the procedure described for preparation of compound **32a**, compound **32f** was prepared from **4** and **31b** as a yellow solid with 33.7% yield. $[\alpha]^{20}_D$ = 20.232 (c 0.060 g/100 mL, MeOH); mp 102-104 °C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.83 (s, 1H), 5.68 (s, 1H), 5.36 (d, *J* = 5.2 Hz, 1H), 5.27 (s, 2H), 4.74 (d, *J* = 2.3 Hz, 1H), 4.60 (s, 1H), 4.21 (dd, *J* = 12.6, 5.9 Hz, 3H), 4.09 (t, *J* = 6.6 Hz, 2H), 3.95 (s, 3H), 3.83 (s, 3H), 3.40 (t, *J* = 7.9 Hz, 1H), 2.99 (d, *J* = 10.0 Hz, 1H), 2.85 (q, *J* = 15.6 Hz, 3H), 2.43 (t, *J* = 7.3 Hz, 4H), 2.24 (q, *J* = 8.2, 7.6 Hz, 2H), 1.99 (q, *J* = 7.6 Hz, 4H), 1.68 (s, 3H), 1.63 (d, *J* = 3.3 Hz, 2H), 1.58 (s, 2H), 1.43 (s, 2H), 1.40 (s, 2H), 1.34 (d, *J* = 7.4 Hz, 3H), 1.29 (s, 3H), 0.96 (s, 3H), 0.93 (s, 3H), 0.85 (s, 3H), 0.74 (s, 3H); ^{13}C NMR (125 MHz, Chloroform-*d*) δ 179.0, 177.3, 173.5, 172.7, 169.8, 160.4, 150.4, 130.0, 129.5, 121.5, 119.5, 109.7, 106.8, 73.2, 72.5, 67.0, 66.2, 64.9, 58.0, 56.5, 56.3, 50.7,

49.3, 48.4, 46.9, 43.3, 42.4, 42.1, 40.7, 38.4, 37.1, 36.3, 33.4, 33.3, 29.7, 25.5, 22.7, 20.3, 19.1, 16.6, 16.0, 14.7, 14.1, 13.6, 11.8; HR-MS (ESI) *m/z*: calculated for $C_{46}H_{63}NO_{10}Na$ $[M + Na]^+$: 812.4344, found: 812.4351.

*4.1.26 Benzyl 3-hydroxy-23-O-[(5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol)-3-methoxyacyl)-butyryl]-lup-20(29)-en-28-oate (**32g**)*

Following the procedure described for preparation of compound **32a**, compound **32g** was prepared from **5b** and **31b** as a yellow solid with 54.8% yield. $[\alpha]^{20}_D$ = 48.033 (c 0.055 g/100 mL, MeOH); mp 106-108 °C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.38 – 7.31 (m, 5 H), 6.82 (s, 1 H), 5.67 (s, 1 H), 5.27 (s, 2 H), 5.17 – 5.04 (m, 2 H), 4.72 (s, 1 H), 4.59 (s, 1 H), 4.14 (d, *J* = 11.5 Hz, 1 H), 3.94 (s, 3 H), 3.81 (s, 4 H), 3.39 (t, *J* = 8.2 Hz, 1 H), 3.01 (td, *J* = 10.8, 4.2 Hz, 1 H), 2.42 (td, *J* = 7.1, 2.1 Hz, 5 H), 2.31 – 2.10 (m, 2 H), 1.67 (s, 3 H), 1.33 (s, 3 H), 0.93 (s, 3 H), 0.83 (s, 3 H), 0.75 (s, 3 H); ^{13}C NMR (75 MHz, Chloroform-*d*) δ 179.0, 177.4, 175.8, 173.3, 172.7, 160.4, 150.6, 136.5, 130.0, 129.5, 128.5, 128.4, 128.3, 128.1, 121.5, 119.5, 109.6, 106.8, 72.4, 67.0, 65.7, 58.1, 56.6, 56.5, 50.7, 49.4, 48.3, 46.9, 42.4, 42.1, 40.6, 38.5, 38.2, 37.1, 36.9, 36.3, 34.0, 33.4, 33.2, 32.1, 30.6, 29.5, 26.3, 25.5, 20.9, 20.2, 19.4, 18.2, 16.6, 15.8, 14.7, 11.8; HR-MS (ESI) *m/z*: calculated for $C_{53}H_{70}NO_{10}$ $[M + H]^+$: 880.4994, found: 880.4995.

*4.1.27 1',4-bipiperidine-1-carbonyl-3-hydroxy-23-O-[(5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol)-3-methoxyacyl)-propionyloxy]-lup-20(29)-en-28-oate (**32h**)*

Following the procedure described for preparation of compound **32a**, compound **32h** was prepared from **23** and **31a** as a yellow solid with 72.9% yield. $[\alpha]^{20}_D$ = 12.066 (c 0.060 g/100 mL, MeOH); mp 114-116 °C; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 6.83 (d, *J* = 5.7 Hz, 1H), 5.67 (s, 1H), 5.34 (d, *J* = 4.9 Hz, 1H), 5.32 – 5.25 (m, 1H), 4.93 – 4.84 (m, 1H), 4.74 (s, 1H), 4.62 (s, 1H), 4.44 (d, *J* = 14.1 Hz, 1H), 4.21 (dd, *J* = 14.7, 8.8 Hz, 1H), 3.94 (s, 3H), 3.82 (s, 3H), 3.50 – 3.28 (m, 2H), 2.99 (t, *J* = 10.4 Hz, 3H), 2.92 – 2.87 (m, 1H), 2.66 (d, *J* = 15.9 Hz, 4H), 2.37 (t, *J* = 8.1 Hz, 1H), 2.23 (dd, *J* = 14.6, 7.0 Hz, 3H), 2.01 (q, *J* = 6.8, 6.4 Hz, 4H), 1.84 (d, *J* = 36.5 Hz, 4H), 1.69 (s, 4H), 1.63 (d, *J* = 11.6 Hz, 3H), 1.44 (d, *J* = 9.2 Hz, 3H), 1.37 (s, 4H), 1.33 (s, 4H), 1.27 (s, 4H), 0.98 (s, 3H), 0.96 (d, *J* = 2.9 Hz, 2H), 0.89 (s, 3H), 0.87 (d, *J* = 6.9 Hz, 3H), 0.74

(s, 2H); ^{13}C NMR (125 MHz, Chloroform-*d*) δ 179.0, 177.4, 175.8, 174.9, 172.1, 160.3, 129.5, 128.5, 128.2, 124.7, 123.4, 121.4, 119.5, 115.9, 109.6, 106.8, 86.0, 66.5, 65.7, 64.4, 61.8, 58.4, 56.5, 52.9, 51.1, 49.4, 46.7, 46.1, 42.5, 40.5, 40.0, 38.2, 36.3, 33.6, 32.3, 31.9, 31.6, 30.3, 29.7, 29.3, 29.1, 28.8, 27.9, 25.5, 24.0, 22.7, 20.9, 17.0, 15.5, 14.1, 12.7, 11.8, 11.4; HR-MS (ESI) *m/z*: calculated for $C_{56}H_{80}N_3O_{11}$ $[M + H]^+$: 970.5787, found: 970.5781.

*4.1.28 N-(3-(dimethylamino) propyl)-N-(ethylcarbamoyl) 3-hydroxy-23- O-[(5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol)-3-methoxyacyl)-propionyloxy]-lup-20(29)-en-28-amid (**32i)***

Following the procedure described for preparation of compound **32a**, compound **32i** was prepared from **22** and **31a** as a yellow solid with 63.7% yield. $[\alpha]^{20}_D$ = -13.353 (c 0.065 g/100 mL, MeOH); mp 108-110 ^{o}C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.84 (s, 1 H), 5.68 (s, 1 H), 5.29 (s, 2 H), 4.72 (s, 1 H), 4.60 (s, 1 H), 4.17 (d, *J* = 11.4 Hz, 1 H), 3.94 (s, 3 H), 3.82 (s, 5 H), 3.72 – 3.58 (m, 1 H), 3.44 – 3.38 (m, 2 H), 3.32 – 3.26 (m, 3 H), 3.02 – 2.88 (m, 2 H), 2.68 (s, 3 H), 2.43 (s, 1 H), 2.34 (s, 3 H), 2.25 (d, *J* = 14.5 Hz, 1 H), 1.91 – 1.78 (m, 2 H), 1.69 (s, 3 H), 1.26 (s, 3 H), 0.97 (s, 3 H), 0.88 (s, 3 H), 0.85 (s, 3 H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ 179.0, 177.4, 172.7, 172.1, 160.3, 156.7, 151.2, 129.9, 129.6, 121.4, 119.4, 109.2, 108.6, 106.8, 72.0, 67.0, 58.4, 58.3, 56.6, 55.7, 53.3, 50.8, 48.2, 46.2, 44.5, 44.4, 42.1, 42.0, 40.7, 38.5, 37.5, 37.1, 36.5, 36.3, 35.5, 34.1, 33.9, 31.9, 31.3, 29.7, 29.2, 29.2, 26.3, 25.6, 21.1, 19.6, 18.1, 16.7, 16.0, 14.8, 11.8; HR-MS (ESI) *m/z*: calculated for $C_{53}H_{79}N_4O_{10}$ $[M + H]^+$: 931.5791, found: 931.5787.

*4.1.29 Ethyl 5-((5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol-3-yl) oxy)-4-oxopentanoate 3, 23-dihydroxy-lup-20(29)-en-28-oate (**32j**).*

Following the procedure described for preparation of compound **32a**, compound **32j** was prepared from **7a** and **31a** as a yellow solid with 48.7% yield. $[\alpha]^{20}_D$ = 30.238 (c 0.050 g/100 mL, MeOH); mp 104-106 ^{o}C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.83 (s, 1 H), 5.67 (s, 1 H), 5.30 (s, 2 H), 4.73 (s, 1 H), 4.60 (m, 1 H), 4.27 – 4.10 (m, 3 H), 3.94 (s, 4 H), 3.82 (s, 5 H), 3.43 – 3.38 (m, 1 H), 3.03 – 2.95(m, 1 H), 2.67 (d, *J* = 3.6 Hz, 4 H), 2.27 – 2.17 (m, 3 H), 1.93 – 1.87 (m, 3 H), 1.68 (s, 3 H), 1.26 (s, 3 H), 0.97

(s, 3 H), 0.91 (s, 3 H), 0.85 (s, 3 H), 0.73 (s, 3 H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ 179.0, 177.4, 176.6, 172.7, 172.1, 160.3, 150.4, 129.9, 129.6, 121.4, 119.5, 109.7, 106.8, 72.1, 67.0, 65.7, 62.6, 61.6, 58.4, 56.6, 50.6, 49.4, 48.1, 47.0, 42.4, 42.2, 40.7, 38.5, 38.3, 37.1, 36.3, 34.0, 30.6, 29.7, 29.3, 29.2, 28.9, 26.3, 25.5, 22.6, 20.9, 19.4, 18.1, 16.7, 15.9, 14.7, 11.8; HR-MS (ESI) *m/z*: calculated for $C_{47}H_{66}NO_{11}$ $[M + H]^+$: 820.4630, found: 820.4640; calculated for $C_{47}H_{65}NO_{11}Na$ $[M + Na]^+$: 842.4450, found: 842.4463; the purity of compound **32j** was 99% (t_R = 6.357 min), which was estimated by HPLC (SHIMADZU Labsolutions, UV detection at λ = 285 nm) analysis on the Aglilent C18 column (4.6 × 150 mm, 5 μm) eluting at 1 mL/min of 90% methanol/10% water.

*4.1.30 Propyl 5-((5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol-3-yl) oxy)-4-oxopentanoate 3, 23-dihydroxy-lup-20(29)-en-28-oate (**32k**).*

Following the procedure described for preparation of compound **32a**, compound **32k** was prepared from **7b** and **31a** as a yellow solid with 46.3% yield. $[\alpha]^{20}_D$ = 21.518 (c 0.050 g/100 mL, MeOH); mp 132-134 ^{o}C; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 6.83 (s, 1 H), 5.67 (s, 1 H), 5.30 (s, 2 H), 4.73 (s, 1 H), 4.62 (s, 1 H), 4.26 – 4.15 (m, 1 H), 3.94 (s, 3 H), 3.82 (s, 4 H), 3.75 – 3.56 (m, 2 H), 3.41 (t, *J* = 7.7 Hz, 2 H), 3.04 – 2.97 (m, 2 H), 2.68 – 2.65 (s, 4 H), 2.23 (t, *J* = 12.2 Hz, 3 H), 2.00 – 1.97 (m, 2 H), 1.62 (s, 3 H), 1.26 (s, 3 H), 0.93 (s, 3 H), 0.86 (s, 3 H), 0.74 (s, 3 H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ 179.0, 177.5, 172.8, 172.1, 172.0, 160.3, 150.0, 130.0, 129.6, 121.4, 119.5, 109.9, 106.8, 72.2, 58.4, 58.0, 56.6, 50.7, 49.2, 46.6, 42.5, 42.2, 40.7, 39.4, 39.4, 38.2, 37.1, 36.3, 36.0, 33.0, 31.7, 30.7, 30.3, 29.7, 29.3, 29.2, 26.9, 26.3, 25.5, 23.5, 22.7, 20.9, 19.4, 16.7, 16.5, 16.1, 14.7, 11.9, 11.3; HR-MS (ESI) *m/z*: calculated for $C_{48}H_{68}NO_{11}$ $[M + H]^+$: 834.4787, found: 834.4788.

*4.1.31 Butyl 5-((5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol-3-yl) oxy)-4-oxopentanoate 3, 23-dihydroxy-lup-20(29)-en-28-oate (**32l**).*

Following the procedure described for preparation of compound **32a**, compound **32l** was prepared from **7c** and **31a** as a yellow solid with 42.9% yield. $[\alpha]^{20}_D$ = -158.450 (c 0.065 g/100 mL, MeOH); mp 140-142 ^{o}C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.83 (s, 1 H), 5.68 (s, 1 H), 5.27 (s, 2 H), 4.73 (s, 1 H), 4.61 (s, 1 H), 4.19 – 4.15 (m, 1 H),

3.94 (s, 4 H), 3.82 (s, 5 H), 3.43 – 3.38 (m, 1 H), 3.11 – 2.80 (m, 2 H), 2.41 (d, *J* = 7.3 Hz, 6 H), 2.25 – 2.19 (m, 2 H), 2.00 – 1.93 (m, 5 H), 1.68 (s, 3 H), 1.28 (s, 3 H), 0.96 (s, 3 H), 0.93 (s, 3 H), 0.86 (s, 3 H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ 179.1, 177.4, 174.2, 173.3, 172.8, 160.4, 149.6, 130.0, 129.7, 129.6, 121.5, 119.4, 106.8, 74.8, 72.4, 58.1, 58.0, 56.6, 50.7, 49.2, 48.3, 46.9, 46.7, 42.6, 42.5, 40.7, 39.4, 38.2, 38.0, 37.1, 36.9, 36.3, 33.6, 33.4, 33.2, 33.0, 31.7, 31.5, 30.7, 30.2, 29.7, 23.5, 22.7, 20.2, 19.4, 18.1, 16.8, 16.6, 16.0, 15.8, 14.7, 11.9; HR-MS (ESI) *m/z*: calculated for $C_{49}H_{70}NO_{11}$ $[M + H]^+$: 848.4943, found: 848.4949.

*4.1.32 N-ethyl-5-((5-methoxy-1-methyl-4, 7-dioxo-4,7-dihydro-1H-indol-3-yl) oxy)-4-oxopentanamide 3, 23-diacetoxy-lup-20(29)-en-28-amide (**32m**).*

Following the procedure described for preparation of compound **32a**, compound **32m** was prepared from **9** and **31a** as a yellow solid with 57.4% yield. $[\alpha]^{20}_D$ = -12.359 (c 0.050 g/100 mL, MeOH); mp 100-102 ^{o}C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.85 (s, 1H), 5.67 (s, 1H), 5.35 (d, *J* = 5.7 Hz, 1H), 5.30 (s, 2H), 4.78 (d, *J* = 4.9 Hz, 1H), 4.72 (d, *J* = 2.5 Hz, 1H), 4.60 (s, 1H), 3.93 (s, 3H), 3.82 (s, 3H), 3.69 (d, *J* = 11.8 Hz, 2H), 3.30 (ddd, *J* = 7.5, 5.4, 2.4 Hz, 2H), 2.88 (dd, *J* = 20.5, 10.2 Hz, 4H), 2.63 (s, 3H), 2.60 (s, 4H), 2.07 (s, 3H), 2.02 (s, 3H), 1.69 (s, 2H), 1.37 (s, 3H), 1.33 (d, *J* = 2.4 Hz, 6H), 1.25 (s, 9H), 1.17 (d, *J* = 7.2 Hz, 3H), 0.97 (s, 2H), 0.89 (s, 3H), 0.88 (d, *J* = 2.6 Hz, 3H), 0.80 (s, 3H); ^{13}C NMR (75 MHz, Chloroform-*d*) δ 179.0, 177.1, 171.1, 170.7, 160.3, 156.9, 156.1, 155.6, 150.8, 129.4, 120.0, 109.5, 106.8, 104.9, 97.6, 74.5, 65.4, 58.2, 56.6, 55.4, 53.3, 50.7, 48.9, 48.1, 47.9, 43.3, 42.0, 40.7, 40.6, 38.0, 37.4, 37.0, 36.4, 36.3, 35.6, 33.9, 32.1, 31.3, 30.3, 29.7, 25.5, 25.4, 23.1, 21.3, 21.0, 19.6, 17.9, 16.7, 15.9, 14.8, 12.9; HR-MS (ESI) *m/z*: calculated for $C_{51}H_{72}N_3O_{11}$ $[M + H]^+$: 902.5161, found: 902.5164.

*4.1.33 (E)-but-2-en-1-yl 5-((5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol-3-yl) oxy)-4-oxopentanoate 3, 23-diacetoxy-lup-20(29)-en-28-oate (**32n**).*

Following the procedure described for preparation of compound **32a**, compound **32n** was prepared from **10** and **31a** as a yellow solid with 41.7% yield. $[\alpha]^{20}_D$ = 20.799 (c 0.055 g/100 mL, MeOH); mp 140-142 ^{o}C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.38 (s, 1H), 6.87 (s, 1H), 5.72 (s, 1H), 5.31 (s, 2H), 4.83 (d, *J* = 11.2 Hz, 1H), 4.25 (t, *J* =

5.8 Hz, 2H), 3.99 (s, 4H), 3.87 (s, 3H), 3.76 (d, *J* = 11.6 Hz, 1H), 3.64 (s, 2H), 3.51 (d, *J* = 6.5 Hz, 2H), 2.70 – 2.65 (m, 4H), 2.52 (d, *J* = 6.7 Hz, 3H), 2.44 (s, 3H), 2.10 (s, 3H), 2.01 (t, *J* = 7.3 Hz, 2H), 1.88 (d, *J* = 7.6 Hz, 2H), 1.76 (d, *J* = 15.8 Hz, 3H), 1.67 (s, 5H), 1.57 (s, 2H), 1.40 (s, 4H), 1.08 (s, 4H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.92 (s, 3H), 0.86 (s, 3H). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 179.9, 179.0, 177.4, 172.8, 172.8, 172.6, 171.0, 169.6, 160.3, 129.7, 128.4, 128.0, 121.5, 119.45, 106.8, 86.0, 77.5, 77.1, 76.7, 74.5, 65.4, 61.4, 57.9, 56.6, 53.3, 52.97, 51.2, 48.3, 46.69, 46.1, 45.1, 41.6, 40.7, 40.6, 39.9, 38.2, 37.1, 36.3, 36.0, 33.6, 33.3, 33.1, 32.3, 31.9, 30.7, 29.6, 28.8, 28.0, 27.8, 26.5, 25.5, 24.0, 23.1, 21.0, 20.1, 17.0, 15.5, 13.5, 13.0. HR-MS (ESI) *m/z*: calculated for $C_{53}H_{72}NO_{13}$ $[M + H]^+$: 930.4998, found: 930.5001.

*4.1.34 Ethyl5-((5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol-3-yl) oxy)-4-oxopentan-3-(4-oxo-4-(piperazin-1-yl) butanoate)-23-acetoxy-lup-20(29)-en-28-oate (**32o**).*

Following the procedure described for preparation of compound **32a**, compound **32o** was prepared from **14a** and **31a** as a yellow solid with 43.5% yield. $[\alpha]^{20}_D$ = -7.527 (c 0.055 g/100 mL, MeOH); mp 112-114 ^{o}C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.35 (d, *J* = 4.3 Hz, 1H), 6.84 (s, 1H), 5.68 (s, 1H), 5.31 (s, 3H), 5.26 (s, 2H), 4.78 (dd, *J* = 11.3, 4.8 Hz, 1H), 4.24 (q, *J* = 5.8, 4.4 Hz, 1H), 3.95 (s, 4H), 3.83 (s, 4H), 3.71 (d, *J* = 11.8 Hz, 1H), 3.63 (s, 2H), 3.53 (d, *J* = 7.1 Hz, 2H), 2.67 (s, 2H), 2.62 (d, *J* = 5.7 Hz, 3H), 2.57 – 2.51 (m, 2H), 2.43 (d, *J* = 7.4 Hz, 2H), 2.41 – 2.36 (m, 2H), 2.06 (s, 4H), 1.97 (q, *J* = 8.1, 7.3 Hz, 2H), 1.85 (dd, *J* = 13.8, 3.2 Hz, 2H), 1.77 (d, *J* = 12.8 Hz, 2H), 1.63 (s, 2H), 1.53 (d, *J* = 4.1 Hz, 2H), 1.43 (d, *J* = 3.3 Hz, 2H), 1.39 (s, 2H), 1.34 (d, *J* = 5.2 Hz, 3H), 1.25 (s, 4H), 1.17 (d, *J* = 4.0 Hz, 2H), 1.03 (s, 3H), 0.96 (s, 3H), 0.91 (s, 3H), 0.89 (s, 3H), 0.87 (s, 3H), 0.82 (d, *J* = 3.2 Hz, 3H); ^{13}C NMR (125 MHz, Chloroform-*d*) δ 179.8, 179.0, 177.3, 172.7, 172.7, 172.6, 170.9, 169.6, 160.3, 136.4, 129.6, 128.4, 128.0, 121.5, 119.5, 106.8, 86.0, 74.5, 65.9, 65.4, 61.2, 57.9, 56.6, 53.2, 52.9, 51.2, 48.3, 46.7, 46.1, 45.0, 40.7, 40.6, 39.9, 38.2, 37.1, 36.3, 36.0, 33.5, 33.2, 33.1, 32.3, 31.9, 29.6, 28.7, 28.0, 27.8, 26.4, 25.5, 23.9, 23.0, 21.0, 20.1, 17.8, 17.0, 15.5, 13.5, 13.0; HR-MS (ESI) *m/z*: calculated for $C_{57}H_{80}N_3O_{14}$ $[M + H]^+$: 1030.5635, found: 1030.5636.

*4.1.35 Ethyl5-((5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol-3-yl) oxy)-4-oxopentan-3-(4-oxo-4-(piperazin-1-yl) butanamide)-23-acetoxy-lup-20(29)-en-28-oate (**32p**).*

Following the procedure described for preparation of compound **32a**, compound **32p** was prepared from **14b** and **31a** as a yellow solid with 39.8% yield. $[\alpha]^{20}_{D}$ = 133.824 (c 0.060 g/100 mL, MeOH); mp 104-106 ^{o}C; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.40 – 7.32 (m, 1H), 6.83 (s, 1H), 5.68 (d, *J* = 1.5 Hz, 1H), 5.38 – 5.32 (m, 1H), 5.27 (d, *J* = 1.7 Hz, 2H), 5.15 – 5.04 (m, 1H), 4.21 (ddd, *J* = 6.9, 5.1, 2.4 Hz, 2H), 3.94 (s, 3H), 3.82 (s, 3H), 3.70 – 3.60 (m, 2H), 3.50 (d, *J* = 20.1 Hz, 2H), 2.77 – 2.70 (m, 1H), 2.69 – 2.61 (m, 3H), 2.53 (s, 2H), 2.45 – 2.41 (m, 3H), 2.40 – 2.35 (m, 2H), 2.22 (t, *J* = 7.6 Hz, 1H), 2.02 – 1.96 (m, 3H), 1.94 (d, *J* = 3.3 Hz, 3H), 1.83 (ddd, *J* = 15.0, 11.5, 3.3 Hz, 2H), 1.66 (d, *J* = 15.5 Hz, 2H), 1.53 (d, *J* = 4.7 Hz, 2H), 1.42 (q, *J* = 3.3 Hz, 3H), 1.40 – 1.37 (m, 3H), 1.35 (d, *J* = 8.4 Hz, 3H), 1.32 (s, 2H), 1.29 (s, 2H), 1.26 (s, 3H), 1.22 – 1.20 (m, 1H), 1.03 (d, *J* = 1.9 Hz, 3H), 0.97 (d, *J* = 3.9 Hz, 3H), 0.93 (d, *J* = 5.9 Hz, 3H), 0.90 (d, *J* = 1.4 Hz, 3H), 0.88 (d, *J* = 2.5 Hz, 2H), 0.86 (d, *J* = 2.8 Hz, 2H), 0.73 (q, *J* = 6.3, 5.2 Hz, 3H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ 180.0, 179.0, 172.8, 169.9, 164.6, 160.3, 150.5, 129.7, 128.4, 128.0, 121.5, 119.5, 106.8, 86.1, 61.3, 58.0, 56.6, 46.7, 46.1, 41.3, 40.5, 39.9, 39.1, 37.0, 36.3, 36.0, 33.5, 33.3, 33.1, 32.3, 31.9, 30.3, 29.7, 29.1, 28.8, 27.9, 27.7, 26.5, 25.5, 24.0, 23.6, 22.6, 20.9, 20.4, 20.1, 19.4, 18.8, 18.1, 16.9, 15.5, 14.3, 14.1, 13.6, 12.7, 11.4; HR-MS (ESI) *m/z*: calculated for $C_{57}H_{81}N_4O_{13}$ $[M + H]^+$: 1029.5795, found: 1029.5786.

*4.1.36 Ethyl5-((5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol-3-yl) oxy)-4-oxopentanoate 3-oxo-23-acetoxy-lup-20(29)-en-28-oate (**32q**)*

Following the procedure described for preparation of compound **32a**, compound **32q** was prepared from **17** and **31a** as a yellow solid with 58.1% yield. $[\alpha]^{20}_{D}$ = -175.188 (c 0.050 g/100 mL, MeOH); mp 114-116 ^{o}C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 6.85 (s, 1 H), 5.68 (s, 1 H), 5.29 (s, 2 H), 4.73 (s, 1 H), 4.60 (s, 1 H), 4.40 (t, *J* = 6.2 Hz, 1 H), 4.30 (s, 3 H), 4.04 (s, 1 H), 3.93 (s, 3 H), 3.83 (s, 4 H), 3.55 – 3.47 (m, 1 H), 3.03 – 2.95 (m, 1 H), 2.69 – 2.67 (m, 5 H), 2.51 – 2.42 (m, 1 H), 2.28 – 2.03 (m, 2 H), 2.03 (s, 2 H), 1.68 (s, 3 H), 1.40 (s, 3 H), 0.99 (s, 3 H), 0.96 (s, 3 H), 0.94 (s, 3 H); ^{13}C NMR

(100 MHz, Chloroform-*d*) δ 214.9, 178.0, 177.3, 175.8, 172.1, 172.0, 170.8, 160.3, 150.3, 129.9, 129.5, 121.3, 119.5, 109.7, 106.7, 67.6, 64.0, 62.6, 61.5, 58.4, 56.6, 56.5, 50.1, 49.6, 49.2, 48.1, 46.9, 42.4, 40.6, 38.3, 38.1, 36.9, 36.5, 36.3, 35.0, 33.3, 31.9, 30.5, 29.5, 28.9, 28.6, 25.5, 21.3, 20.9, 19.5, 19.3, 17.2, 15.8, 15.7, 14.6; HR-MS (ESI) *m/z*: calculated for $C_{49}H_{66}NO_{12}$ $[M + H]^+$: 860.4580, found: 860.4575.

*4.1.37 Ethyl 5-[(5-methoxy-1-methyl-4,7-dioxo-4,7-dihydro-1H-indol-3-yl) oxy)-4-oxopentanoate] 3-hydroxy-23-O-(4-piperidinylformyl)-lup-20(29)-en-28-oate (**32r**)*

Following the procedure described for preparation of compound **32a**, compound **32r** was prepared from **21** and **31a** as a yellow solid with 36.3% yield. $[\alpha]^{20}_D$ = 28.829 (c 0.065 g/100 mL, MeOH); mp 104-106 °C; ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.37 (d, *J* = 4.8 Hz, 1H), 7.34 (d, *J* = 3.9 Hz, 1H), 6.85 (s, 1H), 5.68 (s, 1H), 5.35 (d, *J* = 5.8 Hz, 1H), 5.30 (s, 2H), 5.15 – 5.03 (m, 1H), 4.77 – 4.54 (m, 1H), 4.29 (q, *J* = 7.7, 5.8 Hz, 2H), 4.20 – 4.09 (m, 1H), 3.94 (s, 4H), 3.82 (s, 4H), 3.42 (dd, *J* = 34.1, 10.4 Hz, 2H), 2.97 (s, 3H), 2.73 (s, 2H), 2.67 (s, 3H), 2.21 (td, *J* = 19.4, 17.6, 11.8 Hz, 3H), 2.07 – 1.94 (m, 3H), 1.85 (d, *J* = 15.8 Hz, 2H), 1.67 (s, 3H), 1.53 (d, *J* = 4.0 Hz, 2H), 1.45 – 1.36 (m, 6H), 1.33 (s, 2H), 1.25 (s, 6H), 1.15 – 1.08 (m, 2H), 1.03 (s, 3H), 0.96 (s, 3H), 0.91 (d, *J* = 2.6 Hz, 3H), 0.88 (d, *J* = 2.0 Hz, 2H), 0.83 (s, 2H), 0.74 (d, *J* = 4.1 Hz, 2H); ^{13}C NMR (125 MHz, Chloroform-*d*) δ 179.0, 177.4, 175.8, 174.8, 172.1, 160.3, 129.5, 128.5, 128.2, 124.7, 123.4, 121.4, 119.5, 115.9, 109.6, 106.8, 86.0, 66.5, 65.7, 64.4, 61.8, 58.4, 56.5, 52.9, 51.1, 49.4, 46.7, 46.1, 42.5, 40.5, 40.0, 38.2, 36.3, 33.6, 32.3, 31.9, 31.6, 30.3, 29.7, 29.3, 29.1, 28.8, 27.9, 25.5, 24.0, 22.7, 20.9, 17.0, 15.5, 14.1, 12.7, 11.8, 11.4; HR-MS (ESI) *m/z*: calculated for $C_{53}H_{75}N_2O_{12}$ $[M + H]^+$: 931.5315, found: 931.5318.

4.2 Biology

4.2.1 In vitro anti-proliferative assay

HepG2, A375, A549, HT-29, H596 and HFL-1 cells were purchased from Nanjing KeyGen Biotech Co. Ltd. (Nanjing, China). The cytotoxicity of the test compounds was determined using the MTT assay. Briefly, the cells were incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h in 96-microwell plates. After medium removal,

100 μL of culture medium with 0.1% DMSO containing the test compounds at different concentrations were added to each well and incubated at 37 ºC for another 72 h. The MTT (5 mg/mL in PBS) was added and incubated for another 4 h, the optical density was detected with a microplate reader at 560 nm. The IC_{50} values were calculated according to the dose-dependent curves. All the experiments were repeated in at least three independent experiments.

*4.2.2 Metabolism of **32j** by NQO1*

A 10 mM stock solution of **32j** was prepared in DMSO and stock solutions of 4 mM NADH and 100 μM FAD were prepared in TRIS buffer pH 7.4. The enzyme stock solution was prepared by dissolving 1.5 mg of the lyophilized human DT-Diaphorase (Sigma, ≥100 units; wherein 1 unit is 1.0 μmole cytochrome C reduced per min/mg) in TRIS buffer pH 7.4 to make a solution of 40 mU/μL. The reaction mixture was prepared according the Supplementary Table S2. The reaction mixtures were stirred at 37 ºC and were monitored by HPLC analysis carried out on a Prominence LC-20AT HPLC system equipped with a column (Agilent eclipse XDB C18 5 μm, 4.6*250 mm). The mobile phase used for elution was a linear gradient beginning with 50:50 (v/v) methanol: water at a flow rate of 1 mL/min.

4.2.3 Cell apoptosis analysis

Annexin V-FITC/PI dual staining assay was performed to determine the cells apoptosis. HT-29 cells were incubated with 1, 2, 4 μM compound **32j** in 6-well cell culture plates for 48 h. The cells were washed twice in PBS, then 500 μL binding buffer suspended cells were added. The cells were stained with 5 μL Annexin V-FITC and PI. Then the cells were incubated at room temperature for 20 min without light exposure. Apoptosis was analyzed using a FACS Calibur flow cytometer (Bectone Dickinson, San Jose, CA, USA).

4.2.4 Cell cycle analysis

HT-29 cells were seeded into 6-well plates and incubated at 37 ºC in a humidified 5% CO_2 incubator for 24 h, and then treated with or without **32j** at indicated concentrations for another 48 h. The collected cells were fixed by adding 70% ethanol at 4 ºC for 12 h. Subsequently, the cells were resuspended in PBS containing 100 μL

RNase A and 400 μL of propidium iodide for 30 min. The DNA content of the cells was measured using a FACS Calibur flow cytometer (Bectone Dickinson, San Jose, CA, USA).

4.2.5 Measurement of intracellular ROS generation

Intracellular ROS production was detected by using the peroxide-sensitive fluorescent probe DCF-DA. In brief, after treatment with 0, 1, 2, 4 μM **32j** for 48 h, HT-29 cells were incubated with 10 μM DCF-DA at 37 ºC for 15 min. The intracellular ROS mediated oxidation of DCF-DA to the fluorescent compound 2',7'-dichlorofluorescein (DCF). Then cells were harvested and the pellets were suspended in 1 mL PBS. Samples were analyzed at an excitation wave length of 480 nm and an emission wave length of 525 nm by flow cytometry on an FC500 cytometer (Beckman Coulter)

4.2.6 Mitochondrial membrane potential assay

After treatment with vehicle control 0.1% DMSO, **32j** (1, 2 and 4 μM) for 48 h, the cells were washed in PBS and resuspended in 500 μL JC-1 incubation buffer at 37 ºC for 15 min. The percentage of cells with healthy or collapsed mitochondrial membrane potentials was monitored by flow cytometry analysis (Bectone-Dickinson, San Jose, CA, USA).

4.2.7 Western Blot analysis

HT-29 cells were incubated in the presence of **32j**, and after 48 h, were collected, centrifuged, and washed two times with ice cold PBS. The pellet was then re-suspended in lysis buffer. After the cells were lysed on ice for 20 min, lysates were centrifuged at 13000 g at 4 ºC for 15 min. The protein concentration in the supernatant was determined using the BCA protein assay reagents. Equal amounts of protein (20 μg) were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8-12% acrylamide gels) and transferred to PVDF Hybond-P membrane. Membranes were blocked for 1 h at room temperature. Membranes were then incubated with primary antibodies against Bax, Bcl-xl, cytochrome C and caspase-9, the membrane being gently rotated overnight at 4 ºC. The bound antibodies were detected using horseradish peroxidase (HRP)-conjugated second antibodies and visualized by the enhanced

chemiluminescent reagent.

4.2.8 In vivo anti-tumor evaluation

Five-week-old female Institute of Cancer Research (ICR) mice were purchased from Shanghai SLAC Laboratory Animals Co. Ltd. A total of 1×10^7 HT-29 cells were subcutaneously inoculated into the right flank of ICR mice according to protocols of tumor transplant research, to initiate tumor growth. After incubation for one day, mice were weighted and divided into five groups at random with six animals in each group. The groups treated with **32j** was administered 15, 30 mg/kg in a vehicle of 10% DMSO/2% Tween 80/88% saline every day by intraperitoneal injection, respectively. The positive control groups were treated with 23-HBA (30 mg/kg) every day and 5-fluorouracil (30 mg/kg) every 2 days by intraperitoneal injection, respectively. The negative control group received a vehicle of 10% DMSO/2% Tween 80/88% saline through intraperitoneal injection. The mice were sacrificed after the treatments for a total 21 consecutive days and the tumors were excised and weighed. The inhibition rate was calculated as follows: Tumor inhibitory ratio (%) = (1-average tumor weight of treated group/average tumor weight of control group) × 100%.

Acknowledgments

This study was supported from the National Natural Science Foundation of China (No. 81973167, 82173672, and 82173679), "Double First-Class" University project of China Pharmaceutical University (CPU2018GY04, CPU2018GY35), and Innovation and Entrepreneurship Training Program of China Pharmaceutical University (202110316011Z), for financial support.

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