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Design, synthesis, and biological evaluation of novel nitric oxide releasing dehydroandrographolide derivatives

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[ABSTRACT] A series of new hybrids of dehydroandrographolide (TAD), a biologically active natural product, bearing nitric oxide (NO)-releasing moieties were synthesized and designated as NO-donor dehydroandrographolide. The biological activities of target compounds were studied in human erythroleukemia K562 cells and breast cancer MCF-7 cells. Biological evaluation indicated that the most active compound I-5 produced high levels of NO and inhibited the proliferation of K562 (IC₅₀ 1.55 μ mol·L⁻¹) and MCF-7 (IC₅₀ 2.91 μ mol·L⁻¹) cells, which were more potent than the lead compound TAD and attenuated by an NO scavenger. In conclusion, **I-5** is a novel hybrid with potent antitumor activity and may become a promising candidate for future intensive study.

[KEY WORDS] Tehydroandrographolide; Nitric oxide; Anticancer

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Introduction

Andrographolide (andro) and dehydroandrographolide (TAD) are lactone diterpenoid compounds present in the medicinal plant *Andrographis paniculate*, which is clinically applied for multiple human diseases in Asia and Europe. The pharmacological activities of andro and TAD have been widely demonstrated, including anti-bacterial, anti-inflammatory, anti-tumor, anti-HIV, anti-hyperglycemia, anti-thrombotic, and anti-malarial properties ^[1-3]. In recent years, andro and TAD have been successfully used as an antineoplastic drug in cancer chemotherapy by suppressing growth of cancer cells, inhibiting NF-kappa B, PI3K/AKT and other kinase pathways, and inducing apoptosis^[4-6].

In addition, developing novel agents with multiple effects is one of common strategies in search for new treatments of cancer. The activity of many of these compounds has been improved by the introduction of the second pharmacophores ^[7]. As a result, in the last few decades, investigation of the pathophysiological and pharmacological roles of "gasotransmitters" has represented a challenging research field, which is still widely unexplored [8-9]. All three gases (nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H₂S)) at appropriate concentrations exert variety of physiological effects in different systems. Especially NO, as a signaling molecule, plays a pivotal role in diverse physiological and pathophysiological processes ^[10]. It can relax vascular smooth muscle, inhibit platelet aggregation, reduce neutrophil adherence and activation, mitigate cell death, and promote cell proliferation, etc ^[11-14]. More importantly, it is well-known that high levels of NO generated from NO donors can not only induce apoptosis and inhibit metastasis of tumor cells, but also sensitize tumor cells to chemotherapy, radiation, and immunotherapy in



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vitro and *in vivo*^[15-17]. Several NO-releasing compounds, e.g., oleanolic acid/furoxan ^[18], anilinopyrimidine/furoxan ^[19], and bifendate/furoxan hybrids ^[20], have been studied, exhibiting greater antitumor activity than their respective parent compounds.

On the basis of these investigations, we hypothesized that hybridization of various NO-donor moieties with TAD and its derivatives may release NO to exert synergistic antitumor effects with TAD. To test this hypothesis, a series of novel hybrids of TAD and its derivatives (Fig. 1), bearing NO-releasing moieties connected via ester-type spacer, were designed and synthesized ^[21-22]. These compounds were further evaluated *in vitro* for their NO releasing ability, and their effects on anti-cancer activity.



Fig. 1 Design of NO-dehydroandrographolides

Materials and Methods

Materials

Doxorubicin (DOXO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hemoglobin was purchased from Beyotime (Shanghai, China). All other chemicals were molecular biology grade and obtained from Sigma-Aldrich or Thermo Fisher Scientific (Waltham, MA, USA).

Cell lines and cell culture

Human leukemia cell line K562 and human breast cancer cell line MCF-7 were kindly provided by Dr LI Yun-Man (Department of Physiology, China Pharmaceutical University, Nanjing, China). The cell lines were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified incubator with 5% CO₂. All experiments were performed with cells in exponential growth phase.

MTT assay

K562 cells or MCF-7 cells were incubated in RPMI 1640 medium supplemented with 10 % fetal bovine serum at 37 °C in a 5% CO₂ humidified atmosphere. K562 cells or MCF-7 cells (1×10^4 – 2×10^4 cells per well) were seeded in 96-well plates. After 24 h incubation, the cells were treated with various concentrations of target compounds and DOXO 48 h in an atmosphere of 95% air with 5% CO₂ at 37 °C. Then, MTT was added directly to the cells. After additional incubation for 4 h at 37 °C, the absorbance at 490 nm was read on a microplate reader (Thermo Fisher Scientific). The IC₅₀ values of the compounds for cytotoxicity were calculated by GraphPad Prism 5.0 software from the dose-response curves. Experiments were conducted in triplicate and repeated thrice.

Nitrite measurement in vitro

The levels of NO generated by individual compounds were determined and presented as that of nitrite. Briefly, stock solutions of test compounds (10 mmol \cdot L⁻¹ in DMSO) were prepared, and this solution was diluted with 0.2 mol·L⁻¹ phosphate buffer (pH 7.4) [29 g Na₂HPO₄, 3.0 g NaH₂PO₄ and 394 mg L-cysteine in H₂O (500 mL)] to 100 μ mol·L⁻¹ concentration. The reaction samples were incubated at 37 °C for 150 min; then 2 mL aliquots were taken and mixed with 500 µL of Greiss reagent (5 mL of 85% phosphoric acid, 2.0 g sulfanilamide and 0.1 g N-(1-Naphthyl) ethylenediamine dihydrochloride, diluted to 50 mL with purified water). The mixture was allowed to stand at 37 °C for 10 min, and the absorbance of each sample was determined at 540 nm. Solutions of $0-100 \text{ }\mu\text{mol}\cdot\text{L}^{-1}$ sodium nitrite were used to prepare a nitrite absorbance versus concentration curve under the same experimental conditions. The nitric oxide released (quantitated as nitrite ion) was calculated from the standard nitrite concentration curve.

General chemistry

All starting materials, reagents and solvents were obtained from commercial sources and used without further purification unless otherwise indicated. Column chromatography was carried out on silica gel (200-300 mesh) and monitored by thin layer chromatography performed on GF/UV 254 plates and were visualized by using UV light at 365 and 254 nm. Melting points were measured using a RY-1 melting-point apparatus, which was uncorrected. All of the NMR spectra were recorded on a Bruker ACF-300Q instrument (300 MHz for ¹H NMR spectra), chemical shifts are expressed as values relative to tetramethylsilane as internal standard, and coupling constants (J values) were given in hertz (Hz). HRMS (high-resolution mass spectra) were taken with a O-TofMicromass spectrometer. The TAD derivatives 1-4 were synthesized as described in literature ^[23-25]. The NO donor derivatives were synthesized using literature procedures ^[26]. The characterization data were in agreement with those reported in the literature.

General procedure for the preparation of I-1-9, 13

To a solution of **4** (1.3 g, 2.0 mmol), EDCI (490 mg, 2.4 mmol) and DMAP (catalytic amount) in anhydrous dichloromethane (20 mL) at room temperature were added different NO-donors (1.0 mmol, dissolved in 5 mL anhydrous dichloromethane) dropwise and the reaction mixture was stirred until the end of the reaction. Then, the whole solution was diluted with dichloromethane (20 mL), washed with water (20 mL \times 3), saturated NaHCO₃ aqueous solution (20 ml \times 3), and saturated brine (20 mL \times 3), dried over Na₂SO₄, and evaporated to obtain the crude product. To the solution of crude product (1.0 mmol) in dichloromethane (20 mL) at room temperature was added formic acid (5 mL) dropwise and the reaction mixture was stirred until the end of the reac-



tion. Then, the solution was diluted with dichloromethane (20 mL), washed with saturated NaHCO₃ aqueous solution (20 mL \times 3), and saturated brine (20 mL \times 3), dried over Na₂SO₄, and evaporated to obtain a residue purified by column chromatography.

3-((1-(2-oxido-4-phenyl-1, 2, 5-oxadiazol-3-yl)methoxyl-4- oxobutanoyl)oxy)-dehydroandrographolide (**I-1**)

White solid, Yield (72%), mp 70–71 °C, ¹H NMR (CDCl₃, 300 MHz) δ : 7.72–7.54 (m, 5H, Ar-H), 7.17 (s, 1H, 14-H), 6.89 (dd, *J* = 15.8, 10.0 Hz, 1H, 11-H), 6.12 (d, *J* = 15.8 Hz, 1H, 12-H), 5.17 (s, 2H, COOCH₂), 4.81 (s, 2H, 15-CH₂), 4.80 (s, 1H, 17a-H), 4.68 (t, *J* = 8.6Hz, 1H, 3-H), 4.54 (s, 1H, 17b-H), 4.42 (d, *J* = 11.6 Hz, 1H, 19a-H), 3.43 (d, *J* = 11.6 Hz, 1H, 19b-H), 2.65 (m, 4H, CH₂CH₂), 2.33 (d, *J* = 10.0 Hz, 1H, 9-H), 2.46–1.21 (m, 9H, 1, 2, 5, 6, 7-H), 1.06 (s, 3H, 18-CH₃), 0.83 (s, 3H, 20-CH₃); HRMS (ESI) for [C₃₃H₃₈N₂O₉ + Na]⁺, Calcd. 629.2470, found 629.2474 [M + Na]⁺.

3-((1-(ethoxy-(3-(2-oxido-3-(phenylsulfonyl)-1, 2, 5-oxadiazol-4-yl) oxy) ethoxy)-4-oxobutanoyl)oxy)-dehydroandrographolide (**I**-2)

White solid, Yield (25%), mp 60–62 °C, ¹H NMR (CDCl₃, 300 MHz) 5: 8.09-7.62 (m, 5H, Ar-H), 7.19 (s, 1H, 14-H), 6.91 (dd, J = 15.8, 10.2 Hz, 1H, 11-H), 6.13 (d, J = 15.8 Hz, 1H, 12-H), 4.83 (s, 2H, 15-CH₂), 4.81 (s, 1H, 17a-H), 4.69 (t, J = 8.4 Hz, 1H, 3-H), 4.56 (s, 1H, 17b-H), 4.56 (t, J = 6.0 Hz, 2H, $COOCH_2CH_2OCH_2CH_2O$), 4.45 (t, J = 6.0 Hz, 2H, COOCH₂CH₂OCH₂CH₂O), 4.40 (d, *J* = 11.9Hz, 1H, 19a-H), 4.13 (d, J = 11.9 Hz, 1H, 19b-H), 3.82 (m, 4H, COOCH₂CH₂OCH₂CH₂O), 2.69-2.61 4H, (m, OOCCH₂CH₂COO), 2.34 (d, J = 10.0 Hz, 1H, 9-H), 2.48-1.17 (m, 9H, 1, 2, 5, 6, 7-H), 1.17 (s, 3H, 18-CH₃), 0.84 (s, 3H, 20-CH₃); HRMS (ESI) for $[C_{36}H_{44}N_2O_{13}S + Na]^+$, Calcd. 767.2462, found 767.2458 [M + Na]⁺.

3-((1- (-3-((2-oxido-3-(phenylsulfonyl)-1, 2, 5-oxadiazol-4-yl) oxy) propyl)-4-oxobutanoyl)oxy)-dehydroandrographolide (**I-3**)

White solid, Yield (65%), mp 70–72 °C, ¹H NMR (CDCl₃, 300 MHz) δ : 8.09–7.62 (m, 5H, Ar-H), 7.19 (s, 1H, 14-H), 6.91 (dd, J = 15.8, 10.2 Hz, 1H, 11-H), 6.13 (d, J = 15.8 Hz, 1H, 12-H), 4.83 (s, 2H, 15-CH₂), 4.81 (s, 1H, 17a-H), 4.69 (t, J = 8.4 Hz, 1H, 3-H), 4.56 (s, 1H, 17b-H), 4.53 (t, J = 6.0 Hz, 2H, COO<u>CH₂</u>), 4.40 (d, J = 11.9 Hz, 1H, 19a-H), 4.32 (t, J =6.0 Hz, 2H, CH₂O), 4.13 (d, J = 11.9 Hz, 1H, 19b-H), 2.69– 2.61 (m, 4H, OOCCH₂CH₂COO), 2.34 (d, J = 10.0 Hz, 1H, 9-H), 2.24 (q, 2H, CH₂<u>CH₂</u>CH₂), 2.48–1.17 (m, 9H, 1, 2, 5, 6, 7-H), 1.17 (s, 3H, 18-CH₃), 0.84 (s, 3H, 20-CH₃); HRMS (ESI) for [C₃₅H₄₂N₂O₁₂S + Na]⁺, Calcd. 737.2356, found 737.2363 [M + Na]⁺.

3-((1- (-3-((2-oxido-3-(phenylsulfonyl)-1, 2, 5-oxadiazol-4-yl) oxy)butyl)-4-oxobutanoyl)oxy) -dehydroandrographolide (**I**-4)

White solid, Yield (56%), mp 64–66°C, ¹H NMR (CDCl₃, 300 MHz) δ : 8.09–7.62 (m, 5H, Ar-H), 7.17 (s, 1H, 14-H), 6.92 (dd, J = 15.8, 10.0 Hz, 1H, 11-H), 6.13 (d, J = 15.8 Hz, 1H, 12-H), 4.82 (s, 2H, 15-H), 4.81 (s, 1H, 17a-H), 4.72 (t, J = 8.4 Hz, 1H, 3-H), 4.56 (s, 1H, 17b-H), 4.47 (t, J = 6.0Hz, 2H, COOCH₂), 4.21 (t, J = 6.0 Hz, 2H, CH₂O), 4.15 (d, J = 11.9 Hz, 1H, 19a-H), 3.43 (d, J = 11.9 Hz, 1H, 19b-H), 2.69–2.61 (m, 4H, OOCCH₂CH₂COO), 2.34 (d, J = 10.0 Hz, 1H, 9-H), 2.48–1.17 (m, 13H, 1, 2, 5, 6, 7-H, CH₂(CH₂)₂CH₂), 1.10 (s, 3H, 18-CH₃), 0.84 (s, 3H, 20-CH₃); HRMS (ESI) for [C₃₆H₄₄N₂O₁₂S + Na]⁺, Calcd. 751.2513, found 751.2518 [M + Na]⁺.

3-((3-(1-methyl-3-((2-oxido-3-(phenylsulfonyl)-1, 2, 5-oxadiazol-4-yl) oxy) propoxy)-4-oxobutanoyl)oxy)-dehydroandrographolide (**I-5**)

White solid, Yield (33%), mp 69–70 °C, ¹H NMR (CDCl₃, 300 MHz) δ : 8.09–7.61 (m, 5H, Ar-H), 7.19 (s, 1H, 14H), 6.91 (dd, J = 15.8, 10.0 Hz, 1H, 11-H), 6.15 (d, J = 15.8 Hz, 1H, 12-H), 5.23–5.19 (m, 1H, COOCH), 4.83 (s, 2H, 15-CH₂), 4.80 (s, 1H, 17a-H), 4.62 (t, J = 6.0 Hz, 1H, 3-H), 4.56 (s, 1H, 17b-H), 4.48 (t, J = 6.0 Hz, 2H, OCH₂), 4.12 (d, J = 11.7 Hz, 1H, 19a-H), 3.40 (d, J = 11.7 Hz, 1H, 19b-H), 2.69–2.61 (s, 4H, OOCCH₂CH₂COO), 2.36 (d, J = 10.0 Hz, 1H, 9-H), 2.16 (q, 2H, OCH₂<u>CH</u>₂CH), 2.05–1.09 (m, 9H, 1, 2, 5, 6, 7-H), 1.35 (d, J = 6.3 Hz, 3H, CH<u>CH</u>₃), 1.07 (s, 3H, 18-CH₃), 0.81 (s, 3H, 20-CH₃); HRMS (ESI) for [C₃₆H₄₄N₂O₁₂S + Na]⁺, Calcd. 751.2513, found 751.2520 [M + Na]⁺.

3-((1-(-3-((2-oxido-3-(phenylsulfonyl)-1, 2, 5-oxadiazol-4-yl) oxy) pentyl) -4-oxobutanoyl)oxy)-dehydroandrographolide (**I-6**)

White solid, Yield (51%), mp 62–64 °C, ¹H NMR (CDCl₃, 300 MHz) δ : 8.08–7.62 (m, 5H, Ar-H), 7.18 (s, 1H, 14-H), 6.92 (dd, J = 15.8, 10.0 Hz, 1H, 11-H), 6.14 (d, J = 15.8 Hz, 1H, 12-H), 4.83 (s, 2H, 15-CH₂), 4.81 (s, 1H, 17a-H), 4.71 (t, J = 8.4 Hz, 1H, 3-H), 4.56 (s, 1H, 17b-H), 4.47 (t, J = 6.0Hz, 2H, COOCH₂), 4.31 (t, J = 6.0 Hz, 2H, CH₂O), 4.14 (d, J =11.9 Hz, 1H, 19a-H), 3.42 (d, J = 11.9 Hz, 1H, 19b-H), 2.69–2.61 (m, 4H, OOCCH₂CH₂COO), 2.35 (d, J = 10.0 Hz, 1H, 9-H), 2.42–1.13 (m, 15H, 1, 2, 5, 6, 7-H, CH₂(<u>CH₂)</u>₃CH₂), 1.08 (s, 3H, 18-CH₃), 0.84 (s, 3H, 20-CH₃); HRMS (ESI) for [C₃₇H₄₆N₂O₁₂S + Na]⁺, Calcd. 765.2669, found 765.2674 [M + Na]⁺.

3-((1-(-3-((2-oxido-3-(phenylsulfonyl)-1, 2, 5-oxadiazol-4-yl) oxy) hexyl propoxy)-4-oxobutanoyl)oxy)-dehydroandrographolide (**I**-7)

White solid, Yield (65%), mp 60–62 °C, ¹H NMR (CDCl₃, 300 MHz) δ : 8.09–7.64 (m, 5H, Ar-H), 7.16 (s, 1H, 14-H), 6.91 (dd, J = 15.8, 10.0 Hz, 1H, 11-H), 6.12 (d, J = 15.8 Hz, 1H, 12-H), 4.81 (s, 2H, 15-H), 4.80 (s, 1H, 17a-H), 4.71 (t, J =8.4 Hz, 1H, 3-H), 4.55 (s, 1H, 17b-H), 4.46 (t, J = 6.0Hz, 2H, COOCH₂), 4.20 (t, J = 6.0 Hz, 2H, CH₂O), 4.14 (d, J =11.9 Hz, 1H, 19a-H), 3.43 (d, J = 11.9 Hz, 1H, 19b-H), 2.69–2.61 (m, 4H, OOCCH₂CH₂COO), 2.33 (d, J = 10.0 Hz, 1H, 9-H), 2.48~1.17 (m, 17H, 1, 2, 5, 6, 7-H, CH₂(<u>CH₂)</u>₄CH₂), 1.09 (s, 3H, 18-CH₃), 0.84 (s, 3H, 20-CH₃); HRMS (ESI) for [C₃₈H₄₈N₂O₁₂S + Na]⁺, Calcd. 779.2826, found 779.2833 [M + Na]⁺.

3-((2-(nitrooxy)-ethylamine-4-oxobutanoyl)oxy)-dehydroandr ographolide (**I-8**)

White solid, Yield (45%), mp 60-62 °C, ¹H NMR

(DMSO- d_6 , 300 MHz) δ : 8.15 (s, 1H, CONH), 7.65 (s, 1H, 14-H), 6.76 (dd, J = 15.8, 10.2 Hz, 1H, 11-H), 6.15 (d, J = 15.8 Hz, 1H, 12-H), 4.88 (s, 2H, 15-H), 4.73 (s, 1H, 17a-H), 4.71 (m, 1H, 3-H), 4.50 (s, 2H, O₂NOCH₂), 4.42 (s, 1H, 17b-H), 3.65 (d, J = 11.2 Hz, 1H, 19a-H), 3.54 (d, J = 11.2 Hz, 1H, 19b-H), 3.38 (s, 2H, CH₂NH), 2.49–2.34 (m, 4H, OOCCH₂CH₂COO), 1.98–1.02 (m, 10H, 1, 2, 5, 6, 7, 9-H), 0.90 (s, 3H, 18-CH₃), 0.85 (s, 3H, 20-CH₃); HRMS (ESI) for [C₂₆H₃₆N₂O₉ + Na]⁺, Calcd. 543.2313, found 543.2319 [M + Na]⁺.

3-((isosorbide mononitrate-4-oxobutanoyl)oxy)-dehydroandrographolide (1-9)

White solid, Yield (72%), mp 68–70 °C, ¹H NMR (CDCl₃, 300MHz) & 7.16 (s, 1H, 14H), 6.89 (dd, J = 15.8, 10.0 Hz, 1H, 11-H), 6.12 (d, J = 15.8 Hz, 1H, 12-H), 5.34 (m, 1H, 5'-H), 5.24 (s, 1H, 2'-H), 4.97 (t, J = 5.2 Hz, 1H, 4'-H), 4.81 (s, 2H, 15-CH₂), 4.79 (s, 1H, 17a-H), 4.69 (t, J = 6.0 Hz, 1H, 3-H), 4.54 (s, 1H, 17b-H), 4.49 (d, J = 4.8Hz, 1H, 3'-H), 4.14 (d, J = 11.7 Hz, 1H, 19a-H), 4.04–3.87 (m, 4H, 1'-CH₂, 6'-CH₂), 3.42 (d, J = 11.7 Hz, 1H, 19b-H), 2.63–2.58 (m, 4H, OOCCH₂CH₂COO), 2.53 (d, J = 10.0 Hz, 1H, 9-H), 2.48~ 1.21 (m, 9H, 1, 2, 5, 6, 7-H), 1.12 (s, 3H, 18-CH₃), 0.81 (s, 3H, 20-CH₃); HRMS (ESI) for [C₃₀H₃₉NO₁₂ + Na]⁺, Calcd. 628.2364, found 628.2374 [M + Na]⁺.

General procedure for the preparation of I-10-12

To a solution of 4 (3.0 g, 4.5 mmol), triethylamine (9 mmol) in anhydrous acetone (20 mL) at room temperature was added dibromoalkane (18.0 mmol) dropwise. The reaction mixture was heated to reflux and stirred until the end of the reaction. Then the mixture was evaporated and washed with petroleum ether to remove the excess dibromoalkane. The crude product thus obtained was purified by recrystallization to yield the compound 5 as a white or pale yellow solid. A mixture of the compound 5 (0.5 mmol) and AgNO₃ (0.75 mmol) in CH₃CN (15 mL) was stirred at reflux under the dark condition for about 8 h. Then 20 mL of saturated brine was added and stirred about 10 min to remove the excess AgNO₃. Themixture was concentrated in vacuototoobtain the crude product. To the solution of crude product (1.0 mmol) in dichloromethane (20 mL) at room temperature was added formic acid (5 mL) dropwise and the reaction mixture was stirred until the end of the reaction. Then, the solution was diluted with dichloromethane (20 mL), washed with saturated NaHCO₃ aqueous solution (20 mL \times 3), and saturated brine $(20 \text{ mL} \times 3)$, dried over Na₂SO₄ and evaporated to obtain a residue purified by column chromatography.

3-((2-(nitrooxy)-ethyl-4-oxobutanoyl)oxy)-dehydroandrograp holide (**I-10**)

White solid, Yield (39%), mp 57–59 °C, ¹H NMR (DMSOd₆, 300 MHz) δ : 7.63 (s, 1H, 14-H), 6.65 (dd, J = 15.8, 10.05 Hz, 1H, 11-H), 6.12 (d, J = 15.8 Hz, 1H, 12-H), 4.86 (s, 2H, 15-H), 4.68 (s, 1H, 17a-H), 4.57 (t, J = 6.3 Hz, 2H, O₂NOCH₂), 4.51 (m, 1H, 3-H), 4.35 (s, 1H, 17b-H), 4.11 (t, J = 6.3 Hz, 2H, CH₂OOC), 3.32 (d, J = 9.4 Hz, 1H, 19a-H), 3.02 (d, J = 9.4 Hz, 1H, 19b-H), 2.53–2.50 (m, 4H, OOCCH₂CH₂COO), 2.35–1.18 (m, 10H, 1, 2, 5, 6, 7, 9-H), 1.14 (s, 3H, 18-CH₃), 0.25 (s, 3H, 20-CH₃); HRMS (ESI) for $[C_{23}H_{31}NO_9 + Na]^+$, Calcd. 488.1891, found 488.1898 [M + Na]⁺.

3-((3-(nitrooxy)-propyl-4-oxobutanoyl)oxy)-dehydroandrogra pholide (**I-11**)

White solid, Yield (46%), mp 61–63 °C, ¹H NMR (DMSOd₆, 300 MHz) δ : 7.62 (s, 1H, 14-H), 6.63 (dd, J = 15.8, 10.1 Hz, 1H, 11-H), 6.10 (d, J = 15.8 Hz, 1H, 12-H), 4.86 (s, 2H, 15-H), 4.68 (s, 1H, 17a-H), 4.57 (t, J = 6.3 Hz, 2H, O₂NOCH₂), 4.51–4.47 (m, 1H, 3-H), 4.35 (s, 1H, 17b-H), 4.11 (t, J = 6.3 Hz, 2H, CH₂OOC), 3.32 (d, J = 9.4 Hz, 1H, 19a-H), 3.02 (d, J = 9.4 Hz, 1H, 19b-H), 2.53–2.50 (m, 4H, OOCCH₂CH₂COO), 2.35–1.18 (m, 10H, 1, 2, 5, 6, 7, 9-H), 2.01 (m, 2H, CH₂<u>CH₂CH₂</u>), 1.14 (s, 3H, 18-CH₃), 0.25 (s, 3H, 20-CH₃); HRMS (ESI) for [C₂₄H₃₃NO₉ + Na]⁺, Calcd. 502.2048, found 502.2054 [M + Na]⁺.

3-((4-(nitrooxy)-butyl-4-oxobutanoyl)oxy)-dehydroandrograp holide (**I-12**)

White solid, Yield (38%), mp 58–60 °C, ¹H NMR (DMSOd₆, 300 MHz) δ : 7.62 (s, 1H, 14-H), 6.62 (dd, J = 15.8, 10.2 Hz, 1H, 11-H), 6.10 (d, J = 15.8 Hz, 1H, 12-H), 4.86 (s, 2H, 15-H), 4.68 (s, 1H, 17a-H), 4.57 (t, J = 6.3 Hz, 2H, O₂NOCH₂), 4.51–4.47 (m, 1H, 3-H), 4.35 (s, 1H, 17b-H), 4.05 (t, J = 6.0 Hz, 2H, CH₂OOC), 3.29 (d, J = 9.4 Hz, 1H, 19a-H), 3.02 (d, J = 9.4 Hz, 1H, 19b-H), 2.52 (m, 4H, OOCCH₂CH₂COO), 2.38–1.17 (m, 14H, 1, 2, 5, 6, 7, 9-10 H, CH₂(<u>CH₂)</u>₂CH₂), 1.14 (s, 3H, 18-CH₃), 0.24 (s, 3H, 20-CH₃); HRMS (ESI) for [C₂₅H₃₅NO₉ + Na]⁺, Calcd. 516.2204, found 516.2212 [M + Na]⁺.

3-((2-(nitrooxy)-ethylamine-4-oxobutanoyl)oxy)-19-O-tritylde hydroandrographolide (**I-13**)

To a solution of 4 (1.3 g, 2.0 mmol), EDCI (490 mg, 2.4 mmol), and DMAP (catalytic amount) in anhydrous dichloromethane (20 mL) at room temperature was added 2-aminoethyl nitrate (1.0 mmol, dissolved in 5 mL anhydrous dichloromethane) dropwise and the reaction mixture was stirred until the end of the reaction. Then, the whole solution was diluted with dichloromethane (20 mL), washed with water (20 mL \times 3), saturated NaHCO₃ aqueous solution (20 mL \times 3), and saturated brine (20 mL \times 3), dried over Na₂SO₄, filtered, and concentrated under reduced pressure to obtain a residue purified by column chromatography. White solid, Yield (60%), mp 84–86 °C, ¹H NMR (DMSO- d_6 , 300MHz) δ : 8.13 (s, 1H, CONH), 7.62 (s, 1H, 14-H), 7.40-7.24 (m, 15H, Ar-H), 6.66 (dd, J = 15.8, 10.2 Hz, 1H, 11-H), 6.10 (d, J = 15.8 Hz, 1H)12-H), 4.86 (s, 2H, 15-H), 4.68 (s, 1H, 17a-H), 4.51 (s, 1H, 17b-H), 4.49 (s, 2H, O₂NOCH₂), 4.21 (d, J = 11.0 Hz, 1H, 19a-H), 3.46 (q, 1H, 3-H), 3.38 (s, 2H, <u>CH</u>₂NH), 3.34 (d, J= 11.0 Hz, 1H, 19b-H), 2.51-2.45 (m, 4H, OOCCH₂CH₂COO), 2.31 (d, J = 10.0 Hz, 1H, 9-H), 2.42–1.28 (m, 9H, 1, 2, 5, 6, 7-H), 1.25 (s, 3H, 18-CH₃), 0.25 (s, 3H, 20-CH₃); HRMS (ESI) for $[C_{45}H_{50}N_2O_9 + Na]^+$, Calcd. 785.3409, found



785.3415 [M + Na]⁺.

General procedure for the preparation of I-14-16

A mixture of the compound 5 (0.5 mmol) and AgNO₃ (0.75 mmol) in CH₃CN (15 mL) was stirred at reflux under the dark condition for about 8 h. Then 20 mL of saturated brine was added and stirred about 10 min to remove the excess AgNO₃. The mixture was concentrated in vacuo, dissolved in 15 mL of CH₂Cl₂, filtered, and concentrated under reduced pressure to obtain a residue purified by column chromatography.

3-((2-(nitrooxy)-ethyl-4-oxobutanoyl)oxy)-19-O-trityldehydro andrographolide (**I-14**)

White solid, Yield (31%), mp 60–62 °C, ¹H NMR (DMSO- d_6 , 300 MHz, ppm) δ : 7.62 (s, 1H, 14-H), 7.43–7.22 (m, 15H, Ar-H), 6.63 (dd, J = 15.8, 10.0 Hz, 1H, 11-H), 6.10 (d, J = 15.8 Hz, 1H, 12-H), 4.86 (s, 2H, 15-H), 4.68 (s, 1H, 17a-H), 4.57 (t, J = 6.3 Hz, 2H, O₂NOCH₂), 4.51–4.47 (m, 1H, 3-H), 4.35 (s, 1H, 17b-H), 4.11 (t, J = 6.3 Hz, 2H, CH₂OOC), 3.32 (d, J = 9.4 Hz, 1H, 19a-H), 3.02 (d, J = 9.4 Hz, 1H, 19b-H), 2.53–2.50 (m, 4H, OOCCH₂CH₂COO), 2.35–1.18 (m, 10H, 1, 2, 5, 6, 7, 9-H), 1.14 (s, 3H, 18- CH₃), 0.25 (s, 3H, 20-CH₃); HRMS (ESI) for [C₄₅H₄₉NO₁₀ + Na]⁺, Calcd. 786.3249, found 786.3244 [M + Na]⁺.

3-((3-(nitrooxy)-propyl-4-oxobutanoyl)oxy)-19-O-trityldehydr oandrographolide (**I-15**)

White solid, Yield (76%), mp 60–62 °C, ¹H NMR (DMSOd₆, 300 MHz) δ : 7.61 (s, 1H, 14-H), 7.43–7.22 (m, 15H, Ar-H), 6.63 (dd, J = 15.8, 10.1 Hz, 1H, 11-H), 6.10 (d, J =15.8 Hz, 1H, 12-H), 4.86 (s, 2H, 15-H), 4.68 (s, 1H, 17a-H), 4.57 (t, J = 6.3 Hz, 2H, O₂NOCH₂), 4.51–4.47 (m, 1H, 3-H), 4.35 (s, 1H, 17b-H), 4.11 (t, J = 6.3 Hz, 2H, CH₂OOC), 3.32 (d, J = 9.4 Hz, 1H, 19a-H), 3.02 (d, J = 9.4 Hz, 1H, 19b-H), 2.53–2.50 (m, 4H, OOCCH₂CH₂COO), 2.35–1.18 (m, 10H, 1, 2, 5, 6, 7, 9-H), 2.01 (quint, J = 6.3 Hz, 2H, CH₂CH₂CH), 1.14 (s, 3H, 18-CH₃), 0.25 (s, 3H, 20-CH₃); HRMS (ESI) for [C₄₆H₅₁NO₁₀ + Na]⁺, Calcd. 800.3405, found 800.3412 [M + Na]⁺.

3-((4-(nitrooxy)-butyl-4-oxobutanoyl)oxy)-19-O-trityldehydro andrographolide (**I-16**)

White solid, Yield (78%), mp 59–61 °C, ¹H NMR (DMSOd₆, 300 MHz) δ : 7.62 (s, 1H, 14-H), 7.43–7.22 (m, 15H, Ar-H), 6.62 (dd, J = 15.8, 10.2 Hz, 1H, 11-H), 6.10 (d, J =15.8 Hz, 1H, 12-H), 4.85 (s, 2H, 15-H), 4.68 (s, 1H, 17a-H), 4.56 (t, J = 6.3 Hz, 2H, O₂NOCH₂), 4.51–4.47 (m, 1H, 3-H), 4.35 (s, 1H, 17b-H), 4.05 (t, J = 6.0 Hz, 2H, CH₂OOC), 3.29 (d, J = 9.4 Hz, 1H, 19a-H), 3.02 (d, J = 9.4 Hz, 1H, 19b-H), 2.52 (m, 4H, OOCCH₂CH₂COO), 2.38–1.17 (m, 14H, 1, 2, 5, 6, 7, 9-10 H, CH₂(<u>CH₂)</u>₂CH₂), 1.14 (s, 3H, 18-CH₃), 0.24 (s, 3H, 20-CH₃); HRMS (ESI) for [C₄₇H₅₃NO₁₀ + Na]⁺, Calcd. 814.3562, found 814.3567 [M + Na]⁺.

3-((1-(2-oxido-4-phenyl-1, 2, 5-oxadiazol-3-yl) methoxyl-4oxobutanoyl)oxy)-19-O-formacyldehydroandrographolide (**I-1**7)

To the solution of intermediate **I-1** (1.0 mmol), EDCI (1.2 mmol), and DMAP (catalytic amount) in anhydrous dichloromethane (10 mL) at room temperature was added 5 mL of HCOOH and the reaction mixture was stirred until the end of the reaction. Then, the whole solution was diluted with dichloromethane (20 mL), washed with water (20 mL \times 3), saturated NaHCO₃ aqueous solution (20 mL \times 3), and saturated brine (20 mL \times 3), dried over Na₂SO₄, and evaporated to a residue purified by column chromatography. White solid, Yield (11%), mp 73–75 °C, ¹H NMR (CDCl₃, 300 MHz) δ: 8.05 (s, 1H, HCOO-H), 7.72-7.51 (m, 5H, Ar-H), 7.18 (s, 1H, 14-H), 6.91 (dd, J = 15.8, 10.0 Hz, 1H, 11-H), 6.14 (d, J =15.8 Hz, 1H, 12-H), 5.16 (s, 2H, COOCH₂), 4.81 (s, 2H, 15-H), 4.80 (s, 1H, 17a-H), 4.60 (t, J = 9.0 Hz, 1H, 3-H), 4.56 (s, 1H, 17b-H), 4.45 (d, J = 11.6 Hz, 1H, 19a-H), 4.23 (d, J = 11.6 Hz, 1H, 19b-H), 2.69–2.61 (m, 4H, OOCCH₂CH₂COO), 2.34 (d, J = 10.0 Hz, 1H, 9-H), 2.46–1.13 (m, 9H, 1, 2, 5, 6, 7-H), 1.02 (s, 3H, 18-CH₃), 0.88 (s, 3H, 20-CH₃); HRMS (ESI) for $[C_{34}H_{38}N_2O_{10} + Na]^+$, Calcd. 657.2419, found 657.2425 [M + $Na]^+$.

Results and Discussion

Chemistry

The NO compounds reported herein were developed by using TAD as a scaffold to which NO releasing moieties were coupled with the C-3 positions. The major classes of NO donors include the organic nitrates, furoxans ^[27-29], diazeniumdiolates, metal-NO complexes, S-nitrosothiols, and sydnonimines, etc. The chemical activities and biological applications of major classes of NO donors have been comprehensively reviewed ^[30-32]. In the present study, we used one of the following moieties for NO release, nitrate (-ONO2), isosorbide mononitrate, phenyl-substituted furoxan, and phenylsulfonyl-substituted furoxan, which were attached to the andro through an aliphatic spacer. The synthetic routes employed to prepare the target compounds are outlined in Scheme 1. Compound 2 (TAD) was obtained by refluxing andro (1) in pyridine in the presence of Al₂O₃^[23]. Treatment of 2 with triphenylchloromethane (TrCl) in the presence of N-methylmorpholine in CH₂Cl₂ at room temperature generated triphenylmethyl ether 3. Compound 3 was then treated with succinic anhydride in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP) to give the succinic acid linked intermediate 4. The generated 4 was treated with different NO-donors in the presence of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI)/ DMAP and further deprotected by HCOOH to give the corresponding targets (I-1-12) in moderate yields. Intermediate 4 was also used as the precursor for preparation of I-13 using 2-aminoethyl nitrate in the presence of EDCI/DMAP. Meanwhile, Intermediate 4 coupled with dibromoalkane in the presence of triethylamine were used to yield compound 5. The bromo moiety in compound 5 was then substituted with nitrate using AgNO₃ to give compounds I-14-16. And to compound I-1 was added formic acid in the presence of EDCI/DMAP to afford compound I-17. Their structures were characterized by ¹H NMR and HRMS.





Scheme 1 Synthesis of the target compounds. Reagents and conditions: (a) xylene, pyridine, Al₂O₃, reflux, 88% yield; (b) TrCl, N-Methylmorpholine, CH₂Cl₂, rt, 92% yield; (c) succinic anhydride, DMAP, CH₂Cl₂, reflux, 85% yield; (d) 2-aminoethyl nitrate, EDCI, DMAP, CH₂Cl₂, rt; (e) dibromoalkane, acetone, triethylamine, reflux; (f) AgNO₃, acetonitrile, reflux; (g) NO Donor, EDCI, DMAP, CH₂Cl₂, rt; (h) HCOOH, CH₂Cl₂, rt; (i) HCOOH, EDCI, DMAP, CH₂Cl₂, rt

Cytotoxicity of the target compounds

We initially evaluated the antitumor activity of the target compounds (I-1-17) by MTT assay using human erythroleukemia K562 and breast cancer MCF-7 cell lines. We chose DOXO as the positive control, which is one of the most effective antineoplastic agents in clinical practice ^[33]. As shown in Fig. 2, these NO-releasing compounds were effective in inhibiting the growth of both cell lines. And it was notable that all NO-TAD derivatives (I-1-17) displayed more potent antiproliferative activity than the lead compound TAD, suggesting that the presence of a NO-releasing group may be important to improve TAD's efficacy. Furthermore, the types of the NO donor moiety were important for compounds' activities. Analysis of structure activity relationships (SAR) among these compounds revealed that the 4-phenylsulfonylfuroxan was crucial for the antiproliferative activity of NO-TAD hybrids because the phenylsulfonylfuroxan-substituted NO-TAD (I-2-7) showed stronger cytotoxicity against the tested cells. Moreover, the compounds obtained by coupling triphenylchloromethane (bulky substituent) or formic acid to the 19-OH of NO-TAD derivatives were less active than the same single NO-TAD derivatives (I-1 vs I-17, I-8 vs I-13, I-10 vs I-14, I-11 vs I-15, and I-12 vs I-16). These observations suggested that these varying at 19-OH were not feasible. Next, we selected some of promising candidates for further investigation. We constructed dose-response curves and hence calculated the IC_{50} for cell growth inhibition (Table 1). Indeed,



Fig. 2 The growth inhibition of test compounds at 10 μ mol·L⁻¹ in K562 and MCF-7 cells as determined by MTT method. The data are expressed as means \pm SD of each compound from three independent experiments. **P* < 0.05 *vs* TAD, #*P* < 0.05 *vs* andro

Compound	$IC_{50} (\mu mol \cdot L^{-1})^a$		
	Erythroleukemia K562	Breast cancer MCF-7	
DOXO	$0.67 \pm 0.11^{*,\#}$	$0.29\pm0.07^{\#}$	
TAD	199.36 ± 21.34	ND	
andro	26.85 ± 2.13	37.84 ± 1.76	
I-2	$2.83 \pm 0.32^{*,\#}$	$3.02\pm0.75^{\#}$	
I-3	$1.80\pm 0.18^{*,\#}$	$2.36\pm0.52^{\#}$	
I-4	$2.31 \pm 0.23^{*, \#}$	$4.64\pm0.48^{\#}$	
I-5	$1.55 \pm 0.14^{*,\#}$	$2.91\pm0.72^{\#}$	
I-6	$2.91 \pm 0.49^{*,\#}$	$2.98\pm0.37^{\#}$	
I-7	$2.85 \pm 0.37^{*,\#}$	$2.94\pm0.10^{\#}$	
I-8	$17.30 \pm 1.66^*$	$19.94 \pm 2.02^{\#}$	
NO donor of I-3	$4.28 \pm 1.02^{*,\#}$	$6.85 \pm 0.97^{\#}$	

Table 1 IC_{50} values of the target compounds against K562 and MCF-7 cell lines^a (mean \pm SD, n = 3)

^aThe IC₅₀ values for the target compounds, DOXO, andro and TAD were determined by MTT method. ^{*}P < 0.05 vs TAD, [#]P < 0.05 vs andro

phenylsulfonylfuroxan-substituted NO-TAD compounds (I-2–7) were very potent, and the corresponding IC₅₀ values in K562 and MCF-7 cells ranged from 1.55 to 2.91, and 2.36 to 4.64 μ mol·L⁻¹, respectively. In a fold comparison study of the IC₅₀ values (TAD/NO-TAD), Compound I-5, which was the most potent amongst the phenylsulfonylfuroxan-substituted NO-TADs, was at least 128-fold more potent than TAD in K562 cells.

Role of NO in the antitumor activity of the target compounds

To verify the contribution of NO to the inhibitory activity, we evaluated some of the tested compounds for their NO releasing ability with L-cysteine *in vitro*. In brief, 100 μ mol·L⁻¹ of tested compounds were treated with phosphate buffer (pH 7.4) and L-cysteine at 37 °C for 150 min, and then the levels of produced NO were determined by a Griess assay in the form of nitrite ion (NO₂⁻).

As shown in Fig. 3, treatment with TAD in the medium did not result in more nitrite than the vehicle. In contrast, treatment with individual tested compounds led to variable levels of nitrite. It was observed that phenylsulfonylfuroxan-substituted NO-TADs (I-3 and I-5) released higher levels of NO than phenylfuroxan I-1 and nitrate compounds (I-8, I-9, and I-11). These results were consistent with the above inhibitory effects. And these observations also suggest that, the electron withdrawing moiety phenylsulfonyl as a substituent group might be beneficial to the NO-release ability of furoxan.

To examine whether **I-5** was more potent than its two moieties (TAD and the corresponding NO donor) alone, we conducted additional experiments to determine the inhibitory activity of the corresponding NO donor in **I-5** against MCF-7 and K562 cells. As shown in Table 2, Compound **I-5** was more potent, and the corresponding IC_{50} values in K562 and MCF-7 cells were 1.23 and 2.85 µmol·L⁻¹, respectively. The inhibitory activity of TAD and the corresponding NO donor of **I-5** was far lower than that of compound **I-5**. Meanwhile, to verify the selectivity of compound **I-5**, the inhibitory activity of compound **I-5** against on non-cancer MCF10A cells was tested. The data indicated that compound **I-5** has better selectivity.



Fig. 3 Assessment of NO release from the test compounds using the Griess assay. 100 μ mol·L⁻¹ of each of the tested compounds was treated with phosphate buffer (pH 7.4) and L-cysteine at 37 °C for 150 min, and the concentrations of 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. The data are expressed as means ± SD from three independent experiments. *P < 0.05 vs the vehicle, **P < 0.01 vs the vehicle

Table 2 IC_{50} values of compound I-5 and its two moieties (TAD and the corresponding NO donor)^a

Compound	$IC_{50} \ (\mu mol \cdot L^{-1})^a$			
	Erythroleukemia K562	Breast cancer MCF-7	MCF10A	
DOXO	$0.55\pm0.09^{\ast}$	0.26 ± 0.04	1.67 ± 0.21	
TAD	184.75 ± 13.28	ND	ND	
The NO donor of I-5	$3.78 \pm 0.67^{*}$	4.98 ± 0.74	ND	
I-5	$1.23 \pm 0.11^{*}$	2.85 ± 0.46	12.73 ± 1.28	

^aThe IC₅₀ values were determined by MTT method. Each experiment was carried out three times. *P < 0.05 vs TAD

Following that, **I-3** and **I-5** were tested for their antiproliferative effects in the presence or absence of a NO scavenger, hemoglobin. In the hemoglobin group, the K562 cells were pretreated with 10 μ mol·L⁻¹ of hemoglobin for 1 h and then treated with 10 μ mol·L⁻¹ of tested compounds for 48 h. The effects of different treatments on the growth of K562 cells were determined by the MTT assay using DOXO as a control (Fig. 4). It was observed that treatment with **I-3** or **I-5** alone remarkably inhibited the growth of K562 cells and their inhibitory effects were diminished by pretreatment with hemoglobin. By contrast, the percentage of cell growth inhibition measured in the same cells treated with DOXO did not differ from pretreatment with hemoglobin cells. These results suggested that NO produced by **I-3** and **I-5** might contribute to their inhibition of K562 cell proliferation.





Fig. 4 Effects of hemoglobin on the antiproliferative activity of I-3, I-5, and DOXO. The results are expressed as the percentage of cell growth inhibition relative to control cells. The data are means \pm SD obtained from three independent experiments

Conclusion

A series of hybrid compounds (I-1-17), derived from TAD, were developed and evaluated in the present study. All the compounds showed inhibitory effects on K562 and MCF-7 cells, which were superior to TAD. The most active compound I-5 exhibited strong and preferable antitumor activity against both K562 (IC₅₀ 1.55 μ mol·L⁻¹) and MCF-7 $(IC_{50} 2.91 \ \mu mol \ L^{-1})$ cells. In addition, treatment with I-5 induced high levels of NO production, and its antitumor activity was significantly attenuated by the NO scavenger hemoglobin. Taken together, these data indicated that I-5, a hybrid of TAD and NO donor moiety had more potent antitumor activity preferably against human erythroleukemia and breast cancer cells than TAD. The superior properties of this hybrid may be attributed to the introduction of phenylsulfonyl-substituted furoxan NO-releasing group. Compound I-5 could be a promising candidate for intensive study.

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