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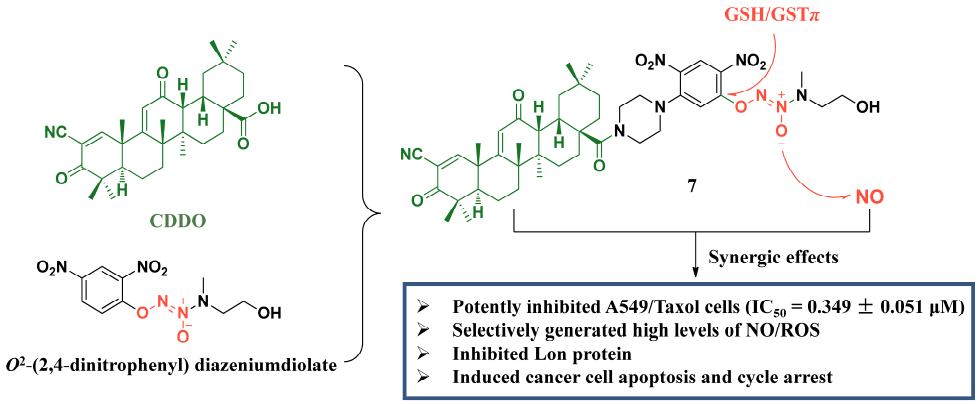
 # These authors contributed equally to this work.

Graphical Abstract



Design and synthesis of new hybrids from 2-cyano-3,12-dioxooleana-9-dien-28-oic acid and O^2-(2,4-dinitrophenyl) diazeniumdiolate for intervention of drug-resistant lung cancer

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Abstract

To search for new drugs for intervention of drug-resistant lung cancer, a series of hybrids **4**-**15** from 2-cyano-3,12-dioxooleana-9-dien-28-oic acid (CDDO) and O^2-(2,4-dinitrophenyl) diazeniumdiolate were designed, synthesized and biologically evaluated. The most active compound **7** produced relatively high levels of nitric oxide (NO) and reactive oxygen species (ROS) in drug-resistant lung cancer A549/Taxol cells which over-express glutathione S-transferase π (GSTπ), and significantly inhibited the cells' proliferation (IC_{50} = 0.349 ± 0.051 μM), superior to the positive controls CDDO-Me, JS-K and Taxol. The inhibitory activity of **7** could be attenuated by an NO scavenger, ROS scavenger or GSTπ inhibitor. In addition, **7** suppressed the Lon protease expression as well as induced cell apoptosis and cycle arrest in A549/Taxol cells more strongly than CDDO-Me or JS-K. Together, our findings suggest that **7** may be worth studying further for intervention of drug-resistant lung cancer.

Keywords: CDDO, O^2-(2,4-dinitrophenyl) diazeniumdiolate, nitric oxide, ROS, drug resistance.

1. Introduction

It has been reported that the synthetic oleanolic acid derivatives, such as 2-cyano-3,12-dioxooleana-9-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me) (Fig. 1) exhibit strong anti-cancer activity by inducing production of intracellular reactive oxygen species (ROS) in cancer cells [1], and that combination of CDDO with other anti-cancer agent(s) may synergistically induce oxidative stress overload [2].

Lon protease (Lon) refers to a highly conserved ATP-dependent serine peptidase that contributes to protein quality control and stress response pathways by selectively degrading misfolded, misassembled, or damaged proteins in mitochondria [3]. The expression of Lon is frequently elevated in cancer cells [3] and down-regulation of Lon may significantly block the cancer cell proliferation and enhance the sensitivity of cancer cells to chemotherapeutic agents by promoting apoptosis without significant toxicity to normal cells [4]. Recently, it has been discovered that CDDO-Me directly and selectively blocks mitochondrial Lon protease activity through an addition reaction between CDDO-Me and Lon, ultimately inhibiting proliferation and inducing apoptosis of the cancer cells [2].

Nitric Oxide (NO) plays an important regulation role in the realm of cancer biology [5]. High levels of NO tend to interact with ROS, such as superoxide anion ($O_2^{\bullet-}$), to generate reactive nitrogen species (RNS), such as peroxynitrite anion ($ONOO^-$) [6], acting as an inducer of cytotoxicity and apoptosis [7] as well as a reversal agent of multidrug resistance (MDR) by nitration of key protein and alteration of various redox-sensitive proteins in cancer cells [8,9]. Given its multifunctional biological effects, NO should be released from NO donors in a controlled manner, preferably in response to a certain stimulus in cancer cells, thus avoiding off-target effects [10]. O^2-(2,4-Dinitrophenyl) diazeniumdiolates, such as PABA/NO and JS-K (Fig. 1), represent an important class of NO donors as they are able to preferentially release NO in cancer cells [11], which are promoted by glutathione S-transferase π (GSTπ),

over-expressed in a large number of cancer cells [12-14].

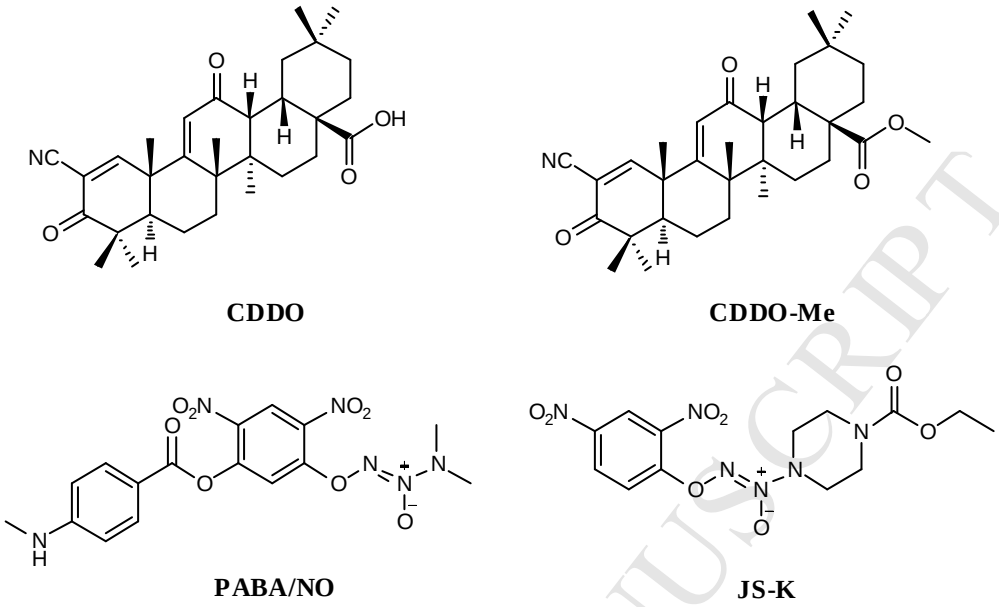


Fig. 1. Chemical structures of CDDO, CDDO-Me, PABA/NO and JS-K.

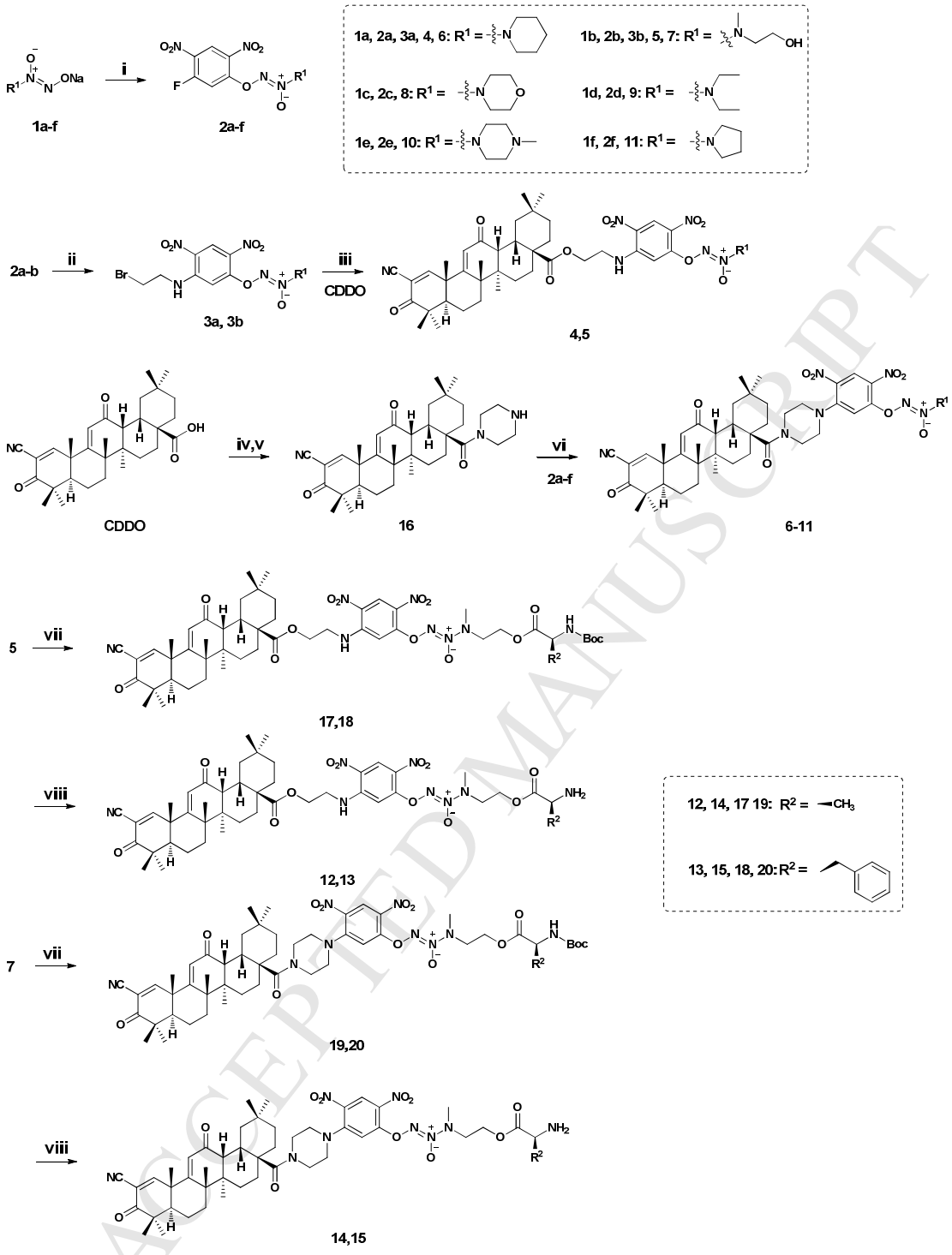
The above investigations led us to hypothesize that hybridization of O^2-(2,4-dinitrophenyl) diazeniumdiolate moiety with the CDDO scaffold would provide a new class of NO-donating CDDO derivatives with ROS/RNS inducing and Lon inhibitory activities against lung cancer resistant cells. To test the hypothesis, we designed, synthesized the hybrids **4-15** by coupling the carboxyl group of CDDO with O^2-(2,4-dinitrophenyl) diazeniumdiolate moiety through various linkers, and evaluated the inhibitory activity of these compounds against drug-resistant lung cancer.

2. Results and discussion

2.1. Chemistry

The synthesis of the target compounds is depicted in Scheme 1. The condensation of diazeniumdiolate sodium salts **1a-f** with 1,5-difluoro-2,4-dinitrobenzene in the mixture of 5% $NaHCO_3$ aqueous and acetone gave arylated diazeniumdiolates **2a-f**.

Next, substitution of one fluorine atom of **2a** or **2b** with 2-bomoethylamine hydrobromide yielded **3a** or **3b**. Finally, conjugation of 28-COOH in CDDO with **3a** and **3b** provided **4** and **5**, respectively. In addition, CDDO was treated with oxalyl chloride and the resulting acylchloride without purification was reacted with anhydrous piperazine to yield compound **16**. The free secondary amine in **16** underwent nucleophilic attack toward **2a-f** in the presence of Na_2CO_3 to furnish **6-11**. Compound **5** or **7** was treated with *N*-Boc-L-alanine or *N*-Boc-L-phenyl alanine to respectively afford the esters **17**, **18** or **19**, **20**, where the Boc group was then removed with boron trifluoride diethyl etherate to generate target compounds **12-15**, respectively.



Scheme 1. The synthetic route of the target compounds **4-15**. Reagents and conditions: (i) 5% $NaHCO_3$ aqueous, 1,5-difluoro-2,4-dinitrobenzene, acetone, N_2, 0 °C→ r.t.; (ii) 2-Bomoethylamine hydrobromide, Na_2CO_3, DMF, 4 h; (iii) CDDO, K_2CO_3, acetone; (iv) $(COCl)_2$, anhydrous CH_2Cl_2, 0 °C → r.t., 12 h; (v) Anhydrous piperazine, TEA, anhydrous CH_2Cl_2, 0 °C → r.t., 12 h; (vi) **2a-f**, Na_2CO_3, DMF; (vii) *N*-Boc-protected L-amino acids, EDCI, DMAP, anhydrous CH_2Cl_2, 25 °C; (viii) $BF_3 \cdot Et_2O$, anhydrous

CH_2Cl_2, 25 °C.

2.2. Expression of GSTπ in various cell lines

Given that our target compounds bear an O^2-(2,4-dinitrophenyl) diazeniumdiolate moiety which could be preferentially promoted by GSTπ to release NO, we initially examined the expression of GSTπ in drug-sensitive human lung adenocarcinoma A549 cells and drug-resistant A549/Taxol cells as well as human lung embryonic fibroblast MRC-5 cells by western blot assay. As shown in Fig. 2, the levels of GSTπ in A549 cells were much higher than that in MRC-5 cells but significantly lower than that in A549/Taxol cells. These results clearly indicated that GSTπ was lower-expressed in normal lung cells, highly-expressed in drug sensitive lung cancer cells, while over-expressed in drug-resistant lung cancer cells.

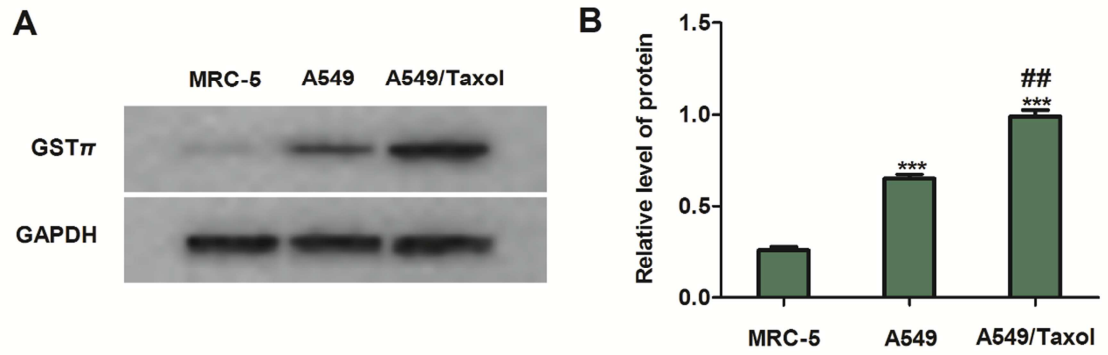


Fig. 2. Expression levels of GSTπ in A549 cells, drug-resistant A549/Taxol cells, as well as non-cancer MRC-5 cells were measured by western blot assay. Data are presented as the mean ± SD (n = 3). ***$P < 0.001$ vs the MRC-5 group, $^{\#\#}$ $P < 0.01$ vs the A549 group.

2.3. Assessment of anti-proliferative activity of the target compounds in drug sensitive and resistant lung cancer cells

To evaluate the anti-cancer activity of the target compounds, we tested their effects

on the proliferation of A549 cells and A549/Taxol cells by the MTT assay using CDDO-Me and JS-K as positive controls. As shown in Fig. 3, all compounds exhibited more potent cell growth inhibitory activity against A549/Taxol cells with over-expression of GSTπ than parental sensitive A549 cell with relatively less expression of GSTπ. Among them, compounds **5**, **7**, **8**, **12** and **14** at 1 μM inhibited proliferation of A549/Taxol cells by > 50%, which were higher than that of CDDO-Me, JS-K and the other seven compounds.

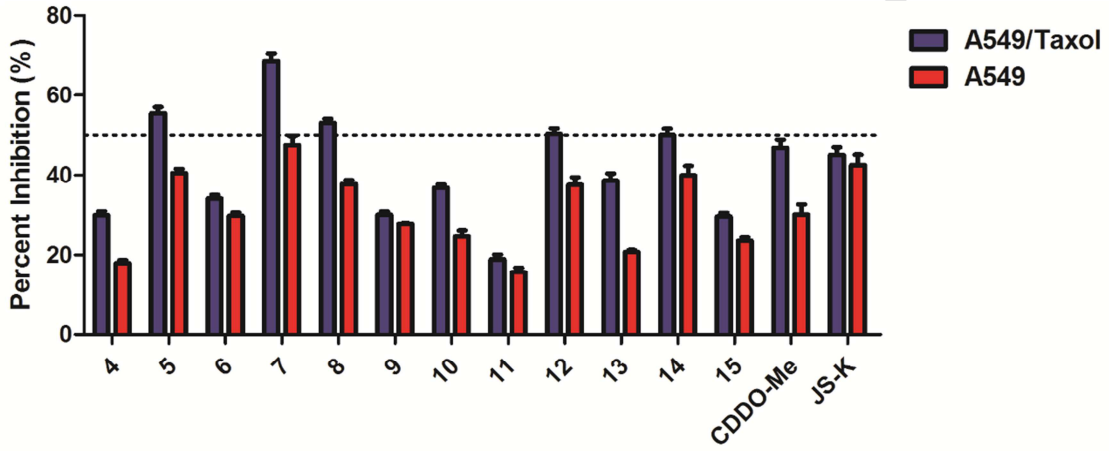


Fig. 3. Target compounds inhibit the proliferation of A549/Taxol and A549 cells. Cancer cell lines were treated with the indicated compounds at 1 μM for 72 h and the cell proliferation was measured by MTT. The inhibition (%) of each compound was determined. Data are presented as the means (%) ± SD of each compound from three independent experiments.

Table 1. Antiproliferative activity of selected compounds.

Compounds	IC_{50} (μM)[a]		RF[b]
	A549/Taxol	A549	
5	0.820 ± 0.010	1.438 ± 0.116	0.6
7	0.349 ± 0.051	1.066 ± 0.041	0.3
8	0.941 ± 0.050	1.078 ± 0.022	0.9
12	1.087 ± 0.029	1.441 ± 0.094	0.8
14	0.697 ± 0.011	1.068 ± 0.019	0.7

CDDO-Me	1.703 ± 0.056	2.074 ± 0.086	0.8
JS-K	1.987 ± 0.033	2.313 ± 0.188	0.9
Taxol	3.112 ± 0.469	0.007 ± 0.073	445

[a]The inhibitory effects of individual compounds on the proliferation of cancer cells were determined by the MTT assay. The data are presented as the means ± SD of each compound from three independent experiments. [b]Resistant factor (RF) was calculated according to the following equation: RF = IC_{50} (corresponding resistant cells)/IC_{50} (parental cells).

Subsequently, the five most potent compounds **5**, **7**, **8**, **12** and **14** were further tested for their anti-cancer activity by measuring the IC_{50} values using MTT assay and the results were summarized in Table 1. We found that these compounds displayed potent inhibitory activity against both A549/Taxol (IC_{50} = 0.349 - 1.087 μM) and A549 (IC_{50} = 1.066 - 1.438 μM) cells, and their antiproliferative activity was more potent than CDDO-Me (IC_{50} = 1.703 and 2.313 μM) and JS-K (IC_{50} = 1.987 and 2.313 μM), respectively. Compound **7** was the most potent to inhibit A549/Taxol cells with IC_{50} value of 0.349 ± 0.051 μM, over ten-fold more active than Taxol (IC_{50} = 3.112 ± 0.469 μM). In contrast, **7** showed approximately 5-fold less toxicity relative to non-cancer MRC-5 cells (IC_{50} = 1.604 ± 0.212 μM, more details see Fig. S1 in the Supporting Information), indicating that **7** preferentially and significantly inhibited the growth of lung cancer resistant cells in vitro, which may be a promising candidate for further investigation.

*2.4. **7** significantly promoted intracellular ROS accumulation in A549/Taxol cells*

To examine ROS produced by **7** in A549/Taxol cells, the cells were treated with various concentrations of **7** for 24 h in triplicate by using CDDO-Me and JS-K as positive controls. Dichlorodihydrofluorescein diacetate (DCFH-DA) probe [15] was then employed to detect the intracellular levels of ROS by measuring the fluorescent

signals using a fluorescence microplate reader. It was observed that **7** dose-dependently generated ROS, and the amounts of ROS were larger than those produced by CDDO-Me and JS-K at the same dose in A549/Taxol cells, indicating that **7** more significantly promoted intracellular ROS accumulation in the cancer resistant cells relative to CDDO-Me and JS-K (Fig. 4).

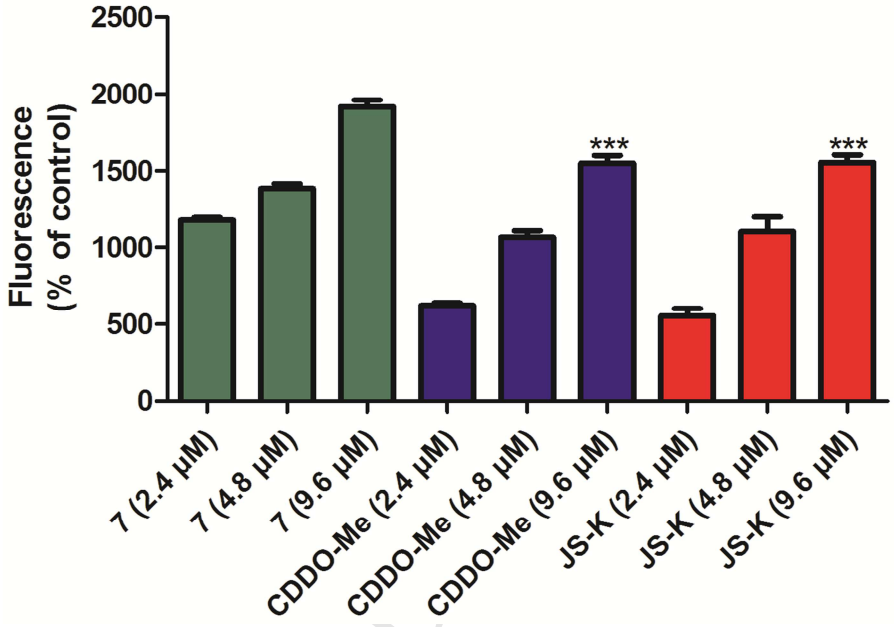


Fig. 4. Treatment with **7**, CDDO-Me and JS-K (2.4, 4.8 and 9.6 μM) induced the accumulation of ROS in A549/Taxol after 24 h. Data are expressed as the mean ± SD of each compound in individual types of cells from three experiments. ***$P < 0.001$ vs the **7** (9.6 μM) group.

*2.5. **7** selectively released high levels of NO in A549/Taxol*

Furthermore, we detected intracellular levels of NO released by **7**, JS-K and CDDO-Me in A549/Taxol cells and MRC-5 cells using Griess assay [16], respectively. As expected, treatment with CDDO-Me resulted in little NO in both A549/Taxol and MRC-5 cells. In contrast, treatment with **7** led to relatively high levels of NO production in A549/Taxol, which were larger than that produced by JS-K under the same conditions. In addition, both **7** and JS-K released small amounts of NO in the

MRC-5 cells, with less amounts from **7** relative to JS-K (Fig. 5). These results indicated that **7** selectively released relatively high amounts of NO in A549/Taxol cells, probably owing to over-expressing GST*π* of the cells.

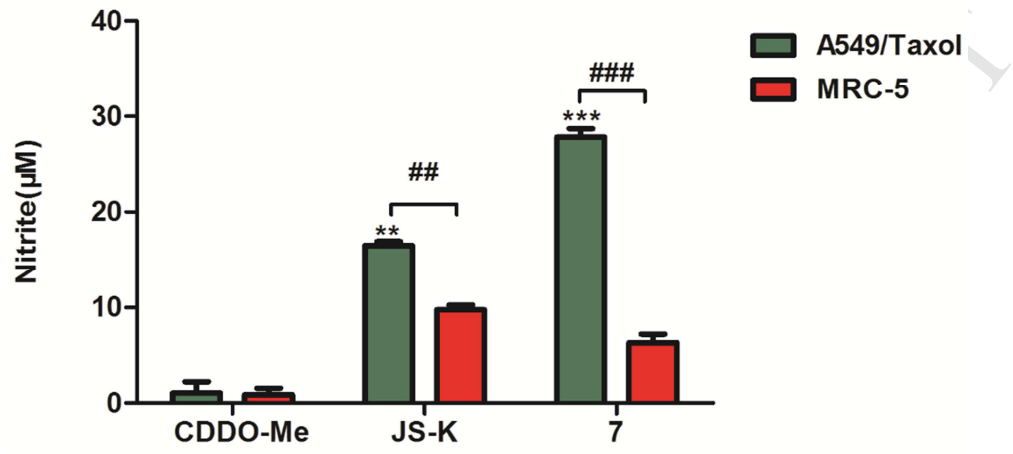


Fig. 5. Variable levels of NO (present as nitrite) produced by the indicated compounds in A549/Taxol and MRC-5 cells. Cells were treated in triplicate with individual compounds at 50 μM for 6 h, and the concentrations of intracellular nitrite were determined by Griess assay. The individual values were determined by measuring the absorbance at 540 nm and calculated according to the standard curve. Data are expressed as the mean ± SD of each compound in individual types of cells from three experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs the CDDO-Me group, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs the MRC-5 group.

*2.6. GSTπ inhibitor, ROS scavenger or NO scavenger diminished inhibitory activity of **7** against A549/Taxol*

To verify the contributions of GST*π*, ROS and NO on the growth inhibitory of **7**, A549/Taxol cells were pretreated with or without GST*π* inhibitor etacrynic acid (EA) [17], ROS scavenger *N*-acetylcysteine (NAC) [18] and NO scavenger hemoglobin (HB) [19] for 1 h and then treated with or without 1 μM of **7** for 72 h using MTT assay, respectively. The results showed that incubation with EA, HB or NAC did not affect A549/Taxol cells while treatment with **7** alone remarkably inhibited the

proliferation of A549/Taxol cells. In sharp contrast, pretreatment with EA, HB or NAC significantly diminished the inhibitory effects of **7** (Fig. 6). These results suggest that the high levels of RNS/ROS and GSTπ may be essential for the potent anti-proliferative activity of **7** against the A549/Taxol cells.

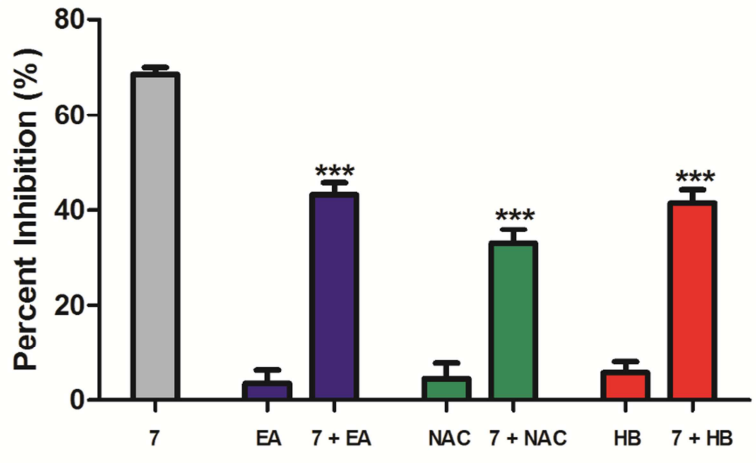


Fig. 6. Effects of GSTπ inhibitor etacrynic acid (EA), ROS scavenger *N*-acetylcysteine (NAC) and NO scavenger hemoglobin (HB) on the cell growth inhibitory activity of **7**. A549/Taxol cells were pretreated with or without EA (10 μM), NAC (10 mM) and HB (20 μM) for 1 h and then treated with or without 1 μM of **7** for 72 h, and the cell inhibition was determined by the MTT assay. Data are the mean ± SD of the cell inhibition (%) from three independent experiments. ***$P < 0.001$ vs the only **7** treated group.

2.7. ***7*** *significantly decreased levels of Lon expression in A549/Taxol cells*

Given that **7** may decrease Lon expression in cancer cells, we first examined the Lon expression in A549, A549/Taxol and MRC-5 cells by Western blot. It was found that lower levels of Lon were expressed in MRC-5 cells, while elevated expression of Lon was observed in lung cancer cells, particularly in drug-resistant A549/Taxol cells (Fig. 7A). These results were consistent with the previous report that high levels of Lon are responsible for the resistance of cancer cells to chemotherapeutics [20]. Significantly, treatment with **7** reduced the levels of Lon in A549/Taxol cells in a dose-dependent

manner, and **7** was stronger than the positive controls CDDO-Me and JS-K at the same concentration (4.8 μM) (Fig. 7B). Previous studies have demonstrated that Lon protease can be inactivated by peroxynitrite under conditions of elevated oxidative stress [21], accordingly, decreased levels of Lon expression by treatment with **7** may be attributed to the synergistic effects of CDDO and O^2-(2,4-dinitrophenyl) diazeniumdiolate which released high levels NO in drug-resistant lung cancer cells.

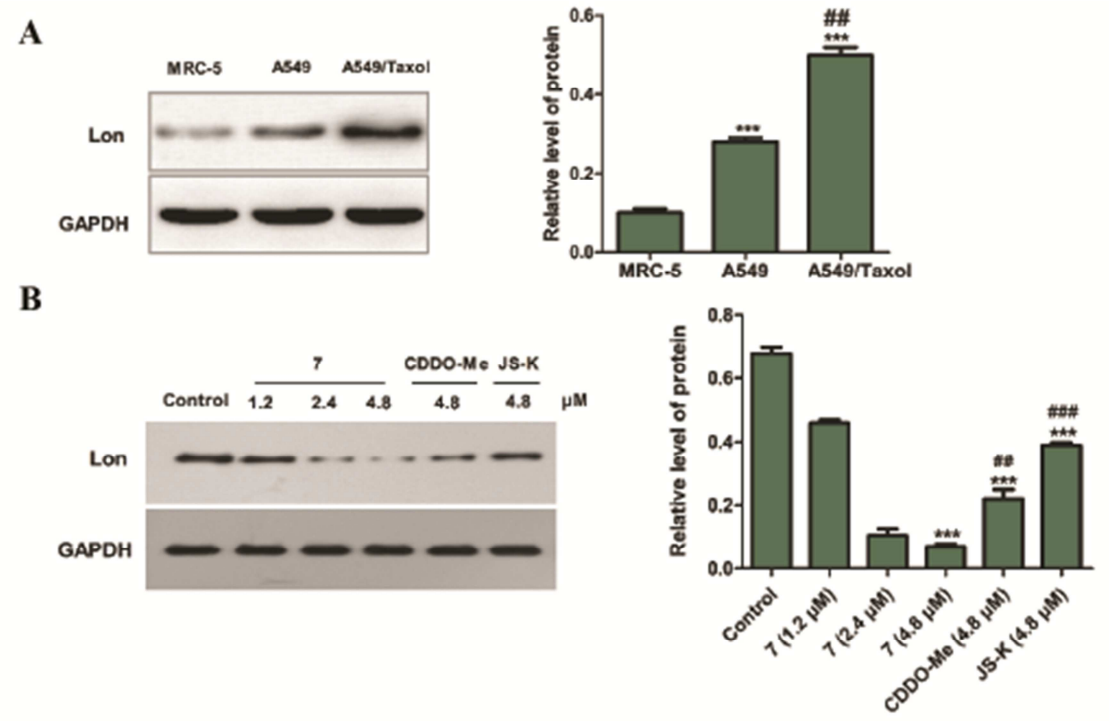


Fig. 7. A. Lon expression in MRC-5, A549, and A549/Taxol cells. MRC-5, A549, and A549/Taxol cells were harvested and lyzed. The relative levels of Lon to control GAPDH in MRC-5, A549, and A549/Taxol cell lysates were determined by Western blot. B. Effect of **7** on the protein expression of Lon in A549/Taxol cells. Cells were treated with, or without, the indicated concentrations of **7**, CDDO-Me and JS-K for 24 h, and the relative levels of Lon protein to GAPDH in cell lysates were determined by Western blotting. Data are presented as the mean ± SD (n = 3). ***$P < 0.001$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$.

*2.8. **7** induced apoptosis of A549/Taxol cells*

Considering that CDDO-Me is able to induce cancer cell apoptosis by modulating the expression of apoptosis-related regulators such as Lon [1,2], and that high levels of NO also induce tumor cell apoptosis [22], the effect of **7** on induction of tumor cells' apoptosis was examined and compared with CDDO-Me and JS-K. A549/Taxol cells were treated with various concentrations of **7**, CDDO-Me, JS-K or vehicle for 24 h. The cells were harvested and stained with Annexin V-FITC and propidium iodide (PI), and the percentages of apoptotic cells were determined by flow cytometry analysis. It was observed that treatment with **7** induced apoptosis in A549/Taxol cells in a dose-dependent manner, and was significantly stronger than treatment with CDDO-Me and JS-K alone at the same doses (Fig. 8). These results suggest that **7** may exert its anti-cancer activity, at least in part, via induction of the cells' apoptosis.

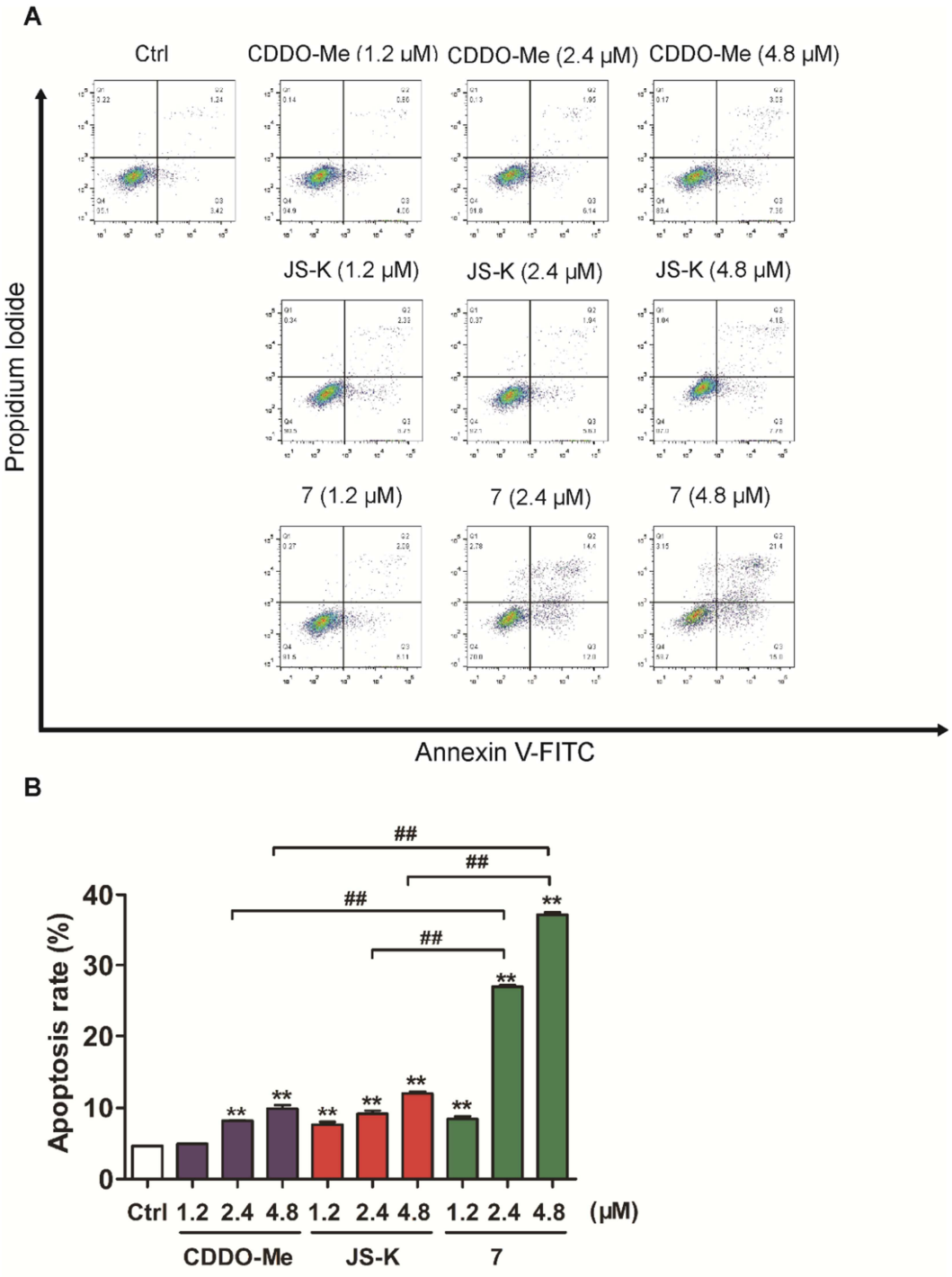


Fig. 8. Apoptosis of A549/Taxol cells treated with **7**, CDDO-Me, and JS-K for 24 h. Data are presented as the mean ± SD (n = 3). $^{**}P < 0.01$ vs the control group, $^{\#\#}P < 0.01$.

*2.9. **7** arrested cell cycle of A549/Taxol cells*

To investigate whether **7** suppressed the cells' growth by arresting cell cycle, we

performed the experiment where cell cycle distribution was analyzed by flow cytometry after staining the DNA with propidium iodide (PI). It was found that treatment with **7** at 1.2, 2.4 and 4.8 μM for 24 h increased the percentage of cells at the G2/M phase from 28.3% to 48.3% while decreased G0/G1-phase cells from 61. 3% to 39.4% in a dose-dependent manner (Fig. 9). Moreover, the blocking effect of **7** were significantly stronger than that of CDDO-Me and JS-K at the same doses (Representative histograms of CDDO-Me and JS-K were shown on pages S2 in the Supporting Information). These results indicated that **7** significantly induced A549/Taxol cell cycle arrest at G0/G1 phase.

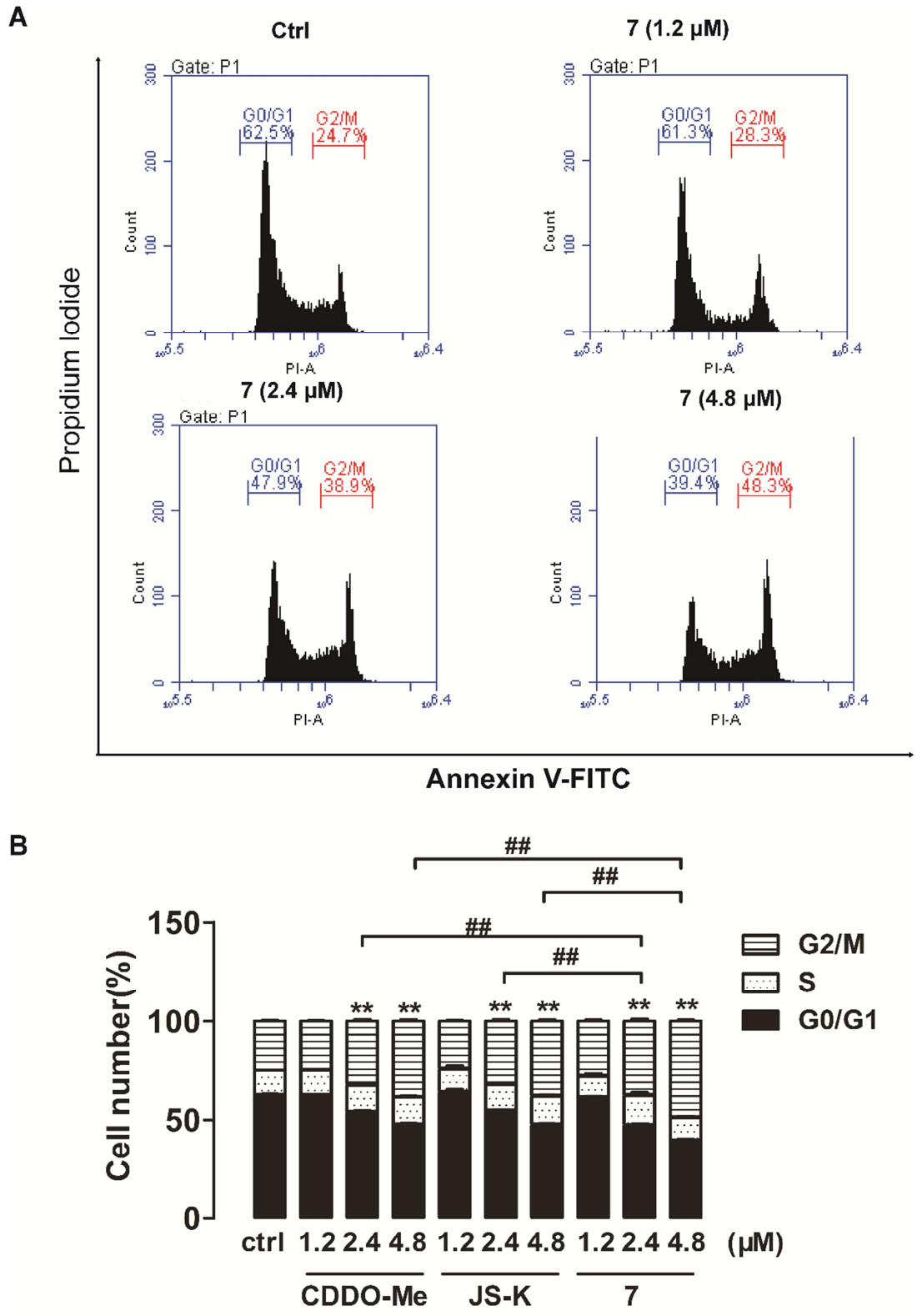


Fig. 9. Effects of **7** on cell cycle of A549/Taxol cells. Cells were treated with the indicated concentrations of **7**, CDDO-Me and JS-K for 24 h and stained with PI, followed by flow cytometry analysis: (A) representative histograms; (more details of CDDO-Me and JS-K were shown on pages S2 in the Supporting Information) (B)

quantitative analysis. Data are representative histograms and expressed as the mean ± SD of each compound from three independent experiments. $^{**}P < 0.01$ vs the control group, $^{\#\#}P < 0.01$.

3. Conclusion

In summary, twelve hybrids from O^2-(2,4-dinitrophenyl) diazeniumdiolate and CDDO were designed and synthesized. The most active hybrid **7** selectively produced high levels of NO and ROS in drug resistant A549/Taxol cells. In addition, **7** significantly inhibited the proliferation of A549/Taxol cells, superior to CDDO-Me and JS-K, and over ten-fold more potent than taxol. In contrast, **7** had approximately 5-fold less effect on non-cancer MRC-5 cells relative to A549/Taxol cells, and its anti-cancer activity was significantly attenuated by a GSTπ inhibitor, ROS scavenger or NO scavenger. Furthermore, **7** suppressed the Lon expression, induced apoptosis and cell cycle arrest in A549/Taxol cells more strongly than CDDO-Me and JS-K. These results suggest that the anti-proliferative activity of **7** could be attributed to the synergic effects of CDDO and O^2-(2,4-dinitrophenyl) diazeniumdiolate moiety, leading to selective generation of high levels of NO/ROS and enhanced inhibition of Lon in drug-resistant lung cancer cells. Therefore, our findings provide a proof of principle in design of new NO-releasing CDDO derivatives for the intervention of drug-resistant lung cancer.

4. Experimental protocols

4.1. Chemical analysis

All commercially available compounds were used without further purification, unless otherwise noted. Anhydrous solvents (CH_2Cl_2) were used as commercially available. Analytical and preparative TLC was performed on silica gel (200 - 300 mesh) GF/UV 254 plates, and the chromatograms were visualized under UV light at 254 and 365 nm. Melting points of individual compounds were determined on a

Mel-TEMP II melting point apparatus and uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) spectrometer at 303 K, using TMS as an internal standard. MS spectra were recorded with a Mariner mass spectrometer (ESI) and high resolution mass spectrometry (HRMS) spectra on an Agilent Technologies LC/MSD TOF instrument. Solutions after reactions and extractions were concentrated using a rotary evaporator operating at a reduced pressure of ~20 Torr. Individual compounds with a purity of > 95% were used for biological experiments. CDDO and acyl chloride of CDDO were synthesized using the reported protocols [23]. Diazeniumdiolate sodium salts **1a-f** were prepared according to the method described previously [13]. *O*^{2}-(2,4-Dinitrophenyl) diazeniumdiolates **2a-f** were synthesized using the reported methods [12].

*4.1.1 General procedure for the preparation of **4** and **5***

*4.1.1.1 Synthesis of intermediates of **3a** and **3b***

To a DMF (2.5 mL) solution of 2-bomoethylamine hydrobromide (1.0 mmol) and **2a** (1.0 mmol) was added Na_2CO_3 (2 mmol), and the resulting solution was stirred at room temperature for 4 h. Then the reaction mixture was diluted with water (15 mL) and extracted with EtOAc (50 mL × 3). The combined organic layer was washed with brine, dried with anhydrous sodium sulfate, filtered, and evaporated in vacuum. The obtained crude product was purified by silica column chromatography (PE/EA = 1:2, v/v) to afford *O*^{2}-(2,4-dinitro-5-(2-bromoethylamino)phenyl) 1-(piperidine-1-yl) diazen-1-ium-1,2-diolate (**3a**) as yellowish liquid, yield 77%. Synthesis of *O*^{2}-(2,4-dinitro-5-(2-bromoethylamino)phenyl) 1-(*N*-methylethanolamino) diazen-1-ium-1,2-diolate (**3b**) was conducted in the similar way with yield 74%.

*4.1.1.2 Synthesis of compounds of **4** and **5***

The solution of **3a** (1.0 mmol) and CDDO (1.0 mmol) in acetone (10 mL) was added K_2CO_3 (1.5 mmol). The mixture was then heated to 45 □ and stirred for 4 h. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The resulting residue was purified by flash chromatography (PE/EA = 3:7,

v/v) to afford the compound **4**. Synthesis of **5** were in the similar method.

*4.1.1.2.1 Compound **4***

O^2-(2,4-Dinitro-5-{2-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-oxo-ethyl-amino}phenyl) 1-(piperidine-1-yl) diazen-1-ium-1,2-diolate. The title compound was obtained in 68% yield as a yellowish solid: mp: 170 - 172 □; ^{1}H NMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 9.08 (s, 1H, Ar-H), 8.73 (t, *J* = 5.1 Hz, 1H, NH-Ar), 8.05 (s, 1H, C_1-H), 6.82 (s, 1H, Ar-H), 5.96 (s, 1H, C_{11}-H), 4.47 - 4.44 (m, 2H, OCH_2), 3.73 - 3.68 (m, 2H, OCH_2), 3.61 (t, *J* = 5.6 Hz, 4H, 2 × NCH_2), 3.07 - 2.97 (m, 1H, C_{13}-H), 2.90 - 2.85 (m, 1H, C_{18}-H), 1.46 (s, 3H, CH_3), 1.28 - 1.24 (m, 8H, CH_3, 3 × CH_2), 1.20 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ 198.0, 196.2, 177.2, 168.0, 165.2, 155.0, 148.0, 127.3, 126.6, 125.8, 123.5, 114.0, 113.9, 97.9, 61.1, 51.3, 49.0, 47.1, 46.8, 45.1, 44.5, 42.0, 41.8, 41.5, 35.1, 33.8, 32.6, 31.1, 30.1, 29.3, 29.0, 28.8, 27.5, 26.4, 26.1, 23.9, 23.8, 22.7, 22.5, 22.1, 21.0, 21.0, 17.6; ESI-MS: 866 $[M + Na]^+$; HRMS: m/z: Calcd. for $C_{44}H_{57}N_7O_{10}$ $[M + Na]^+$ 866.4167, found 866.4050, ppm error 5.0.

*4.1.1.2.2 Compound **5***

O^2-(2,4-Dinitro-5-{2-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-oxo-ethyl-amino}phenyl) 1-(*N*-methyl ethanolamino) diazen-1-ium-1,2-diolate. The title compound was obtained in 74% yield as a yellowish solid: mp: 134 - 136 □; ^{1}H NMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 9.07 (s, 1H, Ar-H), 8.69 (s, 1H, NH-Ar), 8.05 (s, 1H, C_1-H), 6.88 (s, 1H, Ar-H), 5.97 (s, 1H, C_{11}-H), 4.42 - 4.40 (m, 2H, OCH_2), 3.92 - 3.69 (m, 6H, OCH_2, 2 × NCH_2), 3.41 (s, 3H, NCH_3), 2.97 - 2.92 (m, 1H, C_{13}-H), 2.92 - 2.88 (m, 1H, C_{18}-H), 1.46 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.22 (s, 3H, CH_3), 1.17 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ 198.9, 196.6, 178.0, 168.9, 165.8, 155.7, 148.6, 127.8, 126.9, 126.2, 123.9, 114.5, 114.5, 98.1, 61.5, 59.7, 55.7, 49.6, 47.6, 47.4, 45.7, 45.0, 42.6, 42.0, 41.9, 40.6, 35.6, 34.3, 33.1, 32.9, 31.5, 30.6, 29.7, 28.0, 26.9, 26.6, 24.4,

23.0, 22.6, 21.6, 21.4, 18.1; ESI-MS: 834 $[M + H]^+$, 856 $[M + Na]^+$; HRMS: m/z: Calcd. for $C_{42}H_{55}N_7O_{11}$ $[M - H]^-$ 832.3960, found 832.3896, ppm error 5.0.

*4.1.2 General procedure for the preparation of **6-11***

*4.1.2.1 Synthesis of intermediates of **16***

A mixture of CDDO (6.1 mmol) and oxalyl chloride (5 mL) in anhydrous CH_2Cl_2 (50 mL) was stirred at room temperature overnight. The solvent was removed in vacuo, and the residue was coevaporated with anhydrous CH_2Cl_2 three times, and then used for the next reaction without further purification.

To a solution of the residue (0.35 mmol) obtained above in anhydrous CH_2Cl_2 (2 mL) was added a solution of anhydrous piperazine (1.5 mmol). The mixture was stirred at room temperature overnight. The reaction mixture was then diluted with CH_2Cl_2 (15 mL), washed with saturated sodium bicarbonate ($NaHCO_3$) aqueous solution and brine and dried over magnesium sulfate. The solvent was removed and the crude product was purified by flash column chromatography (PE/EA = 1:9, v/v) to obtain 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin (**16**) as an amorphous solid, yield 68%. mp: 198 - 200 °C; ^{1}H NMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.06 (s, 1H, C_1-H), 5.95 (s, 1H, C_{11}-H), 3.70 - 3.68 (m, 4H), 3.36 (s, 1H), 3.09 (d, *J* = 12.9 Hz, 1H), 2.86 - 2.84 (m, 4H),1.48 (s, 3H), 1.32 (s, 3H), 1.26 (s, 3H), 1.17 (s, 3H), 1.02 (s, 3H), 1.01 (s, 3H), 0.91 (s, 3H); ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): *δ* 198.8, 196.2, 174.8, 165.5, 165.3, 123.6, 114.0, 112.9, 62.7, 60.2, 49.1, 47.2, 47.0, 45.5, 45.2, 44.5, 42.0, 41.5, 35.6, 33.7, 32.6, 32.6, 32.5, 31.1, 29.8, 29.7, 27.9, 26.4, 26.4, 26.0, 24.0, 23.5, 22.4, 21.3, 21.0, 17.7; ESI-MS: 560 $[M + H]^+$.

*4.1.2.2 Synthesis of compounds **6-11***

Compounds **6-11** were synthesized using a similar method. A typical procedure can be described as follows using **6** as the example. To a DMF (2.5 mL) solution of **16** (1.0 mmol) and **2a-f** (1.0 mmol) was added Na_2CO_3 (2 mmol), and the resulting solution was stirred at room temperature for 4 h. Then the reaction mixture was

diluted with water (2 mL) and extracted with EtOAc (50 ml × 3). The combined organic layer was washed with brine, dried with anhydrous sodium sulfate, filtered, and evaporated in vacuum. The obtained crude product was purified by silica column chromatography (PE/EA = 2:3, v/v) to afford compounds **6**-**11** as yellowish solid.

*4.1.2.2.1 Compound **6***

O^2-(2,4-Dinitro-5-{4-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin-1-yl}phenyl) 1-(piperidine-1-yl) diazen-1-ium-1,2-diolate. The title compound was obtained in 58% yield as a yellowish solid: mp: 168 - 170 °C; ^{1}HNMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.73 (s, 1H, ArH), 8.05 (s, 1H, C_1-H), 6.94 (s, 1H, ArH), 5.96 (s, 1H, C_{11}-H), 4.09 - 3.93 (m, 4H, 2 × NCH_2), 3.59 (t, J = 5.5 Hz, 4H, 2 × NCH_2), 3.35 - 3.25 (m, 5H, 2 × NCH_2, C_{13}-H), 3.16 - 3.09 (m, 1H, C_{18}-H), 1.86 -1.81 (m, 6H, 3 × CH_2), 1.49 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.17 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ 199.0, 196.5, 175.8, 167.9, 165.9, 165.8, 153.8, 149.8, 133.1, 128.7, 127.2, 124.0, 114.4, 105.6, 51.9, 51.6, 50.5, 49.5, 47.7, 47.6, 45.8, 45.0, 44.9, 42.5, 42.1, 36.1, 34.2, 33.1, 33.0, 31.6, 30.4, 30.4, 29.7, 26.9, 26.5, 24.4, 24.3, 23.9, 23.2, 22.8, 21.7, 21.5, 18.2; ESI-MS: 891 $[M + Na]^+$, 907 $[M + K]^+$;

*4.1.2.2.1 Compound **7***

O^2-(2,4-Dinitro-5-{4-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin-1-yl}phenyl) 1-(*N*-methyl ethanolamino) diazen-1-ium-1,2-diolate. The title compound was obtained in 66% yield as a yellowish solid: mp: 152 - 154 □; ^{1}HNMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.73 (s, 1H, ArH), 8.07 (s, 1H, C_1-H), 6.94 (s, 1H, ArH), 5.96 (s, 1H, C_{11}-H), 3.95 - 3.83 (m, 8H, 4 × NCH_2), 3.37 (m, 3H, NCH_3), 3.26 - 3.09 (m, 6H, NCH_2, OCH_2, C_{13}-H, C_{18}-H), 1.48 (s, 3H, CH_3), 1.30 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 0.93 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ 199.0, 196.1, 175.3, 165.1, 165.1, 153.7, 149.5, 132.5, 126.7, 123.3, 113.9, 113.8, 107.5, 104.8, 59.1, 55.1, 50.0, 49.2, 47.1, 47.0, 45.4, 44.5, 44.5, 42.0, 41.6, 40.1, 35.8, 33.6, 32.6, 32.3, 31.1, 30.0, 29.7, 28.8,

27.8, 26.3, 25.8, 24.0, 23.6, 22.5, 21.6, 21.0, 17.6; ESI-MS: 881 $[M + Na]^+$, 897 $[M + K]^+$; HRMS: m/z: Calcd. for $C_{44}H_{58}N_8O_{10}$ $[M - H]^-$ 857.4276, found 857.4209, ppm error 5.0.

*4.1.2.2.3 Compound **8***

O^2-(2,4-Dinitro-5-{4-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin-1-yl}phenyl) 1-(morpholine-1-yl) diazen-1-ium-1,2-diolate. The title compound was obtained in 60% yield as a yellowish solid: mp: 158 - 160 □; ^{1}HNMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.73 (s, 1H, ArH), 8.05 (s, 1H, C_1-H), 6.93 (s, 1H, ArH), 5.96 (s, 1H, C_{11}-H), 4.03 - 3.97 (m, 8H, 4 × NCH_2), 3.61 (t, *J* = 4.7 Hz, 4H, 2 × OCH_2), 3.30 (s, 4H, 2 × NCH_2), 3.16 - 3.12 (m, 2H, C_{13}-H, C_{18}-H), 1.48 (s, 3H, CH_3), 1.31 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.17 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): *δ* 198.4, 196.0, 175.4, 167.3, 165.1, 153.0, 149.0, 133.0, 126.8, 123.5, 119.5, 114.0, 113.8, 105.3, 65.0, 50.6, 50.0, 49.0, 47.2, 47.1, 45.3, 44.5, 44.4, 44.2, 42.0, 41.7, 35.6, 33.7, 32.5, 32.4, 31.1, 29.9, 29.8, 29.1, 28.8, 27.9, 26.7, 26.1, 23.9, 23.4, 22.5, 21.2, 21.0, 17.7; ESI-MS: 893 $[M + Na]^+$; HRMS: m/z: Calcd. for $C_{45}H_{58}N_8O_{10}$ $[M + Na]^+$ 893.4276, found 893.4193, ppm error 5.0.

*4.1.2.2.4 Compound **9***

O^2-(2,4-Dinitro-5-{4-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin-1-yl}phenyl) 1-(*N*,*N*-diethylamino) diazen-1-ium-1,2-diolate. The title compound was obtained in 58% yield as a yellowish solid: mp: 162 - 164 □; ^{1}HNMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.76 (s, 1H, ArH), 8.06 (s, 1H, C_1-H), 6.98 (s, 1H, ArH), 5.99 (s, 1H, C_{11}-H), 4.02 - 3.87 (m, 4H, 2 × NCH_2), 3.56 - 3.49 (m, 4H, 2 × NCH_2), 3.35 - 3.22 (m, 5H, 2 × NCH_2, C_{13}-H), 3.16 - 3.12 (m, 1H, C_{18}-H), 1.49 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 1.27 - 1.25 (m, 9H, 2 × CH_3, CH_3), 1.17 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): *δ* 199.0, 196.1, 175.8, 167.9, 165.7, 153.8, 149.7, 133.2, 128.7, 127.3, 123.9, 114.6, 114.4, 105.4, 60.3, 50.5, 49.5, 47.8, 47.6, 47.3, 45.8, 45.0, 44.9, 42.5, 42.1, 36.1, 34.2,

33.1, 33.0, 31.6, 30.4, 30.4, 29.6, 28.4, 26.9, 26.5, 24.4, 24.0, 22.9, 21.8, 21.5, 18.2, 14.2, 14.1, 11.6; ESI-MS: 879 $[M + Na]^+$; HRMS: m/z: Calcd. for $C_{45}H_{60}N_8O_9$ $[M + Na]^+$ 879.4483, found 879.4395, ppm error 5.0.

*4.1.2.2.5 Compound **10***

O^2-(2,4-Dinitro-5-{4-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin-1-yl}phenyl) 1-(4-methylpiperazine-1-yl) diazen-1-ium-1,2-diolate. The title compound was obtained in 61% yield as a yellowish solid: mp: 167 - 169 □; ^{1}HNMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.73 (s, 1H, ArH), 8.01 (s, 1H, C_1-H), 6.91 (s, 1H, ArH), 5.96 (s, 1H, C_{11}-H), 4.03 - 3.98 (m, 4H, 2 × NCH_2), 3.67 (s, 4H, 2 × NCH_2), 3.35 - 3.30 (m, 5H, 2 × NCH_2, C_{13}-H), 3.16 - 3.09 (m, 1H, C_{18}-H), 2.71 (t, *J* = 4.5 Hz, 4H, 2 × NCH_2), 2.40 (s, 3H, NCH_3), 1.49 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.17 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): *δ* 199.0, 196.5, 175.9, 167.9, 165.6, 153.6, 149.7, 133.2, 128.8, 127.2, 124.1, 114.5, 114.4, 105.5, 53.2, 50.5, 50.4, 49.5, 47.8, 47.6, 45.8, 45.4, 45.0, 44.9, 42.5, 42.1, 36.2, 36.1, 34.2, 33.1, 32.9, 31.6, 30.4, 30.4, 29.7, 28.4, 26.9, 26.5, 24.5, 24.0, 22.8, 21.8, 21.6, 18.2; ESI-MS: 884 $[M + H]^+$, 906 $[M + Na]^+$, 922 $[M + K]^+$; HRMS: m/z: Calcd. for $C_{46}H_{61}N_9O_9$ $[M + Na]^+$ 906.4592, found 906.4506, ppm error 5.0.

*4.1.2.2.6 Compound **11**.*

O^2-(2,4-Dinitro-5-{4-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin-1-yl}phenyl) 1-(pyrrolidine-1-yl) diazen-1-ium-1,2-diolate. The title compound was obtained in 58% yield as a yellowish solid: mp: 174 - 176 □; ^{1}HNMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.67 (s, 1H, ArH), 8.01 (s, 1H, C_1-H), 6.94 (s, 1H, ArH), 5.93 (s, 1H, C_{11}-H), 4.05 - 3.85 (m, 4H, 2 × NCH_2), 3.74 (s, 4H, 2 × NCH_2), 3.37 - 3.25 (m, 5H, 2 × NCH_2, C_{13}-H), 3.14 - 3.10 (m, 1H, C_{18}-H), 2.08 - 2.06 (m, 4H, 2 × CH_2), 1.47 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 1.21 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): *δ* 199.2, 196.5, 175.8, 165.9, 161.0, 154.2, 150.0, 136.6, 132.4, 127.4, 124.0,

114.5, 107.4, 104.2, 68.2, 50.6, 50.4, 49.5, 47.7, 47.6, 45.8, 45.0, 44.8, 42.5, 42.2, 36.0, 34.2, 34.2, 33.1, 31.6, 31.2, 30.9, 30.4, 28.4, 26.9, 26.5, 24.4, 23.9, 23.5, 22.7, 21.7, 21.6, 19.1, 18.2; ESI-MS: 877 $[M + Na]^+$, 893 $[M +K]^+$; HRMS: m/z: Calcd. for $C_{45}H_{58}N_8O_9$ $[M + Na]^+$ 877.4321, found 877.4236, ppm error 5.0.

*4.1.3 General procedure for the preparation of **12-15***

*4.1.3.1 Synthesis of intermediates of **17**, **18** and **19**, **20***

5 (3.0 mmol), *N*-Boc-protected L-amino acids (*N*-Boc-L-alanine and *N*-Boc-L-phenyl alanine, 3.1 mmol), EDCI (6.0 mmol) and DMAP (0.3 mmol) were added in anhydrous CH_2Cl_2 (5 mL) and stirred for 6 - 12 h at room temperature. The reaction mixture was then diluted with CH_2Cl_2, washed with saturated sodium bicarbonate ($NaHCO_3$) aqueous solution, brine, dried with anhydrous sodium sulfate, filtered, and evaporated in vacuum. The obtained crude product was purified by silica column chromatography to afford compounds **17** or **18** as yellowish solid. Compounds **19** and **20** were synthesized using a similar procedure starting from **7**.

*4.1.3.1.1 Compound **17***

O^2-(2,4-Dinitro-5-{2-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-oxo-ethyl-amino}phenyl) 1-{*N*-methyl-2-[2-(S)-(tert-butoxycarbonylamino)proionyl-1-oxo]ethylamino} diazen-1-ium-1,2-diolate. The title compound was obtained in 84% yield as a yellowish solid: mp: 114 - 116 □; ^{1}H NMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 9.10 (s, 1H, Ar-H), 8.72 (t, *J* = 4.7 Hz, 1H, NH-Ar), 8.03 (s, 1H, C_1-H), 6.87 (s, 1H, Ar-H), 5.96 (s, 1H, C_{11}-H), 4.44 - 4.41 (m, 4H, OCH_2), 4.32 - 4.22 (m, 1H, COCH), 3.92 - 3.91 (m, 2H, NCH_2), 3.71 - 3.70 (m, 2H, NCH_2), 3.36 (s, 3H, NCH_3), 3.03 - 2.99 (m, 1H, C_{13}-H), 2.91 - 2.89 (m, 1H, C_{18}-H), 1.46 (s, 3H, CH_3), 1.43 (s, 9H, 3 × CH_3), 1.25 (s, 3H, CH_3), 1.19 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ197.8, 195.9, 177.1, 167.8, 165.0, 155.0, 147.8, 127.4, 127.3, 126.5, 125.9, 123.4, 114.0, 113.7, 97.8, 61.0, 51.8, 51.7, 49.1, 48.8, 46.9, 45.1, 44.5, 42.0, 41.7, 41.6, 40.2,

35.1, 33.8, 32.6, 32.4, 31.1, 31.0, 30.1, 29.1, 27.8, 27.7, 26.5, 26.1, 23.9, 22.4, 22.1, 21.0, 20.9, 17.6; ESI-MS: 1027 $[M + Na]^+$, 1043 $[M + K]^+$.

*4.1.3.1.2 Compound **18***

O^2-(2,4-Dinitro-5-{2-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-oxo-ethyl-amino}phenyl) 1-{*N*-methyl-2-[2-(S)-(tert-butoxycarbonylamino)-3-phenyl propionyl-1-oxo]ethylamino} diazen-1-ium-1,2-diolate. The title compound was obtained in 82% yield as a yellowish solid: mp: 102 - 104 □; ^{1}H NMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 9.08 (s, 1H, Ar-H), 8.70 (t, *J* = 4.9 Hz, 1H, NH-Ar), 8.03 (s, 1H, C_1-H), 7.32 - 7.24 (m, 3H, 3 × ArH), 7.16 - 7.14 (m, 2H, 2 × ArH), 6.84 (s, 1H, Ar-H), 5.96 (s, 1H, C_{11}-H), 4.39 - 4.31 (m, 4H, OCH_2), 4.16 - 4.09 (m, 1H, COCH), 3.83 - 3.82 (m, 2H, NCH_2), 3.66 - 3.65 (m, 2H, NCH_2), 3.28 (s, 3H, NCH_3), 3.11 - 3.04 (m, 2H, CH_2Ar), 3.02 - 2.95 (m, 1H, C_{13}-H), 2.91 - 2.85 (m, 1H, C_{18}-H), 1.45 (s, 3H, CH_3), 1.40 (s, 9H, 3 × CH_3), 1.25 (s, 3H, CH_3), 1.19 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ197.9, 196.0, 177.3, 167.7, 165.1, 155.1, 147.9, 135.3, 128.7, 128.2, 127.3, 126.7, 126.6, 125.8, 123.5, 114.0, 113.8, 97.7, 61.3, 61,2, 54.1, 51.7, 49.0, 48.6, 47.1, 46.8, 45.2, 44.4, 42.0, 41.6, 41.6, 40.3, 37.7, 35.0, 33.8, 33.4, 32.6, 32.3, 31.0, 31.0, 30.9, 30.0, 29.6, 29.2, 28.8, 27.8, 27.6, 26.4, 26.1, 25.1, 24.4, 23.8, 22.4, 22.1, 21.0, 17.5; ESI-MS: 1103 $[M + Na]^+$, 1119 $[M + K]^+$.

*4.1.3.1.3 Compound **19***

O^2-(2,4-Dinitro-5-{4-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin-1-yl}phenyl) 1-{*N*-methyl-2-[2-(S)-(tert-butoxycarbonylamino)proionyl-1-oxo]ethylamino} diazen-ium-1,2-diolate. The title compound was obtained in 78% yield as a yellowish solid: mp: 134 - 136 □; ^{1}HNMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.73 (s, 1H, ArH), 8.07 (s, 1H, C_1-H), 6.96 (s, 1H, ArH), 5.98 (s, 1H, C_{11}-H), 4.46 - 4.42 (m, 2H, CH_2O), 4.02 - 3.88 (m, 8H, 4 × NCH_2), 3.35 (s, 3H, NCH_3), 3.34 - 3.33 (m, 3H, COCH, NCH_2), 3.19 - 3.12 (m, 2H, C_{13}-H, C_{18}-H), 1.49 (s, 3H, CH_3), 1.42 (s, 9H, 3 × CH_3), 1.32 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.01 (s,

3H, CH_3), 1.00 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ 199.0, 196.4, 176.0, 165.7, 153.9, 149.6, 137.9, 134.2, 133.0, 129.8, 129.5, 128.6, 127.4, 114.5, 114.4, 105.2, 61.6, 58.1, 52.5, 50.5, 49.5, 49.1, 47.8, 45.8, 45.0, 42.5, 42.1, 41.0, 34.2, 33.9, 33.1, 31.6, 30.4, 29.7, 29.3, 28.3, 26.9, 26.5, 25.6, 24.9, 24.4, 23.9, 21.6, 18.2, 15.6, 11.5; ESI-MS: 1052 $[M + Na]^+$, 1068 $[M + K]^+$.

*4.1.3.1.4 Compound **20***

O^2-(2,4-Dinitro-5-{4-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin-1-yl}phenyl) 1-{*N*-methyl-2-[2-(S)-(tert-butoxycarbonylamino)-3-phenyl propionyl-1-oxo]ethylamino) diazen-1-ium-1,2-diolate. The title compound was obtained in 80% yield as a yellowish solid: mp: 121 - 123 □; ^{1}HNMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.74 (s, 1H, ArH), 8.06 (s, 1H, C_1-H), 7.32 - 7.21 (m, 3H, 3 × ArH), 7.17 - 7.15 (m, 2H, 2 × ArH), 6.96 (s, 1H, ArH), 5.96 (s, 1H, C_{11}-H), 4.16 - .09 (m, 2H, CH_2O), 4.04 - 3.70 (m, 8H, 4 × NCH_2), 3.54 - 3.42 (m, 2H, CH_2Ar), 3.31 - 3.28 (m, 3H, COCH, NCH_2), 3. 25 (s, 3H, NCH_3), 3.06 - 3.04 (m, 2H, C_{13}-H, C_{18}-H), 1.48 (s, 3H, CH_3), 1.39 (s, 9H, 3 × CH_3), 1.31 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ 199.0, 196.6, 176.0, 175.6, 167.8, 165.6, 149.5, 148.5, 135.9, 129.2, 129.2, 129.1, 128.7, 127.3, 127.2, 124.0, 114.4, 114.3, 105.6, 61.8, 54.9, 52.4, 50.5, 49.3, 47.8, 47.5, 45.8, 45.0, 42.4, 42.0, 40.8, 38.2, 36.0, 34.3, 33.9, 33.1, 31.6, 30.3, 29.5, 28.3, 26.9, 26.5, 25.6, 24.9, 24.4, 23.8, 21.7, 21.4, 18.2; ESI-MS: 1128 $[M + Na]^+$, 1144 $[M + K]^+$.

*4.1.3.2 Synthesis of compounds **12-15***

Compounds **12-15** were synthesized using a similar method. A typical procedure can be described as follows using **17** as the example. A mixture of **17** (1.0 mmol) and boron trifluoride diethyl etherate (2 mL) in anhydrous CH_2Cl_2 (5 mL) was stirred at room temperature (1.0 mmol) for 1 - 2 h. The reaction mixture was then quenched with saturated sodium bicarbonate ($NaHCO_3$) aqueous solution, diluted with CH_2Cl_2, washed with saturated sodium bicarbonate aqueous solution, brine, dried with

anhydrous sodium sulfate, filtered, and evaporated in vacuum. The obtained crude product was purified by silica column chromatography to afford compounds **12** as yellowish solid. Compounds **13**-**15** were synthesized using a similar procedure starting from **18**, **19** or **20**, respectively.

*4.1.3.2.1 Compound **12***

O^2-(2,4-Dinitro-5-{2-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-oxo-ethyl-amino}phenyl) 1-{*N*-methyl-2-[2-(S)-(tert-butoxycarbonylamino)proionyl-1-oxo]ethylamino} diazen-1-ium-1,2-diolate. The title compound was obtained in 87% yield as a yellowish solid: mp: 153 - 155 □; ^{1}H NMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 9.07 (s, 1H, ArH), 8.74 (t, *J* = 5.3 Hz, 1H, NH-Ar), 8.08 (s, 1H, C_1-H), 6.89 (s, 1H, ArH), 5.98 (s, 1H, C_{11}-H), 4.42 - 4.40 (m, 4H, 2 × CH_2O), 3.97 - 3.94 (m, 2H, NCH_2), 3.74 - 3.72 (m, 2H, NCH_2), 3.64 - 3.57 (m, 1H, COCH), 3.38 (s, 3H, NCH_3), 3.02 - 2.97 (m, 1H, C_{13}-H), 2.92 - 2.89 (m, 1H, C_{18}-H), 2.30 (br s, 2H, NH_2), 1.47 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.22 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ 198.6, 196.6, 177.9, 175.9, 168.5, 165.9, 155.4, 148.5, 127.9, 126.9, 126.3, 124.0, 114.5, 114.5, 98.2, 61.5, 61.2, 52.2, 50.0, 49.5, 47.6, 47.3, 45.6, 45.0, 42.5, 42.0, 40.4, 35.6, 34.3, 33.1, 32.9, 31.6, 31.5, 30.6, 29.3, 28.1, 26.9, 26.6, 24.4, 22.9, 22.6, 21.5, 21.4, 20.5, 18.1; ESI-MS: 905 $[M + H]^+$, 927 $[M + Na]^+$; HRMS: m/z: Calcd. for $C_{45}H_{60}N_8O_{12}$ $[M + H]^+$ 905.4331, found 905.4392, ppm error 5.0.

*4.1.3.2.2 Compound **13***

O^2-(2,4-Dinitro-5-{2-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-oxo-ethyl-amino}phenyl) 1-{*N*-methyl-2-[2-(S)-(tert-butoxycarbonylamino)-3-phenyl propionyl-1-oxo]ethylamino) diazen-1-ium-1,2-diolate. The title compound was obtained in 85% yield as a yellowish solid: mp: 151 - 153 □; ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ 9.08 (s, 1H, ArH), 8.72 (t, *J* = 5.3 Hz, 1H, NH-Ar), 8.04 (s, 1H, C_1-H), 7.27 - 7.17 (m, 5H, 5 × ArH), 6.86 (s, 1H, ArH), 5.96 (s, 1H, C_{11}-H), 4.45 - 4.32 (m, 4H, 2 × CH_2O), 3.92 - 3.85 (m, 2H, NCH_2), 3.78 - 3.65 (m, 3H, NCH_2,

COCH), 3.29 (s, 3H, NCH_3), 3.10 - 3.03 (m, 3H, CH_2Ar, C_{13}-H), 2.90 - 2.89 (m, 1H, C_{18}-H), 1.45 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.20 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 0.90 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ 198.6, 196.7, 177.9, 174.6, 168.5, 165.7, 158.8, 148.5, 129.9, 129.2, 128.7, 127.9, 127.0, 126.4, 124.9, 124.0, 123.6, 116.1, 113.6, 110.8, 98.2, 61.5, 61.2, 55.9, 52.2, 49.5, 47.7, 47.3, 45.6, 45.0, 42.0, 41.1, 40.5, 35.6, 34.3, 33.1, 32.9, 31.5, 30.6, 29.7, 29.3, 28.0, 27.2, 27.0, 26.6, 24.4, 22.9, 22.7, 21.5, 21.4, 18.1; ESI-MS: 981 $[M + H]^+$, 1003 $[M + Na]^+$; HRMS: m/z: Calcd. for $C_{51}H_{64}N_8O_{12}$ $[M + Na]^+$ 1003.4644, found 1003.4526, ppm error 5.0.

*4.1.3.2.3 Compound **14***

O^2-(2,4-Dinitro-5-{4-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin-1-yl}phenyl) 1-{*N*-methyl-2-[2-(S)-(tert-butoxycarbonylamino)proionyl-1-oxo]ethylamino} diazen-1-ium-1,2-diolate. The title compound was obtained in 80% yield as a yellowish solid: mp: 144 - 146 □; ^{1}H NMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.72 (s, 1H, ArH), 8.09 (s, 1H, C_1-H), 6.94 (s, 1H, ArH), 5.97 (s, 1H, C_{11}-H), 4.44 - 4.41 (m, 2H, CH_2O), 3.98 - 3.85 (m, 5H, 2 × $CONCH_2$, $CHNH_2$), 3.41 - 3.25 (m, 9H, NCH_3, 3 × NCH_2), 3.16 - 3.10 (m, 2H, C_{13}-H, C_{18}-H), 1.49 (s, 3H, CH_3), 1.32 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.17 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ 199.2, 196.7, 175.9, 165.9, 153.8, 149.8, 132.8, 132.3, 130.9, 128.9, 128.3, 127.4, 124.0, 114.5, 105.1, 71.8, 61.3, 52.3, 52.2, 50.4, 49.9, 49.5, 47.7, 45.7, 45.0, 42.5, 42.1, 40.7, 40.5, 36.1, 34.2, 33.1, 31.6, 30.4, 29.7, 28.4, 27.7, 26.9, 24.5, 24.4, 23.9, 22.7, 21.8, 21.6, 20.3, 19.2, 18.2; ESI-MS: 930 $[M + H]^+$; HRMS: m/z: Calcd. for $C_{47}H_{63}N_9O_{11}$ $[M + H]^+$ 930.4647, found 930.4735, ppm error 5.0.

*4.1.3.2.4 Compound **15***

O^2-(2,4-Dinitro-5-{4-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]piperazin-1-yl}phenyl) 1-{*N*-methyl-2-[2-(S)-(tert-butoxycarbonylamino)-3-phenyl propionyl-1-oxo]ethylamino) diazen-1-ium-1,2-diolate. The title compound was obtained in 82%

yield as a yellowish solid: mp: 120 - 122 □; ^{1}H NMR (300 M Hz, $CDCl_3$, 25 °C, TMS): δ 8.70 (s, 1H, ArH), 8.08 (s, 1H, C_1-H), 7.23 - 7.12 (m, 5H, 5 × ArH), 6.91 (s, 1H, ArH), 5.96 (s, 1H, C_{11}-H), 4.45 - 4.37 (m, 2H, CH_2O), 4.00 - 3.74 (m, 7H, $CHNH_2$, 2 × $CONCH_2$, $PhCH_2$), 3.38 - 3.16 (m, 10H, NCH_3, 3 × NCH_2, C_{13}-H), 3.05 - 3.02 (m, 1H, C_{18}-H), 2.25 (br s, 2H, NH_2), 1.47 (s, 3H, CH_3), 1.31 (s, 3H, CH_3), 1.25 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 0.92 (s, 3H, CH_3) ppm; ^{13}C NMR (75 M Hz, $CDCl_3$, 25 °C, TMS): δ 199.2, 196.6, 175.9, 166.0, 153.7, 149.8, 136.9, 132.9, 130.9, 129.6, 129.2, 128.8, 128.7, 127.4, 127.0, 126.8, 125.7, 124.0, 114.5, 114.4, 105.0, 71.8, 61.2, 55.9, 52.2, 50.4, 49.5, 47.7, 47.6, 45.8, 45.0, 42.5, 42.1, 41.1, 40.6, 36.1, 34.2, 33.1, 33.0, 31.6, 30.4, 28.4, 27.7, 26.9, 26.5, 24.4, 24.0, 22.8, 21.8, 21.6, 19.2, 18.2; ESI-MS: 1028 $[M + Na]^+$; HRMS: m/z: Calcd. for $C_{53}H_{67}N_9O_{11}$ $[M + Na]^+$ 1028.4960, found 1028.4846, ppm error 5.0.

4.2. Biological assays

4.2.1. Cell lines and reagents

A549/Taxol, A549 and MRC-5 cells were purchased from American Tissue Culture Collection. A549/Taxol, A549 cell lines were maintained in MEN medium, MRC-5 cells were maintained in RPMI1640 medium. Both media were supplemented with 10% fetal bovine serum, and antibiotics [100 IU/mL penicillin and 100 IU/mL streptomycin (Amresco)]. All of the cell lines were grown at 37 °C in a 5% CO_2 atmosphere.

4.2.2. MTT assay

The inhibitory effects on cell proliferation of test compounds were investigated by the MTT method. A549/Taxol, A549 and MRC-5 Cells at a final density (3000 cellls/well) were placed in 96-well cell plates and treated with or without different concentrations of the test compounds for 72 h. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, 20 μL, 5 mg/mL) was added into each well, the cells were incubated for additional 4 h, and the

resulting formazan crystals were dissolved in 150 μL of DMSO and the absorbance was read spectrophotometrically at 570 nm using an enzyme-linked immunosorbent assay plate reader. Experiments were conducted in triplicate. Inhibition rate (%) = [(Acontrol − Atreated)/Acontrol] × 100%.

4.2.3. Nitrite measurement in vitro

The levels of intracellular NO generated by individual compounds were determined by the colorimetric assay using the nitrite colorimetric assay kit (Beyotime, China), according to the manufacturer's instructions. A549/Taxol or MRC-5 cells (1×10^6/well) were treated with 50 μM of each compound for 6 h. Subsequently, the cells were harvested and their cell lysates were prepared and then mixed with Griess reagent for 10 min at 37 °C, followed by measurement at 540 nm by a microplate reader. The cells treated with 0.4% DMSO in medium were used as negative controls for the background levels of nitrite production, while sodium nitrite at different concentrations was prepared as the positive control for the establishment of a standard curve.

4.2.4. Determination of ROS generation in vitro

A549/Taxol cellls treated with different concentrations of each compound (0, 2.4, 4.8, 9.6 μM) for 24 h. After being loaded with 10 μM DCFH-DA at 37 °C for 20 min and washed three times with PBS buffer to remove excess dye. The fluorescence intensity, used as a fluorescent indicator of intracellular ROS, was recorded in a plate reader and measured with the flow cytometer with an excitation/emission (Ex/Em) frequency of 488/540 nm.

4.2.5. Western blotting analysis

Cells were incubated in six-well plates (1×10^6/well) overnight and treated with vehicle DMSO (0.1%, v/v) for 24 h. The cells were harvested and lysed at 4 °C for 30 min in a lysis buffer. The cell lysates were centrifuged at 12000 -16000 g for 5 min at 4 °C, and the supernatants were collected. The protein concentration in the cell lysates

was determined by bicinchonininc acid assay. Protein samples were separated by SDS-polyacrylamide gel electrophoresis (7.5% gel, 20 μg per lane) and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking, the PVDF membranes were washed three times with TBST at room temperature and incubated with primary antibodies for GSTπ (1:1500), MRP1 (1:1000) and LRP (1:1000) at 4°C overnight. After extensive washing, membranes were incubated with secondary peroxidase-labelled goat anti-rabbit IgG (Santa Cruz, USA) for 1 h. After washing four times for 15 min with TBST at room temperature once more, the bands were detected by a Tanon 6000. The flms were scanned and quantitation was carried out with Image pro plus 6.0.

Cells (1×10^6/well) were cultured in six-well plates overnight and treated in triplicate with vehicle DMSO (0.1%, v/v) alone, **7**, CDDO-Me or JS-K at the indicated concentrations for 24 h. The cells were harvested and lysed in a lysis buffer. After being centrifuged, the concentrations of total proteins in the cell lysates were determined by bicinchonininc acid assay. The cell lysates (20 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis (7.5% gel) and transferred onto polyvinylidene difluoride (PVDF) membranes. After the membranes were blocked in 5% fatfree milk for 1 h, the target proteins were probed with anti-LonP1 and anti-GAPDH. The relative levels of target protein to the control or phosphorylated to expressed protein were determined by densitometric analysis using the ImageJ software.

4.2.6. Cell cycle analysis

A549/Taxol cells were treated with **7**, CDDO-Me and JS-K at concentrations of 0, 1.2, 2.4 and 4.8 μM for 24 h, respectively. The cells were harvested, fixed with 70% ethanol for 2 h, and incubated with PI/RNase staining buffer (BD Pharmingen) for 15 min at room temperature. The DNA content in the different groups of cells was assessed by flow cytometry and analyzed by the software MODFIT.

4.2.7. Apoptosis analysis

Cells were incubated in six-well plates (1×10^5/well) and treated with **7**, CDDO-Me and JS-K at concentrations of 0, 1.2, 2.4 and 4.8 μM for 24 h, respectively. The cells were collected, washed with PBS, and stained with FITC-Annexin-V and PI. Apoptosis was determined by flow cytometry.

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Highlights

- **7** significantly inhibited the proliferation of A549/Taxol cells but sparing non-tumor lung cells.
- **7** preferably promoted ROS accumulation in A549/Taxol cells.
- **7** selectively produced higher levels of NO in A549/Taxol cells.
- **7** strongly suppressed the Lon protease expression as well as induced apoptosis and cycle arrest of A549/Taxol cells.