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18β-Glycyrrhetinic acid derivatives induced mitochondrial-mediated apoptosis through reactive oxygen species-mediated p53 activation in NTUB1 cells

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ABSTRACT

Twenty six 18β-glycyrrhetinic acid (GA) (**1**) derivatives **2–27** including twelve new GA derivatives **10**, **11**, **13–17**, **21–25** were synthesized and evaluated for cytotoxicities against NTUB1 cells (human bladder cancer cell lines). *seco*-Compounds **9**, **25**, and **27** are the most potent compounds of this series, inhibiting cell growth of human NTUB1 cells with an IC_{50} values of 2.34 ± 0.28, 4.76 ± 1.15, and 3.31 ± 0.61 μM, respectively. Exposure of NTUB1 to **25** for 24 h significantly increased the production of reactive oxygen species (ROS). Flow cytometric analysis exhibited that treatment of NTUB1 with **25** did not induce cell cycle arrest but accompanied by an increase of apoptotic cell death in a dose-dependant manner after 24 h. Mitochondrial membrane potential (MMP) decreased significantly in a dose-dependant manner when the NTUB1 cells were exposed to **25** for 24 h. Marked collapse of the MMP suggested that dysfunction of the mitochondria may be involved in the oxidative burst and apoptosis induced by **25**. Western blot analysis shows that NTUB1 cells treated with **25** increased the level of *p*-p53 in a dose-dependant manner. Further, NAC treatment prevented p53 phosphorylation stimulated by **25**. These results suggested that **25** induced a mitochondrial-mediated apoptosis in NTUB1 cells through activation of p53, which are mainly mediated ROS generated by **25**.

1. Introduction

Triterpenoids abundantly exist in plant kingdom. The natural triterpenoid, erythrodiol, uvaol, and β-amyrin have been reported to have cytotoxic effect against several human cancer cell lines. These triterpenoids induced cycle cell arrest and promoted apoptosis in human cancer cells through a ROS-dependent mechanism.[1–3] We also reported that a series of ursolic acid derivatives induced cell cycle arrest in NTUB1 cells associated with ROS.[4] Thus it is generally believed that natural or synthetic triterpenoids induce cell death by oxidative stress.

Oxidative stress can induce cell death by apoptosis and apoptotic cell death is a process control by a specific signaling pathway.[5] Several studies have demonstrated that the mitochondria stress and caspase activation are the most typical events required for apoptosis and the mode of cell death induced by oxidative stress differently affected according to the types of cells examined and the modes to generate ROS.[5]

Recently, several 18β-glycyrrhetinic acid (18β-GA) derivatives have been shown to possess cytotoxicity against several human cancer cell lines.[6–8] However, a series of structures and cytotoxic relationships of GA derivatives did not appear in literature. Based on the above reason, we synthesized a series of GA derivatives, evaluated their cytotoxicities against human NTUB1 cells, and discussed their structure and cytotoxic relationships and mechanism of action. In this study, we examined whether GA derivative induces apoptosis using human bladder cancer cell lines, NTUB1. We also investigated the cellular mechanism of GA derivative-induced cell death. We have demonstrated that GA derivative-stimulated ROS production leads to activation of p53 in the cells.

2. Results and discussion

2.1. Chemistry

Compounds **2–27** were synthesized as depicted in Schemes 1–3. Starting material, 18β-GA (**1**) was oxidized to 3-keto compound (**2**)

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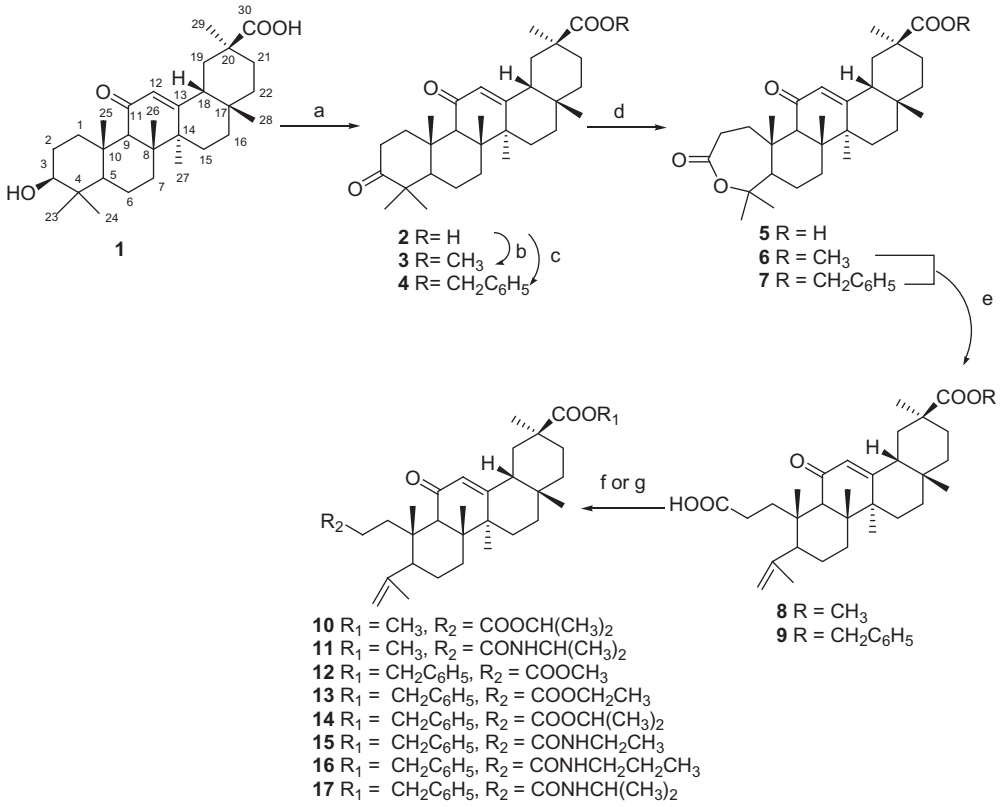
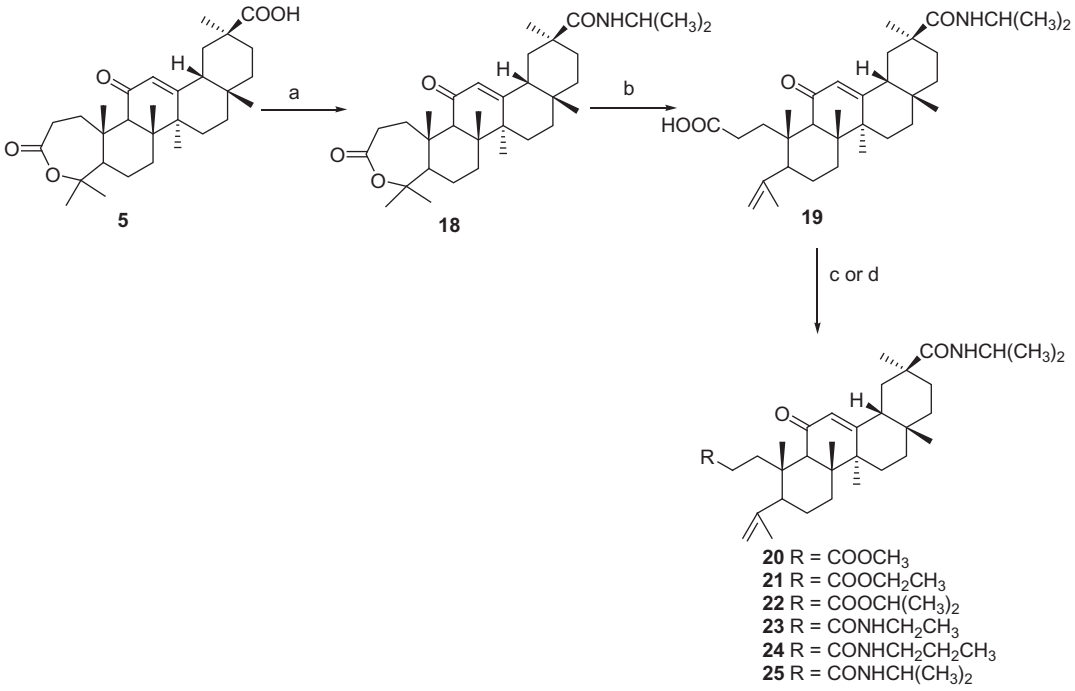
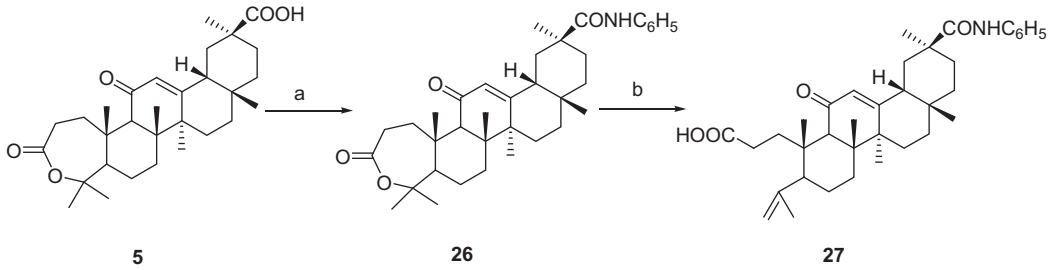


Scheme 1. Reagents and conditions: (a) CrO_3, DMF, rt, 12 h; (b) CH_3OH, H_2SO_4, reflux, 48 h; (c) EDC, DMAP, CH_2Cl_2, ROH; (d) *m*-CPBA, CH_2Cl_2, rt, 12 h; (e) CH_2Cl_2, *p*-TSA, rt, 24 h; (f) various alkyl halides, K_2CO_3, $(CH_3)_2CO$; (g) EDC, DMAP, CH_2Cl_2, R′-NH_2, rt, 24 h.



Scheme 2. Reagents and conditions: (a) EDC, DMAP, CH_2Cl_2, R′-NH_2, rt, 24 h; (b) CH_2Cl_2, *p*-TSA, rt, 24 h; (c) various alkyl halides, K_2CO_3, $(CH_3)_2CO$; (d) EDC, DMAP, CH_2Cl_2, R′-NH_2, rt, 24 h.

using CrO_3 in DMF.[9] The treatment of 3-oxo-derivative **2** with excess of MeOH in the presence of H_2SO_4 as catalyst and benzyl alcohol in the presence of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDCI) and 4-dimethylaminopyridine (DMAP) to yield **3** and **4**, respectively.[9] The treatment of 3-oxo-derivatives **2**, **3**, and **4** with *m*-chloroperbenzoic acid (*m*-CPBA), respectively,



Scheme 3. Reagents and conditions: (a) EDC, DMAP, CH_2Cl_2, R′-NH_2, rt, 24 h; (b) CH_2Cl_2, *p*-TSA, rt, 24 h.

provided lactones **5**, **6**, and **7**.[9] The lactone ring of **6** or **7** was cleaved by treatment of *p*-toluenesulfonic acid (*p*-TSA) in appropriate solvent to give products **8** or **9**. Treatment of *seco*-methyl ester **8** with isopropyl alcohol or isopropylamine in the presence of EDCI as the activating agent and DMAP as the catalyst provided the corresponding 3-ester compound **10** or 3-amide compound **11**. The *seco*-benzylester **9** in acetone reacted with alkyl halide and K_2CO_3 in acetone or treatment with various alkylamines in the presence of EDCI and DMAP to afford *seco*-compounds **12–14** or **15–17** (Scheme 1).

Compound **5** in CH_2Cl_2 reacted with isopropylamine or aniline in the presence of EDCI and DMAP to afford lactone **18** or **26**. The lactone **18** or **26** in CH_2Cl_2 reacted with *p*-TSA to give *seco*-compound **19** or **27**. The *seco*-compound **19** in acetone reacted with various alkyl halides and K_2CO_3 or in CH_2Cl_2 reacted with alkylamine in the presence of EDCI and DMAP to afford **20–22** or **23–25** (Schemes 2 and 3).

The known 18β-GA derivatives **2–9**, **12**, **18**, **19**, **20**, **26**, and **27** were identified by spectroscopic data and compared with those of data reported in literature.[9] The new 18β-GA derivatives were characterized by various spectroscopic methods (Table 1 and Experimental Section) and compared with data reported in literature.[9]

2.2. Biological results and discussion

Cytotoxicity of **1** and its derivatives against NTUB1 cells were studied and the cisplatin was used as positive control (Tables 2–4). As shown in Table 2, several 18β-GA derivatives showed significant cytotoxic activities against NTUB1 cells. The oxidation of C-3 of **1** such as **2** significantly enhanced the cytotoxic activity against NTUB1 cells while esterification of carboxylic acid group at C-30 decreased the cytotoxic activity against NTUB1 cells. The lactonization of **2** and **4** at ring A significantly weakened the cytotoxic activity against NTUB1 cells while the lactonization of **3** at ring A significantly increased the cytotoxic effect against NTUB1 cells. The amidation of **5** with aniline such **26** also significantly increased the cytotoxic effect against NTUB1 cells (Table 4). The cleavage of the lactone ring in CH_2Cl_2 by treatment of *p*-TSA such as **6**, **7**, **18**, and **26** significantly enhanced the cytotoxicity against NTUB1 cells, such as **8**, **9**, **19**, and **27** (Tables 2–4). It clearly indicated that cleaving the lactone ring of 18β-GA derivative in CH_2Cl_2 generally significantly enhanced the cytotoxicity against NTUB1 cells. The above results also indicated that *seco*-compound with a 3,30-dioic acid 30-benzyl ester or 3,30-dioic acid 30-phenylcarbamate, obtained from 18β-GA derivatives such as **9** and **27**, displayed the most potent cytotoxic effect against NTUB1 cells (Tables 3 and 4). As shown in Table 2, the esterification or amidation of C-3 of **9** did not enhance the cytotoxocity against NTUB1 cells.

The esterification of C-3-carboxylic group with methyl halide of **19** enhanced the cytotoxic effect against NTUB1 cells such as **20** while the C-3–carboxylic group of **19** esterified with an alkyl halide longer than methyl halide decreased the cytotoxic effect against NTUB1 cells. The amidation of C-3-carboxylic group of **19** with increasing alkyl chain length of carbamoyl group decreased the cytotoxic effect against NTUB1 cells while the amidation of C-3-carboxylic acid of **19** with branched alkyl chain, such as **25**, significantly increase the cytotoxic effect against NTUB1 cells (Table 4). It indicated that amidation of the two carboxylic acid groups at C-3 and C-30 with isopropylamine significantly enhanced the cytotoxic effect against NTUB1 cells.

The above results and discussion obtained from structure-cytotoxity relationships suggested that *seco*-compounds with a 3,30-dioic acid 30-benzyl ester, 3,30-dioic acid 30-phenylcarbamate, and 3,30-diisopropyl carbamate, such as **9**, **25**, and **27**, obtained from 18β-GA derivatives, exhibited the most potent cytotoxic effect against NTUB1 cells. These three compounds could be used as lead compounds for further design and synthsis of novel anticancer agents.

For further evaluated the cytotoxic effect of 18β-GA derivatives and mechanisms of induced cancer cell death in vitro, cytotoxicities of selective compounds **21**, **23**, and **25** against PC3 (human prostate cancer cell line), SV-HUC1 (immortalized normal human urothelial cells), and NTUB1 were studied and compared with those of cytotoxicities against NTUB1 cells (Fig. 1). As shown in Figure 1, **21**, **23**, and **25** did not exhibited stronger cytotoxic effects against PC3 than those of cytotoxicities against NTUB1 cells while these compounds enhanced the cell growth of SV-HUC1 cells. It suggested that these compounds did not show cytotoxicity against human normal cells. It needs to verify the mechanism related to why these compounds enhanced the cell growth in SV-HUC1 cells.

The MTT method is the most frequently used in the determination of the viable cell number but its inadequacy is evident in presence of compound that cause alterations in mitochondria activity.[10] It is necessary to use another assay to determine the cytotoxicity for supporting the enhancement of cell growth of SV-HUC1 cells induced by **21**, **23**, and **25**.

The antioxidants, such as pyrrolidine dithiocarbamate, epigallocatechin gallate, genistein, and vitamin E, revealed synergistic cytotoxicity to PC3 cells when combined with taxol.[11] In addition, recently we have reported that a low dose of xanthine oxidase (XO) inhibitor, terpenoids, and antioxidant xanthones combined with cisplatin enhanced the cytotoxic effect against NTUB1 cells.[3,12,13]

Cisplatin has been used as a chemotherapeutic agent against malignant solid tumors in the head and neck region. However, there have been several side effects, such as nephrotoxicity and cisplatin resistance, when clinically used for treatment of cancers. For continual evaluation of compounds in combination of cisplatin would enhance the cytotoxicity induced by cisplatin, and attenuate side effect and cisplatin resistance. The authors selected compounds **12** and **20**, previously reported as XO inhibitor and antiinflammatory agents[9], to examine if **12** or **20** in combination of cisplatin would enhance the cytotoxicity induced by cisplatin. As

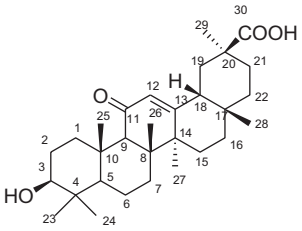
Table 1
^{13}C NMR spectroscopic data for compounds **10**, **11**, **13**–**17** and **21**–**25**

Position	**10**	**11**	**13**	**14**	**15**	**16**	**17**	**21**	**22**	**23**	**24**	**25**
C-1	23.5	23.8	23.8	23.8	23.8	23.8	23.8	23.8	23.8	23.8	23.8	23.8
C-2	31.4	32.2	31.4	31.4	32.0	32.0	32.1	31.4	31.4	32.0	32.1	32.1
C-3	173.4	172.4	173.9	173.4	173.2	173.2	172.3	174.6	173.4	173.2	173.3	172.4
C-4	146.6	146.6	146.6	146.6	146.6	146.6	146.6	146.5	146.5	146.6	146.6	146.6
C-5	38.8	39.0	38.7	38.8	39.0	39.0	39.0	38.8	38.8	39.0	39.0	39.0
C-6	29.7	31.1	28.4	29.7	31.3	31.3	31.1	28.6	29.7	29.5	29.5	29.5
C-7	34.3	35.7	34.4	34.3	35.6	35.7	35.6	34.4	34.3	35.6	35.7	35.7
C-8	43.6	43.7	44.0	44.0	44.0	44.0	44.0	43.3	43.3	43.3	43.3	43.3
C-9	52.8	53.1	52.8	52.7	53.0	53.1	53.1	52.8	52.8	53.1	53.0	53.0
C-10	41.2	41.2	41.2	41.2	41.2	41.2	41.2	42.2	42.1	42.0	41.2	41.2
C-11	199.6	200.4	199.5	199.5	200.4	200.4	200.3	199.6	199.5	200.3	200.3	200.3
C-12	128.5	128.4	128.2	128.3	128.2	128.2	128.2	128.4	128.4	128.4	128.4	128.4
C-13	169.3	170.3	169.2	169.1	170.2	170.3	170.2	169.5	169.4	170.4	170.4	170.3
C-14	45.1	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0	45.0
C-15	26.4	26.4	26.4	26.4	26.3	26.3	26.3	26.4	26.4	26.4	26.4	26.4
C-16	26.5	26.5	26.5	26.5	26.5	26.5	26.5	26.5	26.5	26.6	26.6	26.6
C-17	31.8	31.8	31.7	31.7	31.7	31.7	31.7	31.9	31.9	31.9	31.9	31.9
C-18	48.4	48.3	48.1	48.1	48.2	48.2	48.2	48.2	48.2	48.1	48.1	48.1
C-19	41.2	41.2	43.6	43.6	43.7	43.7	43.7	43.7	41.1	41.1	41.1	41.1
C-20	44.0	44.0	50.7	50.6	50.9	51.0	51.0	43.7	43.7	43.8	43.8	43.7
C-21	31.1	31.3	31.2	31.2	31.1	31.1	31.2	31.5	31.4	31.5	31.5	31.5
C-22	37.7	37.7	37.7	37.7	37.6	37.6	37.6	37.4	37.4	37.4	37.4	37.4
C-23	114.2	114.3	114.2	114.2	114.3	114.3	114.3	114.2	114.2	114.4	114.3	114.3
C-24	23.8	23.5	23.5	23.5	23.5	23.5	23.5	23.5	23.8	23.5	23.5	23.5
C-25	19.5	19.5	19.5	19.5	19.5	19.5	19.4	19.5	19.5	19.6	19.6	19.5
C-26	18.6	18.7	18.4	18.6	18.7	18.7	18.7	18.7	18.6	18.7	18.7	18.7
C-27	23.4	23.5	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3	23.3
C-28	28.6	28.6	28.4	28.5	28.5	28.5	28.5	29.5	29.5	29.5	29.5	29.5
C-29	28.3	28.3	28.3	28.3	28.3	28.3	28.3	29.4	28.6	28.6	28.6	28.6
C-30	176.9	176.5	176.2	176.2	176.2	176.2	176.2	173.9	174.6	174.7	174.7	174.6
OCH_3	51.8	51.8										
OCH_2CH_3			60.2					60.3				
OCH_2CH_3			14.2					14.2				
$OCH_2CH_2CH_3$												
$OCH_2CH_2CH_3$												
$OCH_2CH_2CH_3$												
$OCH(CH_3)_2$	50.6			41.2					50.6			
$OCH(CH_3)(CH_3)$	21.8			21.8					21.8			
$OCH(CH_3)(CH_3)$	21.8			21.8					21.8			
OCH_2			66.2	66.2	66.3	66.2	66.2					
1′			136.1	136.1	136.1	136.1	136.1					
2′			128.3	128.3	128.3	128.3	128.3					
3′			128.6	128.6	128.6	128.6	128.6					
4′			128.5	128.5	128.4	128.4	128.4					
5′			128.6	128.6	128.6	128.6	128.6					
6′			128.3	128.3	128.3	128.3	128.3					
$NH–CH_2CH_3$					34.3					44.9		
$NH–CH_2CH_3$					14.7					14.8		
$NH–CH_2CH_2CH_3$						41.3					42.1	
$NH–CH_2CH_2CH_3$						22.8					31.3	
$NH–CH_2CH_2CH_3$						11.4					11.4	
$NH–CH(CH_3)_2$		50.9					51.0	50.8	50.6	51.0	50.9	50.9
$NH–CH(CH_3)_2$												50.9
$NH–CH(CH_3)(CH_3)$		22.7					22.7	22.8	22.8	22.8	22.8	22.7
$NH–CH(CH_3)(CH_3)$		22.7					22.7	23.0	23.0	22.9	22.9	22.7
$NH–CH(CH_3)(CH_3)$												22.8
$NH–CH(CH_3)(CH_3)$												22.9

shown in Table 5 and Figure 2, the combination of **12** or **20** and cisplatin as the concentration used in Table 5 and Figure 2 significantly enhanced cell death induced by **12** or **20** alone, respectively, except for **20** (30 μM) combined with 5 or 10 μM cisplatin and 50 μM combined with 10 μM cisplatin attenuated the cell death induced by **20** alone, respectively. The above result suggested an additive and possibly a more than additive effect of the most concentration of **12** or **20** combined with cisplatin when compared with **12** or **20** alone. While 50 μM **12** or **20**, each indicating weaker cytotoxic activity than that of 5 μM cisplatin, combined with 5 μM cisplatin enhanced the cell death induced by 5 μM cisplatin. It suggested that the combination of 50 μM **12** or **20** and 5 μM cisplatin or a low dose of cisplatin may enhance the therapeutic efficacy of cisplatin, and reduce the side effect and resistance of cisplatin. It is necessary to use several experimental data for supporting the above results.

ROS induce programed cell death or necrosis, induce or suppress the expression of many genes, and activate cell signaling cascades.[14] Based on the above result, the author selected **25** to determine whether **25** stimulates ROS production in NTUB1 cells. Exposure of cells to 20 μM cisplatin (positive control) and **25** (10 and 25 μM), and 1 mM *N*-acetylcysteine (NAC) (negative control) for 24 h, respectively, caused a significant increase, and decrease in the intracellular generation of ROS, respectively, as determined with the fluorescent dye, H_2DCFDA, which preferentially detected intracellular ROS (Fig. 3A–E). The generation of ROS by **25** was fur-

Table 2
Cytotoxic activity of 18β -glycyrrhetinic acid (**1**) and its derivatives **2–7**



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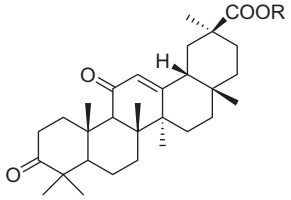
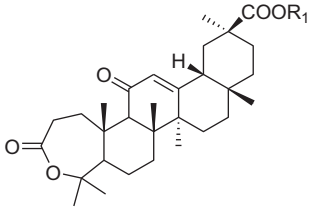


2-4

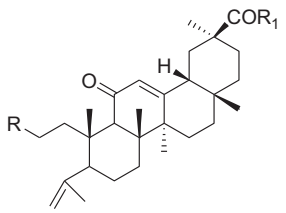


5-7

Compd	R	R_1	$IC_{50} \pm SD^a$ (μM) NTUB1[b]
Cisplatin			3.27 ± 0.10
1			27.31 ± 5.17
2	H		18.57 ± 1.64
3	CH_3		56.5 ± 14.35
4	$CH_2C_6H_5$		20.23 ± 5.10
5		H	73.54 ± 35.12
6		CH_3	24.28 ± 3.98
7		$CH_2C_6H_5$	57.61 ± 8.80

[a] Data represent mean value (SD) of five independent determinations.
[b] Human bladder cancer cell line.

Table 3
Cytotoxic activity of 18β -glycyrrhetinic acid derivatives **8–17**



8-17

Compd	R	R_1	$IC_{50} \pm SD^a$ (μM) NTUB1[b]
8	COOH	OCH_3	20.72 ± 4.97
9	COOH	$OCH_2C_6H_5$	2.34 ± 0.28
10	$COOCH(CH_3)_2$	OCH_3	25.30 ± 2.28
11	$COOCH(CH_3)_2$	OCH_3	31.35 ± 0.55
12	$COOCH_3$	$OCH_2C_6H_5$	9.41 ± 2.72
13	$COOCH_2CH_3$	$OCH_2C_6H_5$	27.76 ± 6.48
14	$COOCH(CH_3)_2$	$OCH_2C_6H_5$	18.20 ± 1.58
15	$CONHCH_2CH_3$	$OCH_2C_6H_5$	42.74 ± 11.15
16	$CONHCH_2CH_2CH_3$	$OCH_2C_6H_5$	49.39 ± 11.52
17	$CONHCH(CH_3)_2$	$OCH_2C_6H_5$	30.12 ± 2.41

[a] Data represent mean value (SD) of five independent determinations.
[b] Human bladder cancer cell line.

ther supported by the finding that cells treated with antioxidant, NAC, attenuated the oxidation of H_2DCFDA. These results indicate the **25** is able to generate intracellular ROS and subsequently induced cell death.

The effect of positive control cisplatin, negative control NAC, and **25** on cell cycle progression was determined by using cell sorting analysis in propidium iodide-stained NTUB1 cells. As shown in Figure 4A–G, treatment with 25, 50, and 75 μM **25** for 24 h did not induce cell cycle arrest but induce the population of sub-G_1 phase in dose-dependent manner. Exposure of cells to **25** resulted in the increase of sub-G1 phase cells which mean apoptotic cells. Expose of cells to **25** also resulted in a notable reduction of cells in S phase. It means that the cell exits from cell cycle and apoptosis occurs. These results suggested that **25** induced cell death is mainly due to apoptosis. Exposure of cells to NAC and H_2O_2 (positive control) caused a attenuated and enhanced the population of sub-G_1 phase, respectively. These results also support that the induction of apoptosis by **25** mediated through ROS.

It has been suggested that ROS overproduction induced a reduction in the mitochondrial membrance potential (MMP) ($\Delta\psi_m$) as well as mitochondrial dysfunction.[2] Thus, to detect the change of MMP, the specific fluorescent probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolocarbocyanine iodide) was used. As shown in Figure 5A–E, positive control CCCP (carbonyl cyanide 3-chlorophenylhydrazone) and various concentrations of **25** significantly increased the number of JC-1 monomers (R2) and decreased that of JC-1 aggregates (R1). It suggested that **25** decreased $\Delta\psi_m$ dramatically in the NTUB1 cells. Marked collapse of the MMP suggested that dysfunction of mitochondria may be involved in the oxidative burst and apoptosis induced by **25**. Here, we may demonstrate that **25** induce cell death of NTUB1 cells mainly by apoptosis through mitochondria-mediated pathway.

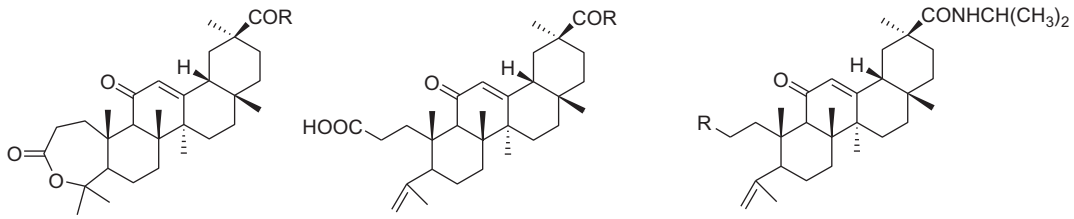
p53 is an important regulator for apoptosis induced by ROS.[5] There is a close relationship among ROS, DNA damage, and p53 activation.[5] The most important p53 function is its ability to activate apoptosis, and disruption of this process can promote tumor progression and chemoresistance.[15] We investigated the effect of **25** on p53 phosphorylation in the cells. Western blot analysis displays that NTUB1 cells treated with **25** increased the level of *p*-p53 in a dose–dependent manner (Fig. 6A). Further, NAC treatment prevented p53 phosphorylation stimulated by **25** (Fig. 6B). These data strongly suggested that the generation of ROS played an important role in p53 activation.

Since the major enzyme involved in apoptosis is caspase-3, activation of caspase-3 in **25**-treated cells was examined. NTUB1 cells were treated with 10, 25, and 50 μM **25**, respectively. After treatment, cells were assessed for the occurrence of activated caspase-3 by western blotting. There was no caspase-3 activity induced by **25** (data not shown).

3. Conclusion

Here we report the design, synthesis, and biological evaluation of 18β-GA derivatives which exhibited cytotoxic activity against

Table 4
Cytotoxic activity of 18β -glycyrrhetinic acid derivatives **18–27**



18, 26 **19, 27** **20-25**

Compd	R	IC_{50} ± SD[a] (μM) NTUB1[b]
18	$NHCH(CH_3)_2$	25.66 ± 1.81
19	$NHCH(CH_3)_2$	17.47 ± 2.11
20	$COOCH_3$	13.12 ± 5.27
21	$COOCH_2CH_3$	19.44 ± 19.20
22	$COOCH\ (CH_3)_2$	29.17 ± 26.61
23	$CONHCH_2CH_3$	12.75 ± 2.10
24	$CONHCH_2CH_2CH_3$	>50
25	$CONHCH(CH_3)_2$	4.76 ± 1.15
26	NHC_6H_5	15.95 ± 5.30
27	NHC_6H_5	3.31 ± 0.61

[a] Data represent mean value (SD) of five independent determinations.
[b] Human bladder cancer cell line

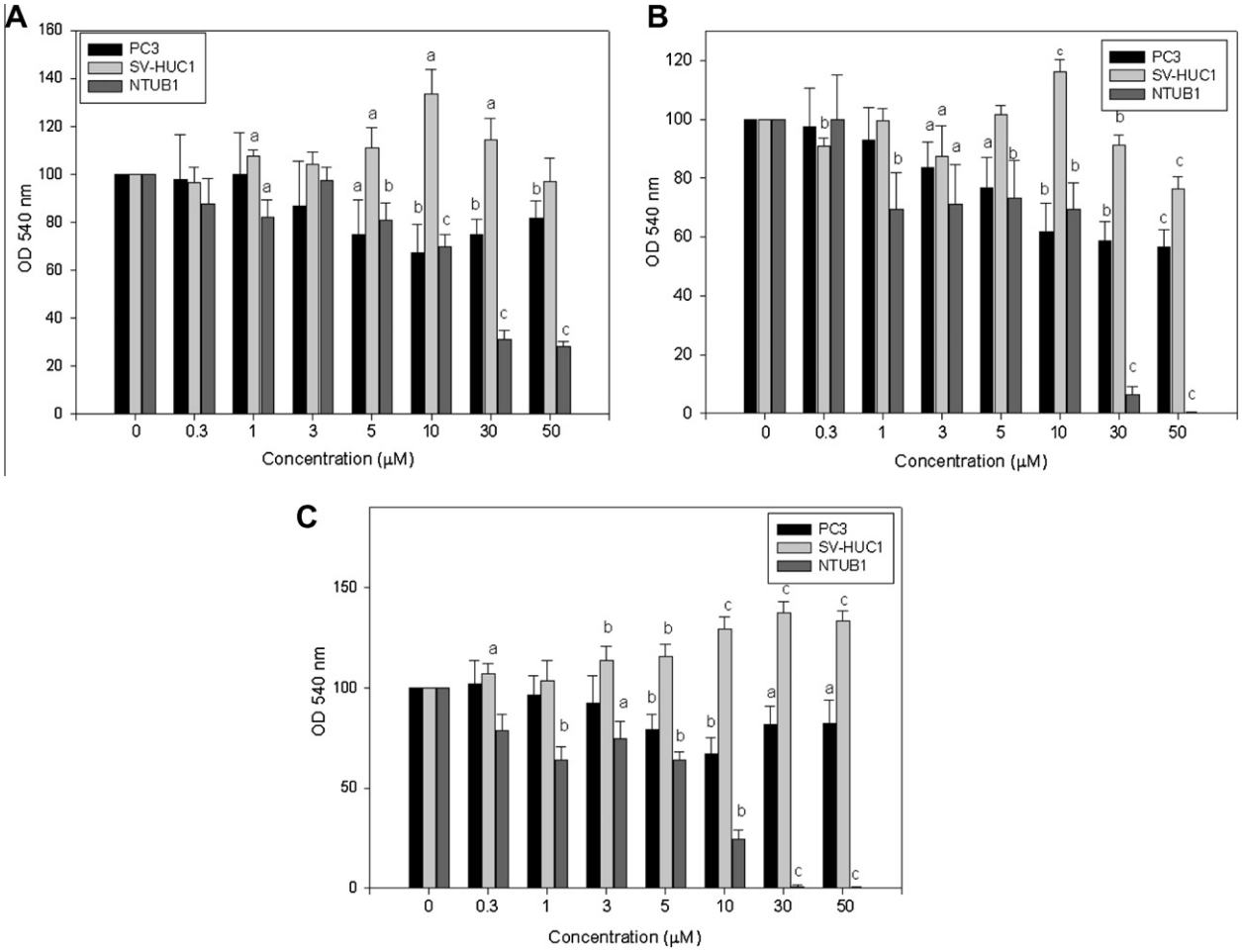


Figure 1. Cytotoxities of **21** (A), **23** (B), and **25** (C) against PC3, SV-HUC1, and NTUB1 cells. Cell viability was assessed by the MTT assay after treating with different concentrations of compounds for 72 h. The data shown represent mean ± SD ($n = 5$) for one experiment performed in triplicate. $P < 0.05$ (a), $P < 0.01$ (b), and $P < 0.001$ (c) compared to the control value, respectively.

Table 5
Percent cytotoxic inhibition (compared to untreated control cells) of different concentrations of **12**, **20**, and **12** or **20** combined with 5 or 10 μM cisplatin, respectively, against NTUB1 cells.

μM	1	3	5	10	30	50
Cisplatin			72	75		
12	32	22	20	50	66	68
12 + 5 μM Cisplatin	50	59	56	58	70	78
12 + 10 μm cisplatin	58	54	56	56	67	70
20	40	25	24	27	67	71
20 + 5 μM Cisplatin	56	59	59	57	66	74
20 + 10 μM Cisplatin	58	60	59	59	55	67

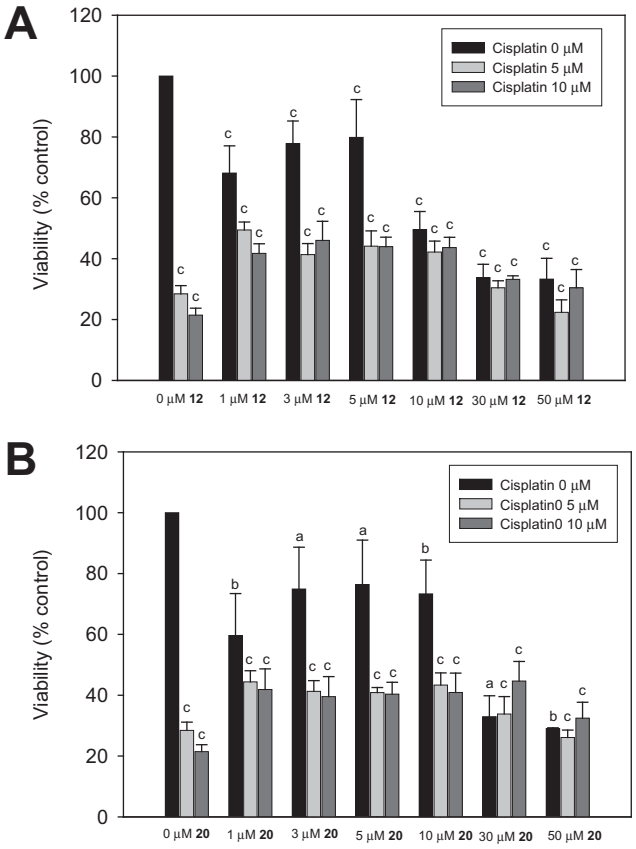


Figure 2. Cisplatin and combination of cisplatin with **12** (A) or **20** (B) induced cell death. Cell viability was assessed by MTT assay for 72 h after treatment with different concentrations of cisplatin (0, 5, and 10 μM) and each concentration of cisplatin combined with different concentrations (0, 1, 3, 5, 10, 30 or 50 μM) of **12** or **20**. P <0.05 (a), P <0.01 (b), and P <0.001 (c) compared to the control value, respectively.

human NTUB1 cells. *seco*-Compounds **9**, **25**, and **27** are the most potent compounds of this series of 18β-GA derivatives with an IC_{50} values of 2.34 ± 0.28, 4.76 ± 1.15, and 3.31 ± 0.61 μM, respectively. Selective compound **25** induces cell death mainly by apoptosis through mitochondria-mediated and caspase-3-independent pathway, where ROS-mediated p53 activation acted as upstream signaling. It needs further to elucidate the detailed mechanism of this series of compounds induced inhibition of tumor cell growth. Several compounds were identified as potential p53 activity modulators.[16–18] The activities of these compounds may be due to inhibition of p53-MDM2 (murin double minute-2) interaction and subsequent p53 release and activation. Compound **25** exhibited different mechanism of induced cell death from the above reported compounds.

4. Experimental

4.1. Chemistry

Reagents, starting material, and solvents were purchased from commercial suppliers. Cisplatin was obtained from Pharmacia Upjohn, Milan, Italy. All culture reagents were obtained from Gibco BRL. Optical rotations were recorded with a JASCO-370 polarimeter using $CHCl_3$ as solvent. IR spectra were determined with a Perkin–Elmer system 2000 FTIR spectrophotometer. ^1H (400 MHz) and ^{13}C (100 MHz) NMR were recorded on a Varian UNITY-400 spectrometer. Low-resolution mass spectra and high-resolution mass spectra data were obtained on a JMX-HX 100 mass spectrometer. Chromatography was performed using a flash-column technique on Silica Gel 60 supplied by E. Merck.

4.2. General procedure for esterification and amidation of *seco*-compounds

To a solution of *seco*-compound in dry acetone (10 mL) were added an equivalent mole of potassium carbonate (2 mmol) followed by alkyl halides (2 mmol). The reaction mixture was stirred at room temperature or refluxed overnight and concentrated in the vacuum. To the crude product was added 10% HCl solution, partitioned with CH_2Cl_2 for three times, and concentrated. The residue was purified by chromatography with ethyl acetate/*n*-hexane or ethyl acetate/CH_2Cl_2 to yield pure product. To a solution of *seco*-compound in dry CH_2Cl_2 (10 mL) were added EDCI (2 mmol) and DMAP (catalytic amount) followed by various amines (2 mmol). The reaction mixture was stirred at room temperature for 2–4 h and concentrated in the vacuum. The residue was purified by chromatography with ethyl acetate/*n*-hexane or ethyl acetate/CH_2Cl_2 to yield pure product.

4.2.1. 3,4-*seco*-11-Oxo-18β-olean-4(23),12-dien-3,30-dioic acid 3-isopropyl 30-methyl, ester (10)

Compound **10** was prepared from **8** (80 mg, 0.16 mmol) following the general procedure described for esterification using isopropyl iodide as alcohol moiety. Compound **10** was obtained as white amorphous powder (86.2 mg, 0.16 mmol, 100%). $[\alpha]_D^{25}$ 8 (*c* 0.1, $CHCl_3$). IR (film on NaCl) cm^{-1} 1731, 1657. ^1H NMR ($CDCl_3$) δ 0.82 (3H, s, Me-28), 1.15 (3H, s, Me-29), 1.16 (3H, s, Me-25), 1.17 (3H, s, Me-26), 1.21 (6H, d, J = 6.4 Hz, –CH(CH_3)$_2$), 1.38 (3H, s, Me-27), 1.76 (3H, s, Me-24), 2.60 (1H, td, J = 13.4, 6.0 Hz, Hβ -18), 3.70 (3H, s, OCH_3), 4.70 (1H, br s, H-23), 4.90 (1H, br s, H-23), 4.95 (1H, m, –C*H*(CH_3)$_2$), 5.69 (1H, s, H-12). ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) m/z (% rel. int.), 540 $[M]^+$ (63). HR-EI-MS m/z calcd for $C_{34}H_{52}O_5$, 540.3815; found: 540.3814.

4.2.2. 3-Isopropylcarbamoyl-11-oxo-18β-3,4-*seco*-olean-4(23), 12-diene 30-methyl ester (11)

Compound **11** was prepared from **8** (212 mg, 0.43 mmol) following the general procedure described for amidation using isopropyl amine as amine moiety. It was obtained as white amorphous powder (201.6 mg, 0.37 mmol, 86.0%). $[\alpha]_D^{25}$ 10 (*c* 0.1, $CHCl_3$). IR (film on NaCl) cm^{-1} 3299, 1727, 1661. ^1H NMR ($CDCl_3$) δ 0.81 (3H, s, Me-28), 1.10 (3H, d, J = 6.8 Hz, –CH(CH_3)(CH_3)), 1.11 (3H, d, J = 6.8 Hz, –CH(CH_3)(CH_3)), 1.14 (6H, s, Me-26 and 29), 1.17 (3H, s, Me-25), 1.37 (3H, s, Me-27), 1.77 (3H, s, Me-24), 2.47 (1H, td, J = 13.2, 6.0 Hz, Hβ-18), 3.69 (3H, s, OCH_3), 4.01 (1H, m, –C*H*(CH_3)$_2$), 4.73 (1H, br s, H-23), 4.90 (1H, br s, H-23), 5.48 (1H, d, J = 7.6 Hz, NH), 5.69 (1H, s, H-12). ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) m/z (% rel. int.), 539 $[M]^+$ (70). HR-EI-MS m/z calcd for $C_{34}H_{53}NO_4$, 539.3974; found: 539.3956.

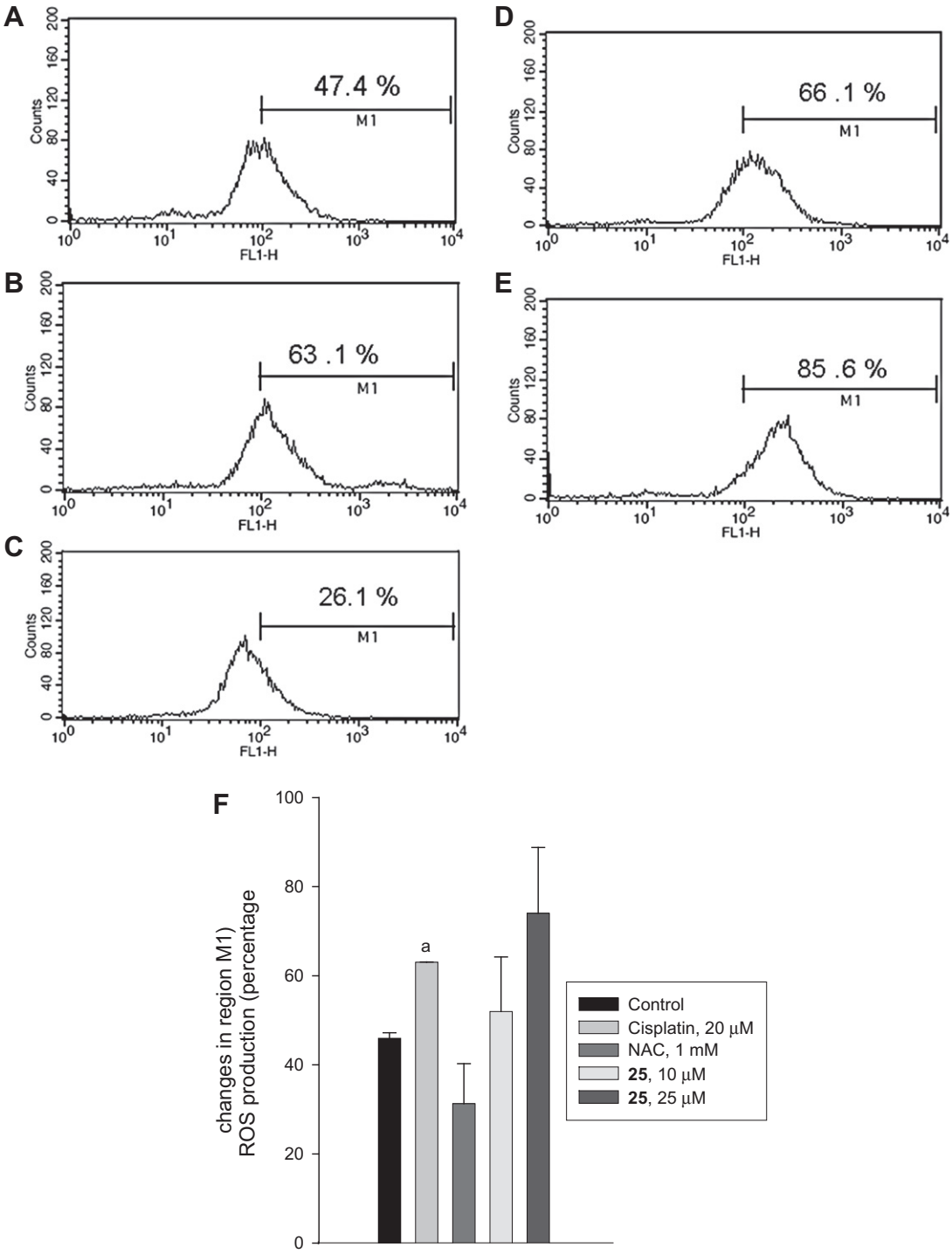


Figure 3. Effect of **25** on the production of ROS in NTUB1 cells: control (A), 20 μM cisplatin (B), 1 mM NAC (C), 10 μM **25** (D), and 25 μM **25** (E) for 24 h. The amount of ROS was assayed by H_2DCFDA staining. Each sampling measured the fluorescence intensity of region M1 of 3×10^5 cells/6 cm dish. The control cells were treated with medium. Results were repeated by three independent experiments. Bar graph (F) shows percentage changes in intracellular ROS generation of region M1 of NTUB1 cells. Data as mean ± SD, $n = 3$. [a]$p < 0.05$ compared to the control value.

4.2.3. 3,4-*seco*-11-Oxo-18β-olean-4(23),12-dien-3,30. dioic acid 3-ethyl,30-benzyl ester (13)

Compound **13** was prepared from **9** (112 mg, 0.20 mmol) following the general procedure described for esterification using ethyl iodide as alcohol moiety. It was obtained as white amorphous powder (81.6 mg, 0.13 mmol, 65.0%). $[\alpha]_D^{25}$ 3 (*c* 0.5, $CHCl_3$). IR (film on NaCl) cm^{-1} 1727, 1657, 1579. ^1H NMR ($CDCl_3$) δ 0.74 (3H, s, Me-28), 1.15 (3H, s, Me-29), 1.16 (6H, s, Me-25 and 26), 1.22 (3H, t, $J = 7.2$ Hz, $-CH_2CH_3$), 1.37 (3H, s, Me-27), 1.75 (3H, s, Me-24), 2.60 (1H, td, $J = 13.2$, 6.0 Hz, Hβ-18), 4.10 (2H, q, $J = 7.2$ Hz, $-CH_2CH_3$), 4.69 (1H, br s, H-23), 4.90 (1H, br s, H-23), 5.09 (1H, d, $J = 12.4$ Hz, –OC*H*H–), 5.20 (1H, d, $J = 12.0$ Hz, –OCH*H*–), 5.57 (1H, s, H-12), 7.37 (5H, m, aromatic proton). ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) *m*/*z* (% rel. int.), 602 $[M]^+$ (21). HR-EI-MS *m*/*z* calcd for $C_{39}H_{54}O_5$, 602.3971; found: 602.3971.

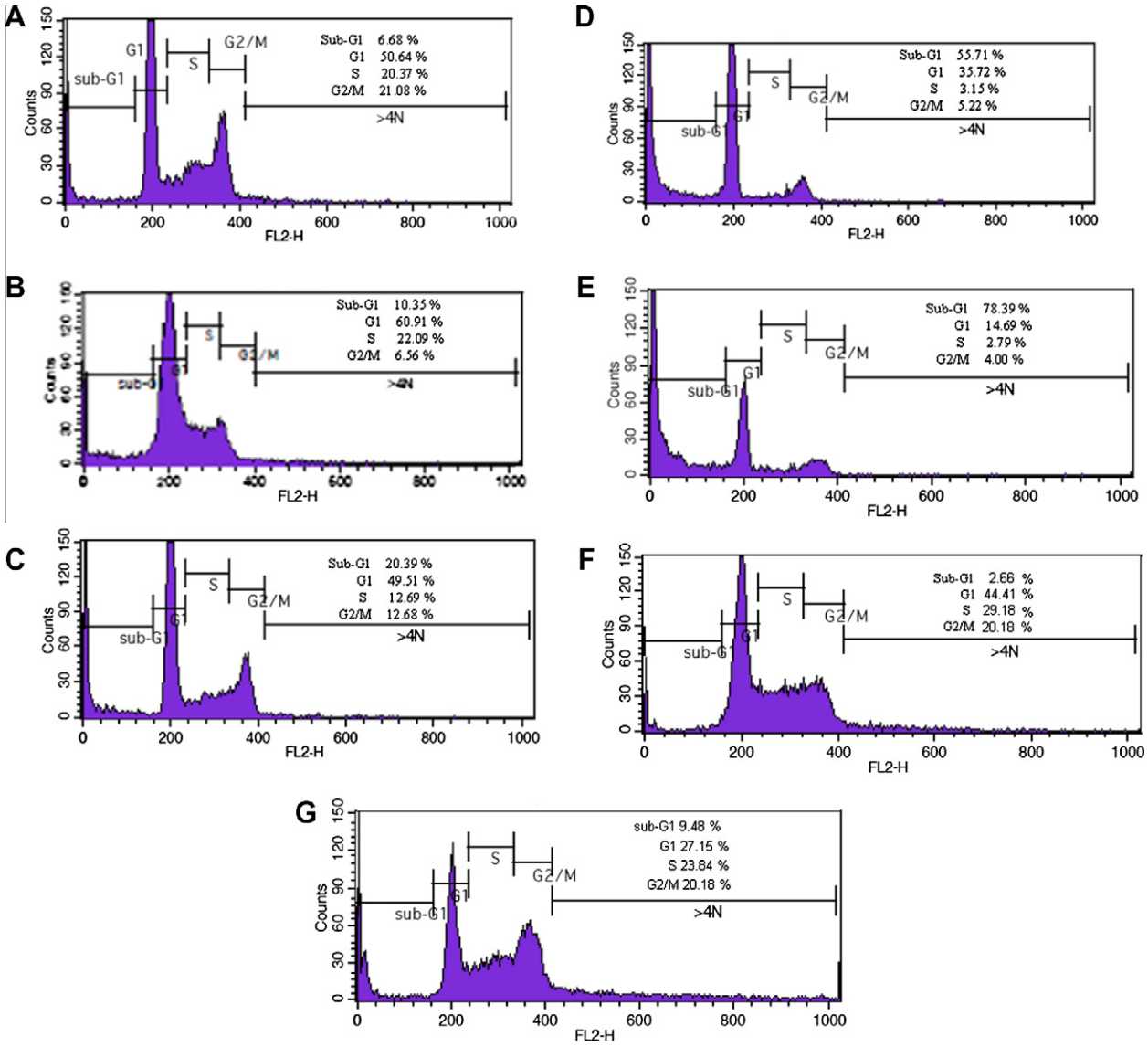


Figure 4. Flow cytometry analysis of cisplatin and **25**-treated NTUB1 cells. (8×10^5 Cell/10 cm dish) were treated with absence of cisplatin and **25** (control A), 20 μM cisplatin (B), 25 μM **25** (C), 50 μM **25** (D), 75 μM **25** (E), 1 mM NAC (F), and 50 μM H_2O_2 (G), for 24 h. At the indicated time, cells were stained with propidium iodide (PI), DNA contents were analyzed via flow cytometry and the amount of apoptosis was measured by accumulation of sub-G1 DNA contents in the cells. The control cells were treated with medium. Results are representative of three independent experiments.

4.2.4. 3,4-*seco*-11-Oxo-18β-olean-4(23),12-dien-3,30. dioic acid 3-isopropyl,30-benzyl ester (14)

Compound **14** was prepared from **9** (100 mg, 0.17 mmol) following the general procedure described for esterification using isopropyl iodide as alcohol moiety. It was obtained as white amorphous powder (49.4 mg, 0.08 mmol, 47.1%). $[\alpha]_D^{25}$ 16 (*c* 0.05, $CHCl_3$). IR (film on NaCl) cm^{-1} 1727, 1657, 1631. ^1H NMR ($CDCl_3$) δ 0.74 (3H, s, Me-28), 1.15 (3H, s, Me-29), 1.16 (6H, s, Me-25 and 26), 1.19 (6H, d, *J* = 6.4 Hz, –CH(CH_3)$_2$), 1.39 (3H, s, Me-27), 1.76 (3H, s, Me-24), 2.58 (1H, td, *J* = 13.2, 6.4 Hz, Hβ-18), 4.69 (1H, br s, H-23), 4.90 (1H, br s, H-23), 4.95 (1H, m, –C*H*(CH_3)$_2$), 5.09 (1H, d, *J* = 12.4 Hz, –OC*H*H–), 5.20 (1H, d, *J* = 12.4 Hz, –OCH*H*–), 5.57 (1H, s, H-12), 7.38 (5H, m, aromatic proton). ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) *m*/*z* (% rel. int.), 616 $[M]^+$ (33). HR-EI-MS *m*/*z* calcd for $C_{40}H_{56}O_5$, 616.4127; found: 616.4136.

4.2.5. 3-Ethylcarbamoyl -11-oxo-18β-3,4-*seco*-olean-4(23),12-diene 30-benzyl ester (15)

Compound **15** was prepared from **9** (100 mg, 0.17 mmol) following the general procedure described for amidation using ethyl amine as amine moiety. It was obtained as white amorphous powder (58.0 mg, 0.097 mmol, 57.1%). $[\alpha]_D^{25}$ 11 (*c* 0.35, $CHCl_3$). IR (film on NaCl) cm^{-1} 3299, 1727, 1657, 1553. ^1H NMR ($CDCl_3$) δ 0.75 (3H, s, Me-28), 1.10 (3H, t, *J* = 7.2 Hz, –CH_2CH_3), 1.14 (3H, s, Me-29), 1.15 (3H, s, Me-25), 1.16 (3H, s, Me-26), 1.36 (3H, s, Me-27), 1.77 (3H, s, Me-24), 2.49 (1H, td, *J* = 13.2, 6.0 Hz, Hβ-18), 3.23 (2H, m, –CH_2CH_3), 4.74 (1H, br s, H-23), 4.90 (1H, br s, H-23), 5.09 (1H, d, *J* = 12.0 Hz, –OC*H*H–), 5.20 (1H, d, *J* = 12.0 Hz, –OCH*H*–), 5.58 (1H, s, H-12), 5.67 (1H, br s, NH), 7.36 (5H, m, aromatic proton). ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) *m*/*z* (% rel. int.), 601 $[M]^+$ (36). HR-EI-MS *m*/*z* calcd for $C_{39}H_{55}NO_4$, 601.4131; found: 601.4131.

4.2.6. 3-Propylcarbamoyl -11-oxo-18β-3,4-*seco*-olean-4(23), 12-diene 30-benzyl ester (16)

Compound **16** was prepared from **9** (125 mg, 0.22 mmol) following the general procedure described for amidation using propyl amine as amine moiety. It was obtained as white amorphous powder (105.2 mg, 0.17 mmol, 77.3%). $[\alpha]_D^{25}$ 15 (*c* 0.5, $CHCl_3$). IR (film on NaCl) cm^{-1} 3439, 1724, 1657, 1513. ^1H NMR ($CDCl_3$) δ 0.75 (3H, s,

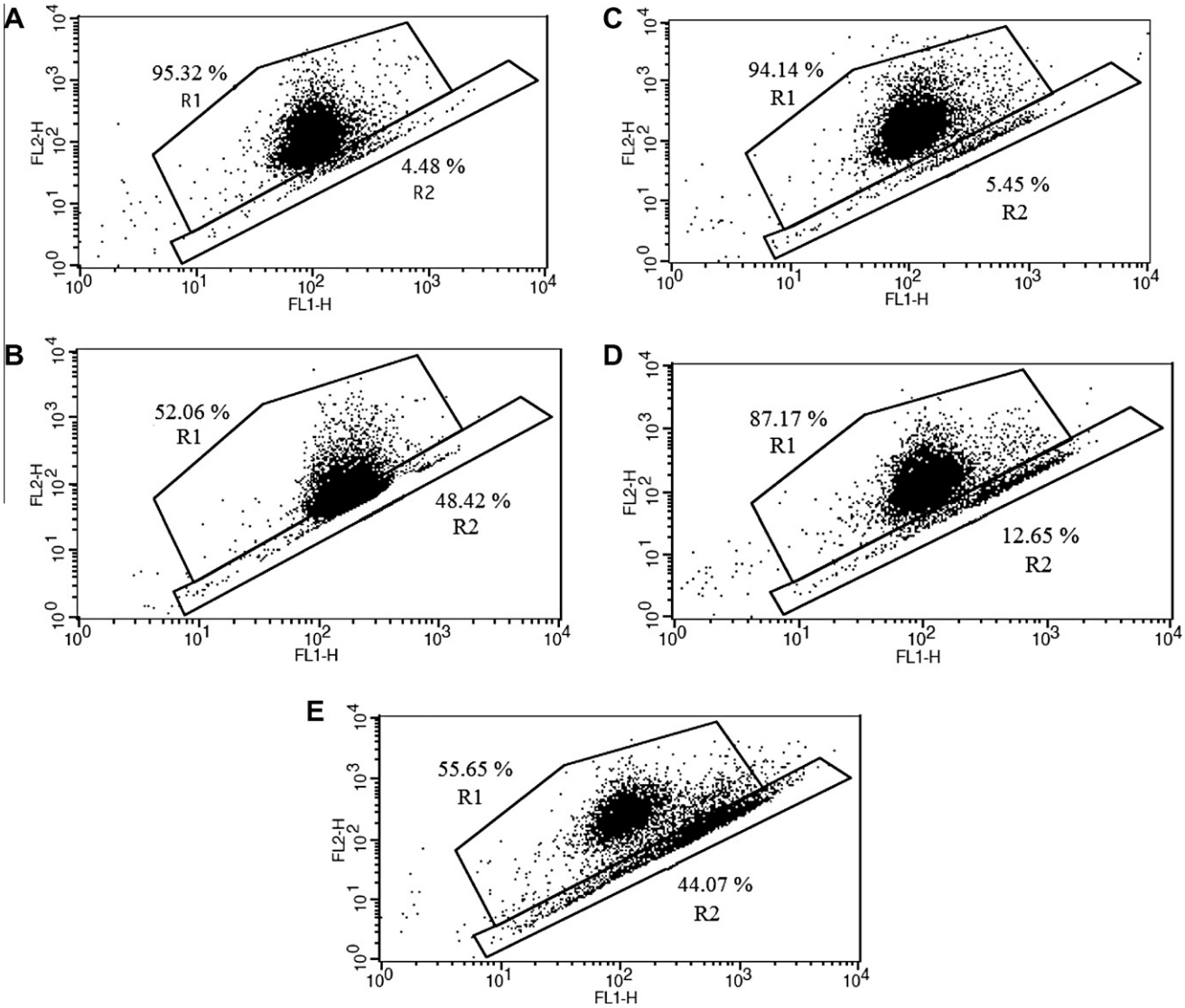


Figure 5. Collapse of MMP induced by CCCP and **25** in the NTUB1 cells were treated with absence CCCP and **25** (control A), 10 μM CCCP (B), 10 μM **25** (C), 25 μM **25** (D), 50 μM **25** (E), for 24 h. Appropriate gates were made to define JC-1 aggregates (R2) and JC-1 monomers (R1).

Me-28), 0.90 (3H, t, J = 7.6 Hz, –$CH_2CH_2CH_3$), 1.14 (3H, s, Me-29), 1.15 (3H, s, Me-25), 1.16 (3H, s, Me-26), 1.36 (3H, s, Me-27), 1.49 (2H, q, J = 7.2 Hz, –$CH_2CH_2CH_3$), 1.77 (3H, s, Me-24), 2.49 (1H, td, J = 13.2, 6.0 Hz, Hβ-18), 3.16 (2H, dd, J = 13.6, 6.0 Hz, –$CH_2CH_2CH_3$), 4.74 (1H, br s, H-23), 4.90 (1H, br s, H-23), 5.09 (1H, d, J = 12.4 Hz, –OC*H*H–), 5.20 (1H, d, J = 12.4 Hz, –OCH*H*–), 5.73 (1H, br s, NH), 5.58 (1H, s, H-12), 7.36 (5H, m, aromatic proton). ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) m/z (% rel. int.), 615 $[M]^+$ (36). HR-EI-MS m/z calcd for $C_{40}H_{57}NO_4$, 615.4287; found: 615.4283.

4.2.7. 3-Isopropylcarbamoyl-11-oxo-18β-3,4-*seco*-olean-4(23), 12-diene 30-benzyl ester (17)

Compound **17** was prepared from **9** (100 mg, 0.17 mmol) following the general procedure described for amidation using isopropyl amine as amine moiety. It was obtained as white amorphous powder (92.9 mg, 0.15 mmol, 88.2%). $[\alpha]_D^{25}$ 7 (*c* 0.25, $CHCl_3$). IR (film on NaCl) cm^{-1} 3306, 1727, 1657, 1535. ^{1}H NMR ($CDCl_3$) δ 0.75 (3H, s, Me-28), 1.10 (3H, d, J = 6.8 Hz, –CH(CH_3)(CH_3)), 1.12 (3H, d, J = 6.8 Hz, –CH(CH_3)(CH_3)), 1.14 (3H, s, Me-29), 1.15 (3H, s, Me-25), 1.16 (3H, s, Me-26), 1.36 (3H, s, Me-27), 1.77 (3H, s, Me-24), 2.46 (1H, td, J = 13.2, 6.0 Hz, Hβ-18), 4.00 (1H, m, –*CH*(CH_3)$_2$), 4.74 (1H, br s, H-23), 4.91 (1H, br s, H-23), 5.09 (1H, d, J = 12.4 Hz, –OC*H*H–), 5.20 (1H, d, J = 12.4 Hz, –OCH*H*–), 5.56 (1H, br s, NH), 5.58 (1H, s, H-12), 7.36 (5H, m, aromatic proton). ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) m/z (% rel. int.), 615 $[M]^+$ (42). HR-EI-MS m/z calcd for $C_{40}H_{57}NO_4$, 615.4288; found: 615.4286.

4.2.8. 30-Isopropylcarbamoyl-11-oxo-18β-3,4-*seco*-olean-4(23), 12-diene 3-ethyl ester (21)

Compound **21** was prepared from **19** (100 mg, 0.19 mmol) following the general procedure described for esterification using ethyl iodide as alcohol moiety. It was obtained as white amorphous powder (27.3 mg, 0.049 mmol, 25.8%). $[\alpha]_D^{25}$ 9 (*c* 0.25, $CHCl_3$). IR (film on NaCl) cm^{-1} 3365, 1735, 1650. ^{1}H NMR ($CDCl_3$) δ 0.82 (3H, s, Me-28), 1.11 (3H, s, Me-26), 1.14 (3H, d, J = 6.4 Hz, –CH(CH_3)(CH_3)), 1.16 (3H, s, Me-25),, 1.16 (3H, d, J = 6.4 Hz, –CH(CH_3)(CH_3)), 1.17 (3H, s, Me-29), 1.23 (3H, t, J = 7.2 Hz, –CH_2 CH_3), 1.39 (3H, s, Me-27), 1.76 (3H, s, Me-24), 2.61 (1H, td, J = 13.2, 6.4 Hz, Hβ-18), 4.08 (2H, q, J = 7.2 Hz, –CH_2CH_3), 4.12 (1H, m, –*CH*(CH_3)$_2$), 4.70 (1H, br s, H-23), 4.90 (1H, br s, H-23), 5.33 (1H, d, J = 8.0 Hz, NH), 5.65 (1H, s, H-12). ^{13}C NMR ($CDCl_3$)

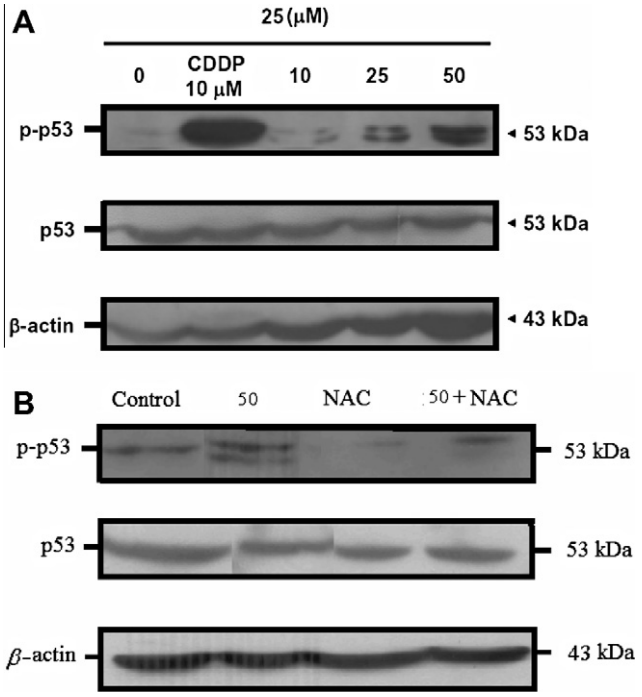


Figure 6. Compound **25** induced p53 phosphorylation in NTUB1 cell. NTUB1 cells were incubated with **25** at different concentrations (0 – 50 μM) and 10 μM CDDP (cisplatin), respectively, for 24 h (A) or the cells were exposed to 50 μM **25** in the absence and presence of 1 mM NAC, and 50 μM **25** combined with 1 mM NAC, respectively, for 24 h (B) and then cellular proteins were analyzed by western blotting. A representative result from triplicate experiments was shown.

see Table 1. EI-MS (70 eV) *m*/*z* (% rel. int.): 553 $[M]^+$ (62). HR-EI-MS *m*/*z* calcd for $C_{35}H_{55}NO_4$, 553.4131; found: 553.4133.

4.2.9. 30-Isopropylcarbamoyl-11-oxo-18β-3,4-*seco*-olean-4(23), 12-diene 3-isopropyl ester (22)

Compound **22** was prepared from **19** (80 mg, 0.15 mmol) following the general procedure described for esterification using isopropyl iodide as alcohol moiety. It was obtained as white amorphous powder (73.3 mg, 0.13 mmol, 86.7%). $[\alpha]_D^{25}$ 1 (*c* 1.0, $CHCl_3$). IR (film on NaCl) cm^{-1} 3373, 1727, 1661. ^1H NMR ($CDCl_3$) δ 0.82 (3H, s, Me-28), 1.11 (3H, s, Me-29), 1.14 (3H, d, *J* = 6.8 Hz, –CH(CH_3)(CH_3)), 1.16 (3H, s, Me-25), 1.16 (3H, d, *J* = 6.8 Hz, –CH(CH_3)(CH_3)), 1.17 (3H, s, Me-26), 1.20 (6H, d, *J* = 6.4 Hz, –CH(CH_3)$_2$), 1.39 (3H, s, Me-27), 1.76 (3H, s, Me-24), 2.59 (1H, td, *J* = 13.2, 6.4 Hz, Hβ-18), 4.12 (1H, m, –C*H*(CH_3)$_2$), 4.69 (1H, br s, H-23), 4.90 (1H, br s, H-23), 4.95 (1H, m, –C*H*(CH_3)$_2$), 5.34 (1H, d, *J* = 8.0 Hz, NH), 5.64 (1H, s, H-12). ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) *m*/*z* (% rel. int.), 567 $[M]^+$ (64). HR-EI-MS *m*/*z* calcd for $C_{36}H_{57}NO_4$, 567.4288; found: 567.4269.

4.2.10. 30-Isopropylcarbamoyl-11-oxo-18β-3,4-*seco*-olean-4(23), 12-diene 3-ethyl carbamate (23)

Compound **23** was prepared from **19** (100 mg, 0.19 mmol) following the general procedure described for amidation using ethyl amine as amine moiety. It was obtained as white amorphous powder (59.3 mg, 0.11 mmol, 58.0%). $[\alpha]_D^{25}$ 2 (*c* 0.25, $CHCl_3$). IR (film on NaCl) cm^{-1} 3292, 1731, 1653. ^1H NMR ($CDCl_3$) δ 0.82 (3H, s, Me-28), 1.10 (3H, t, *J* = 7.2 Hz, –CH_2CH_3), 1.11 (3H, s, Me-26), 1.15 (3H, d, *J* = 6.8 Hz, –CH(CH_3)(CH_3)), 1.16 (3H, d, *J* = 6.8 Hz, –CH(CH_3)(CH_3)), 1.16 (3H, s, Me-25), 1.17 (3H, s, Me-29), 1.39 (3H, s, Me-27), 2.51 (1H, td, *J* = 13.2, 6.0 Hz, Hβ-18), 3.23 (2H, m, –CH_2CH_3), 4.12 (1H, m, –C*H*(CH_3)$_2$), 4.74 (1H, br s, H-23), 4.90 (1H, br s, H-23), 5.34 (1H, t, *J* = 8.4 Hz, NH), 5.68 (1H, t, *J* = 4.0 Hz, –N*H*CH_2CH_3), 5.68 (1H, s, H-12). ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) *m*/*z* (% rel. int.), 552 $[M]^+$ (47). HR-EI-MS *m*/*z* calcd for $C_{35}H_{36}N_2O_3$, 552.4291; found: 552.4288.

4.2.11. 30-Isopropylcarbamoyl-11-oxo-18β-3,4-*seco*-olean-4(23), 12-diene 3-propyl carbamate (24)

Compound **24** was prepared from **19** (100 mg, 0.19 mmol) following the general procedure described for amidation using propyl amine as amine moiety. It was obtained as white amorphous powder (46.1 mg, 0.081 mmol, 42.6%). $[\alpha]_D^{25}$ 4 (*c* 0.25, $CHCl_3$). IR (film on NaCl) cm^{-1} 3424, 1735, 1653. ^1H NMR ($CDCl_3$) δ 0.83 (3H, s, Me-28), 0.91 (3H, t, *J* = 7.2 Hz, –$CH_2CH_2CH_3$), 1.11 (3H, s, Me-26), 1.15 (3H, d, *J* = 6.8 Hz, –CH(CH_3)(CH_3)), 1.16 (3H, d, *J* = 6.8 Hz, –CH(CH_3)(CH_3)), 1.16 (3H, s, Me-25), 1.17 (3H, s, Me-29), 1.38 (3H, s, Me-27), 1.77 (3H, s, Me-24), 2.23 (2H, m, –$CH_2CH_2CH_3$), 2.51 (1H, td, *J* = 13.2, 6.0 Hz, Hβ-18), 3.16 (2H, dd, *J* = 13.2, 6.4 Hz, –$CH_2CH_2CH_3$), 4.12 (1H, m, –C*H*(CH_3)$_2$), 4.74 (1H, br s, H-23), 4.91 (1H, br s, H-23), 5.33 (1H, d, *J* = 8.4 Hz, NH), 5.67 (1H, s, H-12), 5.68 (1H, t, *J* = 4.0 Hz, –N*H*$CH_2CH_2CH_3$) ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) *m*/*z* (% rel. int.), 566 $[M]^+$ (95). HR-EI-MS *m*/*z* calcd for $C_{36}H_{58}N_2O_3$, 566.4447; found: 566.4439.

4.2.12. 3,4-*seco*-11-Oxo-18β-olean-4(23),12-dien-3,30-diisopropyl carbamate (25)

Compound **25** was prepared from **19** (100 mg, 0.19 mmol) following the general procedure described for amidation using isopropyl amine as amine moiety. It was obtained as white amorphous powder (61.4 mg, 0.11 mmol, 58.0%). $[\alpha]_D^{25}$ 4 (*c* 0.25, $CHCl_3$). IR (film on NaCl) cm^{-1} 3439, 1727, 1650. ^1H NMR ($CDCl_3$) δ 0.80 (3H, s, Me-28), 1.11 (3H, s, Me-26), 1.12 (6H, d, *J* = 6.8 Hz, –CH(CH_3)$_2$), 1.15 (3H, s, Me-29), 1.15 (6H, d, *J* = 6.8 Hz, –CH(CH_3)$_2$), 1.17 (3H, s, Me-25), 1.38 (3H, s, Me-27), 1.79 (3H, s, Me-24), 2.48 (1H, td, *J* = 13.2, 6.4 Hz, Hβ-18), 4.02 (1H, m, –C*H*(CH_3)$_2$), 4.12 (1H, m, –C*H*(CH_3)$_2$), 4.74 (1H, br s, H-23), 4.90 (1H, br s, H-23), 5.34 (1H, d, *J* = 7.6 Hz, NH), 5.46 (1H, d, *J* = 7.6 Hz, NH), 5.65 (1H, s, H-12). ^{13}C NMR ($CDCl_3$) see Table 1. EI-MS (70 eV) *m*/*z* (% rel. int.), 566 $[M]^+$ (89). HR-EI-MS *m*/*z* calcd for $C_{36}H_{58}N_2O_3$, 566.4447; found: 566.4444.

4.3. Cell culture and MTT assay for cell viability

NTUB1, a human bladder carcinoma cell and PC3 were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin-G, 100 μg/mL streptomycin, and 2 mM L-glutamine. SV-HUC1, immortalized normal human urothelial cell line, was obtained from Amerrican type Culture Collection (Rockville, USA) and maintained in F12 medium supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin-G, 100 μg/mL streptomycin, and 2 mM L-glutamine. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. For evaluating the cytotoxic effect of tested compounds, **12** and **20** combined with cisplatin, respectively, and positive control cisplatin, a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) assay was performed.[19] Briefly, the cells were plated at a density of 1800 cells/well in 96-well plates and incubated at 37 °C overnight before drug exposure. Cells were then cultured in the presence of graded concentrations of tested compounds, **12** and **20** combined with 5 and 10 μM cisplatin, (Pharmacia & Upjohn), respectively, 5 and 10 μM cisplatin at 37 °C for 72 h. At the end of the culture period, 50 μL of MTT (2 mg/mL in PB) was added to each well and allowed to react for 3 h. Following centrifugation of plates at 1000*g* for 10 min, media were removed and 150 μL DMSO were added to each well. The proportions of surviving cells were determined by absorbance spectrometry at 540 nm using MRX (DYNEXCO) microplate reader. The cell viability was expressed as a percentage to the viable cells

of control culture condition. The IC_{50} values of each group were calculated by the median-effect analysis and presented as mean ± standard deviation (SD).

4.4. Quantitative analysis of intracellular reactive oxygen species (ROS)

Production of ROS was analyzed by flow cytometry as described previously.[20] Briefly, cells were plated and treated as indicated conditions. 10 μM 2,7-dichlorodihydrofluorescein diacetate (H_2DCFHDA; Molecular Probes, Eugene, OR) was added to the treated cells 30 min prior harvest. The cells were collected by trypsinization and washed with PBS. The green fluorescence of intracellular DCF (2′,7′-dichlorofluorescein) was then analyzed immediately by FACScan flow cytometer with a 525 nm band pass filter (Becton Dickinson).

4.5. Flow cytometry analysis

DNA content was determined following propidium iodide (PI) staining of cells as previously described.[21] Briefly, 8×10^5 cells were plated and treated with 10 μM cisplatin, various concentrations of **25**, 1 mM NAC, and 50 μM H_2O_2 for 24 h, respectively. These cells were harvested by trypsinization, washed with 1× PBS, and fixed in ice-cold MeOH at – 20 °C. After overnight incubation, the cells were washed with PBS and incubated with 50 μg/mL propidium iodide (Sigma, Co) and 50 μg/mL RNase A (Sigma, Co) in PBS at room temperature for 30 min. The fractions of cells in each phase of cell cycle were analyzed using FACScan flow cytometer and Cell Quest software (Becton Dickinson).

4.6. Measurement of mitochondrial membrane potential (MMP)

NTUB1 cells (3×10^5) treated with 10 μM CCCP and different concentrations of **25** (10, 25, and 50 μM) were incubated for 30 min with 1 μM of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidaolylcarbocyanine iodide (JC-1) (Moleecular Probes, Eugene, OR, USA) in culture medium. The resulting cells were washed 3 times PBS and dislodged with trypsin-EDTA. Cells were collected in PBS/2% BSA, washed twice by centrifugation (800*g*; 5 min), and resuspended in 0.3 mL PBS/2% BSA for analysis by a FACSCalibur flow cytometer (Becton Dickinson). Cytometer settings were optimized for green (R1) and red (R2) fluorescence and the ratio of JC-1 aggregates (red fluorescence) to JC-1 monomers (green fluorescence) was calculated to represent MMP.[22]

4.7. Western blot analysis

Cells were harvested by trypsinization and resuspended with suitable amount of PBS adjusted with the cell numbers. The cells were mixed with equal volume of 2× sample buffer and boiled for 10 min twice to denature the proteins. Cell extracts were separated by SDS–PAGE. The proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) using a semi-dry blotter. The blotted membranes were treated with 5% (w/v) skimmed milk in TBST buffer (100 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.1% Tween-20). The membranes were incubated with specific antibodies at 4 °C overnight. The membranes were washed with TBST buffer and incubated with secondary antibody at room temperature for another 1 h. Signals were detected by chemiluminescence ECL reagent after TBST wash and visualized on Fuji SuperRX film.

Monoclonal antibody specific for β-acctin (NB600-501) was purchased from Novus Biologicals (Littleton, CO, USA). *p*-p53 (Ser 15) (9284) polyclonal antibody was purchased from Cell Signaling (Beverly, MA, USA). The monoclonal antibody specific to p53 (DO1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.8. Statistical analysis

Data were expressed as mean ± SD. Statistical analysis were performed using the Bonferroni *t*-test method after ANOVA for multigroup comparison and the student's *t*-test method for two group comparison, with $p < 0.05$ was considered to be statistically significant.

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