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PRECLINICAL STUDIES

Inhibition of topoisomerase II α activity and induction of apoptosis in mammalian cells by semi-synthetic andrographolide analogues

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Summary Topoisomerase II α enzyme plays a critical role in DNA replication process. It controls the topologic states of DNA during transcription and is essential for cell proliferation. Human DNA topoisomerase II α (hTopo II α) is a promising chemotherapeutic target for anticancer agents against a variety of cancer types. In the present study, andrographolide and its structurally modified analogues were investigated for their inhibitory activities on hTopo II α enzyme. Five out of nine andrographolide analogues potently reduced hTopo II α activity and inhibited cell proliferation in four mammalian cell lines (Hela, CHO, BCA-1 and HepG2 cells). IC_{50} values for cytotoxicity of analogues 3A.1, 3A.2, 3A.3, 1B and 2C were 4 to 7 μM. Structure-activity relationship studies revealed that both core structure of andrographolide and silicon based molecule of functional group were important for the inhibition of hTopo II α activity whereas position C-19 of analogues was required for anti-proliferation. In addition, the analogue 2C at 10 μM concentration inhibited hTopo II α, and induced apoptosis with nuclear fragmentation and formation of apoptotic bodies in HepG2 cells. The analogue 2C may, therefore, have a therapeutic potential as effective anticancer agent targeting the hTopo II α functions.

Keywords Andrographolide analogues · Apoptosis · Cancer · DNA topoisomerase II α inhibitor

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Introduction

DNA topoisomerases are one of the promising molecular targets for developing anticancer drugs [1]. Inhibitors of DNA topoisomerase have been proposed as the most effective anticancer agents such as campthothecin, etoposide and doxorubicin [2]. They are chosen for the first treatment of many cancers including cervical adenocarcinoma and ovarian cancers [3, 4]. DNA topoisomerases are ubiquitous and highly conserved enzymes involved in mainly genomic integrity of cells. These enzymes are responsible for the relaxation of DNA during critical cellular processes including replication, recombination, transcription and repair system by transiently breaking one or two strands of DNA. Recently, a number of semi-synthetic compounds derived from natural products have been demonstrated to greatly improve anticancer activities [5]. Modified natural compounds could, therefore, render greater potency and efficacy for the inhibition of cancer growth and proliferation [5–10]. For example, etoposide, a synthetically modified compound

derived from *Podophyllum peltatum,* is used for treatment of lung and testicular cancers with much higher potency and efficacy than its parent form [5].

Andrographis paniculata (Burm.f.) Nees (Acanthaceae) is a medicinal plant widely used in many Asian countries including China, India, Taiwan, and Thailand. Its major biological active compound is Andrographolide, a diterpenoid lactone which has been reported to have various biological activities including anti-cancer activity [5]. It inhibits HepG2 (hepatocellular carcinoma) cell proliferation and induces caspase independent cell death [11]. Recently, a number of andrographolide analogues have been developed and demonstrated to have much greater therapeutic potency for cancer treatments [5–10]. They also induced apoptosis in human cervical, breast and liver cancer cell lines [12, 13]. Apoptosis is a highly regulated and a crucial process found in all multi-cellular organisms. Apoptotic cell death is not only implicated in regulatory mechanisms of cells but also an important way of anti-cancer treatments as the process eliminates cancer cells without inducing inflammation [14].

In the present study we semi-synthesized andrographolide analogues and investigated their inhibitory activity on human topoisomerase II α (hTopo II α) enzyme in vitro, anti-proliferation effects on different types of cell lines, and induction of apoptosis in HepG2 cells. This is the first report showing direct inhibition of recombinant hTopo II α in vitro by andrographolide analogues.

Materials and methods

Reagents

MEM medium, antibiotic-antimicotic and trypsin were purchased from Gibco Invitrogen Co (Scotland, UK). Proteinase K and APO-BrdU™ TUNEL Assay Kit were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was purchased from Thermoscientific (CramLington, UK). Etoposide, sulforhodamine B (SRB) dye were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and commercially obtained.

Isolation of andrographolide and semi-synthesis of andrographolide analogues

Andrographolide **(1)** was isolated from dried aerial part and root of *Andrographis paniculata* plant, which were harvested from Pak Tho district, Ratchaburi province, Thailand (September, 2008). The dried raw material was stored at room temperature for 110 days and was extracted with methanol at room temperature for 4 days. The concentrated extract was first fractionated between hexane and water. Then, the water layer was re-extracted with ethyl acetate several times. The combined ethyl acetate extracts were concentrated *in vacuo*. The crude sample was recrystallized in methanol to give pure andrographolide-1 as a white solid compound (9.4484 g, 0.94 % yield). The structure of andrographolide obtained from the plant extract was identified based on ^{1}H- and ^{13}C-NMR spectral data comparing with commercial andrographolide (purchased from Sigma-Aldrich, CAS No. 5588-58-7).

Andrographolide analogues derived from the natural substituents were semi-synthesized by changing the core structure and functional groups at C-3, C-12, C-17, and C-19. The methods for modifying the structure of andrographolide analogues in structure 1 have been described previously [9]. Modifications of andrographolide analogues in structure 2 and 3 were shown in Fig. 1. Structures of andrographolide analogues were identified by using IR, NMR and HRMS (ESI).

Andrographolide (1)

White solid, yield 0.94 %; IR (Neat): 3196, 3097, 1646, 1403, 1178, 1023 cm^{-1}; ^{1}H NMR (400 MHz, CD3OD): δ 6.75 (1H, td, *J*=6.0, 1.0 Hz, H-12), 4.92 (1H, d, *J*=6.0 Hz, H-14), 4.79 (1H, brs, H-17b), 4.57 (1H, brs, H-17a), 4.37 (1H, dd, *J*=10.0, 6.0 Hz, H-15b), 4.07 (1H, dd, *J*=10.0, 2.0 Hz, H-15a), 4.02 (1H, d, *J*=11.0 Hz, H-19b), 3.27 (1H, d, *J*=11.0 Hz, H-19a), 3.23−3.17 (1H, m), 2.58−2.43 (2H, m), 2.33 (1H, dt, *J*=7.0, 2.0 Hz), 1.98−1.64 (5H, m), 1.35−1.15 (4H, m), 1.12 (3H, s, H-18), 0.65 (3H, s, H-20); 13C NMR (100 MHz, CD3OD): δ 172.72 (C-16), 149.48 (C-12), 148.76 (C-8), 129.74 (C-13), 109.24 (C-17), 80.92 (C-3), 76.17 (C-15), 66.64 (C-14), 64.96 (C-19), 57.38 (C-9), 56.32 (C-5), 43.68 (C-4), 39.96 (C-10), 38.96 (C-7), 38.13 (C-1), 29.00 (C-2), 25.73 (C-6), 25.20 (C-11), 23.37 (C-18), 15.54 (C-20).

19-TBDPS-andrographolide (1A)

Andrographolide (**1**) (10.0 mg, 0.0285 mmol) in pyridine (200 μL) was stirred and *tert*-butyldiphenylsilyl chloride (TBDPSCl) (100 μL, 0.391 mmol) was added at room temperature with continuous stirring for 1 h. Then, the reaction mixture was diluted with EtOAc (20 mL) and quenched with H_2O (20 mL), followed by extraction with EtOAc (3×10 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (50 % EtOAc/*n*-hexane) to give 19-TBDPS-Andrographolide (**1A**) in quantitative yield (16.8 mg) as a white solid, R_f 0.40 (50 % EtOAc/*n*-hexane). Mp: 139–143 °C; IR (Neat): 3412, 2933, 1759, 1674, 1428, 1050, 703 cm^{-1};^{1}H NMR (400 MHz, $CDCl_3$): δ 7.68−7.62 (4H, m, PhH), 7.48−7.38 (6H, m, PhH), 6.91

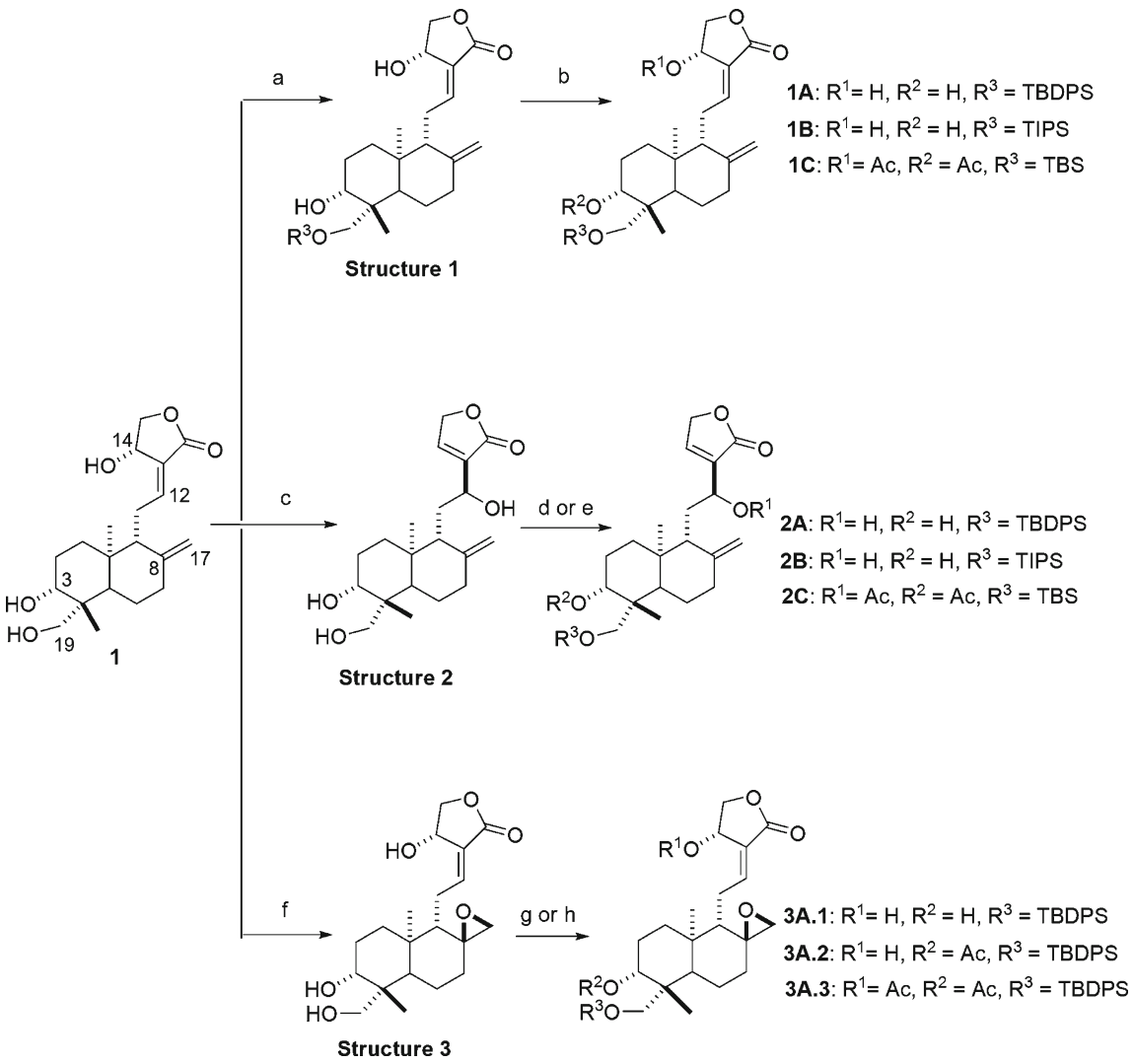

Fig. 1 Three groups of andrographolide analogues. Reagents and conditions: **a** R_3SiCl, pyridine for 1A and 1B; **b** i) TBSCl, pyridine, ii) Ac_2O for **1C**; **c** i) 2,2-dimethoxy propane, PPTS, acetone, ii) PDC, CH_2Cl_2, iii) $AcOH/H_2O$ (7:3); **d** R_3SiCl, pyridine for **2A** and **2B**; **e** i) TBSCl, pyridine, ii) Ac_2O for **2C**; **f** mCPBA, CH_2Cl_2/MeOH; **g** TBDPSCl, pyridine for **3A.1**; **h** i) TBDPSCI, pyridine, ii) Ac_2O, reflux for **3A.2** and **3A.3**

(1H, td, *J*=6.0, 1.0 Hz, H-12), 4.98 (1H, brd, *J*=6.0 Hz, H-14), 4.78 (1H, brs, H-17b), 4.51 (1H, brs, H-17a), 4.42 (1H, dd, *J*=10.5, 6.0 Hz, H-15b), 4.22 (1H, dd, *J*=10.5, 2.0 Hz, H-15a), 4.17 (1H, d, *J*=10.0 Hz, H-19b), 3.37 (1H, d, *J*= 10.0 Hz, H-19a), 3.35 (1H, dd, *J*=12.0, 4.0 Hz, H-3), 2.52 (1H, ddd, *J*=16.0, 7.5, 3.0 Hz, H-11b), 2.43 (1H, ddd, *J*= 16.0, 11.0, 6.5 Hz, H-11a), 2.29 (1H, ddd, *J*=12.0, 3.5, 2.0 Hz, H-9), 1.94−1.57 (5 H, m), 1.31 (3H, s, H-18), 1.28−1.12 (4H, m), 1.04 (9H, s, $SiC(CH_3)_3$), 0.45 (3H, s, H-20); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.97 (C-16), 148.62 (C-12), 146.47 (C-8), 135.68 (Ph), 135.53 (Ph), 132.38 (Ph), 131.92 (Ph), 130.03 (Ph), 130.00 (Ph), 128.03 (C-13), 127.87 (Ph), 127.84 (Ph), 108.60 (C-17), 80.30 (C-3), 74.30 (C-15), 66.08 (C-14), 65.91 (C-19), 56.02 (C-9), 55.27 (C-5), 42.78 (C-4), 38.86 (C-10), 37.65 (C-7), 37.09 (C-1), 28.49 (C-2), 26.79 ($SiC(CH_3)_3$), 24.55 (C-11), 23.62 (C-6), 23.11 (C-18), 19.05 ($SiC(CH_3)_3$), 15.22 (C-20); HRMS (ESI) *m/z* calcd for $C_{36}H_{48}O_5SiNa$ $[M+Na]^+$ 611.3169, found 611.3162.

19-TIPS-andrographolide (1B)

Andrographolide (**1**) (81.1 mg, 0.231 mmol) in pyridine (500 μL) was stirred and triisopropyl chloride (TIPSCl) (250 μL, 1.48 mmol) was added at room temperature with continuous stirring for 4 h. Then, the reaction mixture was diluted with EtOAc (20 mL), quenched with H_2O (20 mL), and extracted with EtOAc (3×20 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (40 % EtOAc/*n*-hexane) to give 19-TIPS-Andrographolide (**1B**) in 65 % yield (75.9 mg) as a white solid, R_f 0.49 (40 % EtOAc/*n*-hexane). Mp: 62–65 °C; IR (Neat): 3377, 2941,

1745, 1674, 1460, 1054, 755 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 6.97 (1H, td, *J*=7.0, 2.0 Hz, H-12), 5.04 (1H, brd, *J*=6.0 Hz, H-14), 4.89 (1H, brs, H-17b), 4.59 (1H, brs, H-17a), 4.52 (1H, d, *J*=7.0 Hz, OH), 4.46 (1H, dd, *J*=10.5, 6.0 Hz, H-15b), 4.31 (1H, d, *J*=10.0 Hz, H-19b), 4.26 (1H, dd, *J*=10.5, 2.0 Hz, H-15a), 3.49 (1H, d, *J*=10.0 Hz, H-19a), 3.32−3.29 (1H, m, H-3), 2.62−2.38 (3H, m), 2.07 (1H, d, *J*=7.0 Hz, OH), 2.02−1.65 (5H, m), 1.29 (3H, s, H-18), 1.30−1.20 (4H, m), 1.06 (18H, s, 3 x (SiCH(CH_3)$_2$)), 1.06 (3H, s, 3 x (Si*C*H(CH_3)$_2$)), 0.69 (3H, s, H-20); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.97 (C-16), 148.67 (C-8), 146.58 (C-12), 128.10 (C-13), 108.73 (C-17), 80.26 (C-3), 74.34 (C-15), 66.15 (C-14), 65.72 (C-19), 56.14 (C-9), 55.23 (C-5), 42.73 (C-4), 38.97 (C-10), 37.78 (C-7), 37.15 (C-1), 28.64 (C-2), 24.70 (C-11), 23.85 (C-6), 23.01 (C-18), 17.96 (3 x (SiCH(*C*H_3)$_2$)), 15.53 (C-20), 11.66 (3 x (Si*C*H (CH_3)$_2$)); HRMS (ESI) *m/z* calcd for $C_{29}H_{50}O_5SiNa$ $[M+Na]^+$ 529.3325, found 529.3325.

19-TBS-3,14-Ac-andrographolide (1C)

Andrographolide (1) (10.0 mg, 0.0285 mmol) in pyridine (200 μL) was stirred and *tert*-butyldimethylsilyl chloride (TBDMSCl) (50.0 mg, 0.332 mmol) was added at room temperature with continuous stirring for 1 h. Then, the reaction mixture was diluted with EtOAc (20 mL), quenched with H_2O (20 mL), and extracted with EtOAc (3×20 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (50 % EtOAc/*n*-hexane) to give 19-TBS-Andrographolide in 92 % yield as a white solid. To a stirred solution of 19-TBS-Andrographolide (78.9 mg, 0.170 mmol) in acetic anhydride (1.0 mL) was heated to 70 °C. After the stirring was continued at 70 °C for 18 h, the reaction mixture was diluted with EtOAc (20 mL) and quenched with saturated $NaHCO_3$, washed with H_2O, and extracted with EtOAc (3×20 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (30 % EtOAc/*n*-hexane) to give 19-TBS-3,14-Ac-Andrographolide (**1C**) in 44 % yield (40.6 mg) as a white solid, R_f 0.78 (50 % EtOAc/*n*-hexane). Mp: 97–103 °C; IR (Neat): 3237, 2953, 1760, 1737, 1432, 1248, 751 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.02 (1H, td, *J*=7.0, 1.5 Hz, H-12), 5.92 (1H, d, *J*=6.0 Hz, H-14), 4.88 (1H, brs, H-17b), 4.58 (1H, dd, *J*=11.0, 5.5 Hz, H-3), 4.54 (1H, dd, *J*=11.0, 6.0 Hz, H-15b), 4.49 (1H, brs, H-17a), 4.24 (1H, dd, *J*= 11.0, 1.5 Hz, H-15a), 3.82 (1H, d, *J*=10.5 Hz, H-19b), 3.60 (1H, d, *J*=10.5 Hz, H-19a), 2.48 (1H, ddd, *J*=16.5, 6.5, 3.0 Hz, H-9), 2.44−2.32 (2H, m, H-11), 2.12 (3H, s, COCH_3), 2.05 (3H, s, COCH_3), 1.96−1.60 (5H, m), 1.36−1.24 (4H, m), 0.94 (3H, s, H-18), 0.89 (9H, s, SiC(CH_3)$_3$), 0.81 (3H, s, H-20), 0.05 (6H, s, Si(CH_3)$_2$); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.76 (*C*OCH_3), 170.51 (*C*OCH_3), 169.08 (C-16), 150.65 (C-12), 147.31 (C-8), 123.83 (C-13), 108.36 (C-17), 80.12 (C-3), 71.59 (C-15), 67.85 (C-14), 63.73 (C-19), 56.11 (C-9), 55.57 (C-5), 42.47 (C-4), 39.05 (C-10), 38.27 (C-7), 37.40 (C-1), 29.70 (C-2), 25.89 (SiC(*C*H_3)$_3$), 25.32 (C-11), 24.40 (C-6), 23.30 (C-18), 21.27 (CO*C*H_3), 20.73 (CO*C*H_3), 18.25 (SiC(CH_3)$_3$), 14.37 (C-20), -5.90 (Si(*C*H_3)$_2$); HRMS (ESI) *m/z* calcd for $C_{30}H_{48}O_7SiNa$ $[M+Na]^+$ 571.3067, found 571.3065.

12-hydroxy-14-dehydroandrographolide (2)

Andrographolide (**1**) (400 mg, 1.14 mmol) in acetone (45.0 mL) was stirred and 2,2-dimethoxypropane (1.26 mL, 10.3 mmol) was added, followed by addition of pyridinium *p*-toluenesulfonate (PPTS) (14.3 mg, 0.0571 mmol). The reaction mixture was stirred at room temperature for 2 h. Then, the mixture was diluted with EtOAc (50 mL), quenched with saturated $NaHCO_3$, washed with H_2O, and extracted with EtOAc (3× 50 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (30 % EtOAc/*n*-hexane) to give 3,19-isopropylideneandrographolide in 97 % yield as a white solid.

To a stirred solution of 3,19-isopropylideneandrographolide (71.8 mg, 0.20 mmol) in CH_2Cl_2 (3.0 mL), pyridinium dichromate (PDC) (7.5 mg, 0.02 mmol) was added. The reaction mixture was continuously stirred at room temperature for 6 h, then was diluted with EtOAc (20 mL) and quenched with saturated $NaHCO_3$, washed with H_2O, and extracted with EtOAc (3×50 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (100 % EtOAc) to give 12-hydroxy-14-hydro-3,19-isopropylidene- andrographolide in 84 % yield as a white solid.

12-hydroxy-14-dehydro-3,19-isopropylideneandrographolide (409 mg, 1.05 mmol) was added into a mixture of CH_3COOH/H_2O (7:3) (10 mL). The reaction was stirred at room temperature for 30 min. After the reaction was completed, the reaction mixture was diluted with EtOAc (50 mL) and quenched with saturated $NaHCO_3$, washed with H_2O, and extracted with EtOAc (3×50 mL). The combined organic layer was washed with brine (20 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (100 % EtOAc) to give 12-hydroxy-14-dehydroandrographolide **(2)** (313 mg) (?% yield) as a white

solid, R_f 0.49 (100 % EtOAc). Mp: 136–140 °C; IR (Neat): 3351, 2937, 1746, 1448, 1034 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.28 (1H, brd, *J*=1.0 Hz, H-14), 4.91 (1H, brs, H-17b), 4.83 (2H, brs, H-15), 4.73 (1H, brs, H-17a), 4.52 (1H, brt, *J*=7.0 Hz, H-12), 4.15 (1H, d, *J*=11.0 Hz, H-19b), 3.45 (1H, dd, *J*=10.0, 6.0 Hz, H-3), 3.29 (1H, d, *J*=11.0 Hz, H-19a), 2.66 (1H, brs, OH), 2.42 (1H, ddd, *J*=14.0, 3.5, 2.5 Hz, H-7b), 2.03 (1H, ddd, *J*=14.0, 9.0, 1.5 Hz), 1.97−1.75 (6H, m), 1.56 (1H, d, *J*=11.0 Hz), 1.34−1.05 (3H, m), 1.22 (3H, s, H-18), 0.63 (3H, s, H-20); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.94 (C-16), 148.22 (C-8), 145.27 (C-14), 136.09 (C-13), 107.86 (C-17), 80.53 (C-3), 70.46 (C-15), 67.61 (C-12), 64.09 (C-19), 55.33 (C-5), 53.25 (C-9), 42.92 (C-4), 39.21 (C-10), 38.21 (C-7), 36.73 (C-1), 30.29 (C-11), 28.20 (C-2), 23.98 (C-6), 22.67 (C-18), 15.42 (C-20); HRMS (ESI) *m/z* calcd for $C_{20}H_{30}O_5Na$ $[M+Na]^+$ 487.2856, found 487.2858.

19-Tr-12-hydroxy-14-dehydroandrographolide (2A)

12-hydroxy-14-dehydroandrographolide (**2**) (41.9 mg, 0.01 mmol) in pyridine (500 μL) was stirred and *tert*-butyldiphenylsilyl chloride (TBDPSCl) (150 μL, 0.57 mmol) was added at room temperature with continuous stirring for 1 h. The reaction mixture was diluted with EtOAc (20 mL) and quenched with H_2O (20 mL), and extracted with EtOAc (3×10 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (60 % EtOAc/*n*-hexane) to give 19-TBDPS-12-hydroxy-14-dehydroandrographolide (**2A**) in 82 % yield (70.4 mg) as a white solid, R_f 0.51 (50 % EtOAc/*n*-hexane). Mp: 71–73 °C; IR (Neat): 2933, 2859, 1753, 1054, 750 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.68−7.61 (4H, m, PhH), 7.48−7.37 (6H, m, PhH), 7.24 (1H, brs, H-14), 4.83 (2H, brs, H-15), 4.81 (1H, brs, H-17b), 4.65 (1H, brs, H-17a), 4.58 (1H, dd, *J*=12.5, 6.5 Hz, H-12), 4.40 (1H, d, *J*=7.5 Hz, OH), 4.15 (1H, d, *J*=10.0 Hz, H-19b), 3.33 (1H, d, *J*=10.0 Hz, H-19a), 3.36−3.29 (1H, m, H-3), 2.54 (1H, d, *J*=6.5 Hz, OH), 2.29 (1H, ddd, *J*=13.0, 3.5, 2.5 Hz, H-7b), 2.02−1.71 (5H, m), 1.68−1.59 (2H, m), 1.51 (1H, d, *J*=10.0 Hz), 1.30 (3H, s, H-18), 1.18−0.94 (3H, m), 1.04 (9H, s, $SiC(CH_3)_3$), 0.39 (3H, s, H-20); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.94 (C-16), 148.05 (C-8), 145.07 (C-14), 136.05 (C-13), 135.70 (2xPh), 135.54 (2xPh), 132.38 (Ph), 132.05 (Ph), 129.99 (2xPh), 127.88 (2xPh), 127.81 (2xPh), 107.64 (C-17), 80.29 (C-3), 70.42 (C-15), 67.38 (C-12), 65.89 (C-19), 55.40 (C-5), 53.19 (C-9), 42.83 (C-4), 39.17 (C-10), 38.12 (C-7), 36.88 (C-1), 30.21 (C-11), 28.49 (C-2), 26.80 ($SiC(CH_3)_3$), 23.81 (C-6), 23.11 (C-18), 19.07 ($SiC(CH_3)_3$), 15.38 (C-20); HRMS (ESI) *m/z* calcd for $C_{26}H_{44}O_5SiNa$ $[M+Na]^+$ 487.2856, found 487.2858.

19-TIPS-12-hydroxy-14-dehydroandrographolide (2B)

12-hydroxy-14-dehydroandrographolide **(2)** (51.0 mg, 0.14 mmol) in pyridine (500 μL) was stirred and triisopropyl chloride (TIPSCl) (300 μL, 1.40 mmol) was added at room temperature with continuous stirring for 8 h. The reaction mixture was diluted with EtOAc (20 mL) and quenched with H_2O (20 mL), and extracted with EtOAc (3×20 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (40 % EtOAc/*n*-hexane) to give 19-TIPS-12-hydroxy-14-dehydro andrographolide (**2B**) in 67 % yield (73.8 mg) as a white solid, R_f 0.47 (60 % EtOAc/*n*-hexane). Mp: 95–98 °C; IR (Neat): 3380, 2942, 2867, 1750, 1460, 1056, 883 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.27 (1H, brs, H-14), 4.90 (1H, brs, H-17b), 4.84 (2H, brs, H-15), 4.73 (1H, brs, H-17a), 4.56–4.49 (1H, m, H-12), 4.47 (1H, d, *J*=7.0 Hz, OH), 4.29 (1H, d, *J*=10.0 Hz, H-19b), 3.47 (1H, d, *J*=10.0 Hz, H-19a), 3.30 (1H, ddd, *J*=13.0, 7.0, 5.0 Hz, H-3), 2.62 (1H, brd, *J*=5.0 Hz, OH), 2.41 (1H, dm, *J*=13.0 Hz, H-7b), 2.03 (1H, ddd, *J*=14.0, 8.0, 2.0 Hz), 1.96−1.76 (5H, m), 1.74−1.62 (1H, m), 1.57 (1H, d, *J*=10.0 Hz, H-9), 1.27 (3H, s, H-18), 1.26−1.00 (6H, m), 1.07 (18H, d, *J*=5.0 Hz, 3xSiCH(CH_3)), 0.64 (3H, s, H-20); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.98 (C-16), 148.11 (C-8), 145.11 (C-14), 136.07 (C-13), 107.72 (C-17), 80.20 (C-3), 70.44 (C-15), 67.31 (C-12), 65.65 (C-19), 55.32 (C-5), 53.22 (C-9), 42.74 (C-4), 39.23 (C-10), 38.21 (C-7), 36.91 (C-1), 30.27 (C-11), 28.58 (C-2), 24.05 (C-6), 22.95 (C-18), 17.91 (3xSiCH(CH_3)$_2$), 15.64 (C-20), 11.63 (3xSi*C*H(CH_3)$_2$),; HRMS (ESI) *m/z* calcd for $C_{26}H_{44}O_5SiNa$ $[M+Na]^+$ 487.2856, found 487.2858.

19-TBS-3,12-Ac-14-dehydroandrographolide (2C)

12-hydroxy-14-dehydroandrographolide **(2)** (40.8 mg, 0.12 mmol) in pyridine (500 μL) was stirred and *tert*-butyldimethylsilyl chloride (TBDMSCl) (91.7 mg, 0.58 mmol) was added at room temperature with continuous stirring for 1 h. The reaction mixture was diluted with EtOAc (20 mL) and quenched with H_2O (20 mL) and extracted with EtOAc (3×20 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (50 % EtOAc/*n*-hexane) to give 19-TBS-12-hydroxy-14-dehydroandrographolide in 77 % yield (43.0 mg) as a white solid.

To a stirred solution of 19-TBS-12-hydroxy-14-dehydroandrographolide (20.1 mg, 0.04 mmol) in acetic anhydride (1.0 mL) was heated to 145 °C. Thereafter the stirring was continued at 145 °C for 1.5 h, the reaction mixture was diluted with EtOAc (20 mL) and quenched with saturated

$NaHCO_3$, washed with H_2O and extracted with EtOAc (3× 20 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (40 % EtOAc/*n*-hexane) to give 19-TBS-3,12-Ac-14-dehydro andrographolide **(2C)** in 83 % (22.9 mg) as a white solid, R_f 0.42 (40 % EtOAc/*n*-hexane). Mp: 104–107 °C; IR (Neat): 2934, 1756, 1738, 1254, 1028, 752 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.35 (1H, brs, H-14), 5.66 (1H, dd, *J*=9.5, 4.5 Hz, H-12), 4.90 (1H, brs, H-17b), 4.83 (2H, brs, H-15), 4.75 (1H, brs, H-17a), 4.56−4.53 (1H, m, H-3), 3.79 (1H, d, *J*=10.5 Hz, H-19b), 3.57 (1H, d, *J*=10.5 Hz, H-19a), 2.36 (1H, dm, *J*=12.0 Hz, H-7b), 2.20 (1H, ddd, *J*=13.5, 9.0, 1.0 Hz), 2.06 (3H, s, $COCH_3$), 2.04 (3H, s, $COCH_3$), 1.94 (1H, *J*=13.5, 11.0, 5.0 Hz), 1.85−1.74 (3H, m), 1.73−1.63 (3H, m), 1.47 (1H, d, *J*=10.0 Hz, H-9), 1.21−1.11 (2H, m, H-5, H-11a), 0.91 (3H, s, H-18), 0.87 (9H, s, $SiC(CH_3)_3$), 0.76 (3H, s, H-20), 0.01 (6H, s, $Si(CH_3)_2$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.44 (C-16), 170.77 ($COCH_3$), 170.00 ($COCH_3$), 148.26 (C-8), 147.05 (C-14), 132.63 (C-13), 107.56 (C-17), 80.17 (C-3), 70.04 (C-15), 68.31 (C-12), 63.59 (C-19), 55.61 (C-5), 52.31 (C-9), 42.51 (C-4), 39.25 (C-10), 38.59 (C-7), 37.06 (C-1), 27.11 (C-11), 25.85 ($SiC(CH_3)_3$) 25.53 (C-2), 24.34 (C-6), 23.06 (C-18), 21.27 ($COCH_3$), 21.12 ($COCH_3$), 18.20 ($SiC(CH_3)_3$), 14.43 (C-20); HRMS (ESI) *m/z* calcd for $C_{26}H_{44}O_5SiNa$ $[M+Na]^+$ 487.2856, found 487.2858.

8,17-epoxy andrographolide (3A)

Andrographolide **1** (301.5 mg, 0.86 mmol) in a mixture of CH_2Cl_2/methanol (5:1) (12.0 mL) was stirred and *m*-chloroperoxybenzoic acid (*m*-CPBA) (297.7 mg, 1.72 mmol) was added. Thereafter, the stirring was continued at room temperature for 19 h, the solvent was then removed by evaporator. The reaction mixture was diluted with EtOAc (50 mL) and quenched with saturated $NaHCO_3$, washed with H_2O, and extracted with EtOAc (3×50 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (100 % EtOAc) to give 8,17-epoxy andrographolide **(3A)** in 72 % yield (225.5 mg) as a white solid, R_f 0.42 (100 % EtOAc). Mp: 147–149 °C; IR (Neat): 3282, 2927, 1739, 1673, 1456, 1035 cm^{-1};^1H NMR (400 MHz, $CDCl_3$): δ 6.79 (1H, ddd, *J*=11.0, 5.5, 1.5 Hz, H-12), 5.01−4.96 (1H, m, H-14), 4.36 (1H, dd, *J*=9.5, 6.0 Hz, H-15b), 4.26 (1H, dd, *J*=9.5, 1.5 Hz, H-15a), 4.17 (1H, d, *J*=11.0 Hz, H-19b), 3.52 (1H, dd, *J*=11.0, 4.0 Hz, H-19a), 3.35 (1H, t, *J*=9.0 Hz, H-3), 2.88 (1H, dd, *J*=3.5, 1.5 Hz, H-17b), 2.80 (1H, brd, *J*=8.5 Hz, OH), 2.68 (1H, d, *J*=3.5 Hz, H-17a), 2.42 (1H, brs, OH), 2.06−1.72 (8H, m), 1.50−1.41 (2H, m), 1.35−1.15 (2H, m), 1.27 (3H, s, H-18), 1.26−1.16 (2H, m), 0.82 (3H, s, H-20); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.20 (C-16), 145.87 (C-12), 129.20 (C-13), 80.13 (C-3), 73.67 (C-15), 65.12 (C-19), 63.83 (C-14), 60.24 (C-8), 54.54 (C-5), 53.36 (C-9), 50.94 (C-17), 42.67 (C-4), 39.73 (C-10), 36.78 (C-1), 35.85 (C-7), 27.09 (C-2), 22.78 (C-11), 22.70 (C-6), 21.26 (C-18), 15.10 (C-20); HRMS (ESI) *m/z* calcd for $C_{26}H_{44}O_5SiNa$ $[M+Na]^+$ 487.2856, found 487.2858.

19-TBDPS-8,17-epoxy andrographolide (3A.1)

8,17-epoxy andrographolide (**3A**) (10.0 mg, 0.03 mmol) in pyridine (500 μL) was stirred and *tert*-butyldiphenylsilyl chloride (TBDPSCl) (200 μL, 0.76 mmol) was added at room temperature. Thereafter, the stirring was continued at room temperature for 1 h. The reaction mixture was diluted with EtOAc (20 mL) and quenched with H_2O (20 mL), and extracted with EtOAc (3×10 mL). The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (50 % EtOAc/*n*-hexane) to give 19-TBDPS-8,17-epoxy andrographolide (**3A.1**) in 81 % yield (67.5 mg) as a white solid, R_f 0.36 (50 % EtOAc/*n*-hexane).Mp: 135–137 °C; IR (Neat): 2931, 1750, 1671, 1472, 1275, 1048, 749 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.70−7.63 (4H, m, PhH), 7.49−7.38 (6H, m, PhH), 6.76 (1H, ddd, *J*=10.5, 5.5, 1.5 Hz, H-12), 4.94 (1H, brd, *J*=5.5 Hz, H-14), 4.34 (1H, dd, *J*=10.0, 5.5 Hz, H-15b), 4.23 (1H, dd, *J*=10.0, 1.5 Hz, H-15a), 4.15 (1H, d, *J*=10.0 Hz, H-19b), 3.40 (1H, d, *J*=10.0 Hz, H-19a), 3.40−3.34 (1H, m, H-3), 2.76 (1H, dd, *J*=3.5, 1.5 Hz, H-17b), 2.53 (1H, d, *J*=3.5 Hz, H-17a), 2.02−1.87 (2H, m), 1.82−1.60 (6H, m), 1.34 (3H, s, H-18), 1.31−1.12 (4H, m), 1.06 (9H, s, $SiC(CH_3)_3$), 0.56 (3H, s, H-20); ^{13}C NMR (100 MHz, $CDCl_3$): δ170.01 (C-16), 145.78 (C-12), 135.69 (2xPh), 135.56 (2xPh), 132.35 (Ph), 131.95 (Ph), 130.05 (Ph), 130.03 (Ph), 129.11 (C-13), 127.90 (2xPh), 127.86 (2xPh), 79.93 (C-3), 73.59 (C-15), 65.57 (C-14), 65.14 (C-19), 60.20 (C-8), 54.65 (C-5), 53.39 (C-9), 50.74 (C-17), 42.66 (C-4), 39.77 (C-10), 36.69 (C-1), 35.86 (C-7), 27.70 (C-2), 26.77 ($SiC(CH_3)_3$), 23.08 (C-11), 22.69 (C-6), 21.22 (C-18), 19.05 ($SiC(CH_3)_3$), 15.23 (C-20); HRMS (ESI) *m/z* calcd for $C_{26}H_{44}O_5SiNa$ $[M+Na]^+$ 487.2856, found 487.2858.

19-TBDPS-3-Ac-8,17-epoxy andrographolide (3A.2) and 19-TBDPS-3,12-AC-8,17-epoxy andrographolide (3A.3)

19-TBDPS-8,17-epoxy andrographolide (3A.1) (80.0 mg, 0.13 mmol) in acetic anhydride (1.0 mL) was stirred and heated to 70 °C. Thereafter, the stirring was continued at 70 °C for 12 h. The reaction mixture was diluted with EtOAc (20 mL) and quenched with saturated $NaHCO_3$, washed with H_2O and extracted with EtOAc (3×20 mL).

The combined organic layer was washed with brine (10 mL), dried over sodium sulfate, and then concentrated under reduced pressure. The residue was purified by column chromatography (30 % EtOAc/*n*-hexane) to give 19-TBDPS-3-Ac-8,17-epoxy andrographolide (**3A.2**) in 11 % yield (9.70 mg) as a white solid, R_f 0.20 (30 % EtOAc/*n*-hexane) and 19-TBDPS-3,14-Ac-8,17-epoxy andrographolide (**3A.3**) in 77 % yield (70.0 mg) as a white solid, R_f 0.49 (30 % EtOAc/*n*-hexane).

19-TBDPS-3-Ac-8,17-epoxy andrographolide (3A.2) Mp: 81–84 °C; IR (Neat): 2935, 1763, 1675, 1466, 1245, 1083, 704 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.70−7.64 (4H, m, PhH), 7.47−7.35 (6H, m, PhH), 6.77 (1H, ddd, *J*= 10.5, 5.0, 1.5 Hz, H-12), 4.94 (1H, brd, *J*=5.5 Hz, H-14), 4.56 (1H, dd, *J*=12.0, 4.5 Hz, H-3), 4.35 (1H, dd, *J*=10.5, 5.5 Hz, H-15b), 4.26 (1H, dd, *J*=10.5, 1.5 Hz, H-15a), 3.13 (1H, brs, OH), 3.85 (1H, d, *J*=10.0 Hz, H-19b), 3.73 (1H, d, *J*=10.0 Hz, H-19a), 2.88 (1H, d, *J*=3.0 Hz, H-17b), 2.65 (1H, d, *J*=3.0 Hz, H-17a), 2.05−1.97 (1H, m), 1.91 (3H, s, $COCH_3$), 1.87−1.48 (7H, m), 1.45−1.19 (4H, m), 1.07 (9H, s, $SiC(CH_3)_3$), 1.05 (3H, s, H-18), 0.79 (3H, s, H-20); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.52 (C-16), 169.99 ($COCH_3$), 145.72 (C-12), 135.86 (2xPh), 135.77 (2xPh), 133.47 (Ph), 133.28 (Ph), 129.70 (2xPh), 129.16 (C-13), 127.64 (2xPh), 127.58 (2xPh), 79.62 (C-3), 73.61 (C-15), 65.57 (C-14), 63.85 (C-19), 60.65 (C-8), 54.86 (C-5), 53.38 (C-9), 50.80 (C-17), 42.85 (C-4), 39.98 (C-10), 36.95 (C-1), 36.38 (C-7), 26.95 ($SiC(CH_3)_3$), 23.53 (C-2), 22.86 (C-11), 22.79 (C-6, $COCH_3$), 21.09 (C-18), 19.27 ($SiC(CH_3)_3$), 14.53 (C-20); HRMS (ESI) *m/z* calcd for $C_{26}H_{44}O_5SiNa$ $[M+Na]^+$ 487.2856, found 487.2858.

19-TBDPS-3,12-Ac-8,17-epoxy andrographolide (3A.3) Mp: 92–94 °C; IR (Neat): 2939, 1734, 1241, 1079, 749 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.71−7.63 (4H, m, PhH), 7.47−7.34 (6H, m, PhH), 7.08 (1H, td, *J*=7.0, 1.0 Hz, H-12), 5.86 (1H, brd, *J*=5.5 Hz, H-14), 4.53 (1H, dd, *J*=11.0, 4.0 Hz, H-3), 4.50 (1H, dd, *J*=11.5, 5.5 Hz, H-15b), 4.22 (1H, dd, *J*= 11.5, 1.5 Hz, H-15a), 3.84 (1H, d, *J*=11.0 Hz, H-19b), 3.37 (1H, d, *J*=11.0 Hz, H-19a), 2.60 (1H, d, *J*=4.0 Hz, H-17b), 2.53 (1H, d, *J*=4.0 Hz, H-17a), 2.11 (3H, s, $COCH_3$), 2.15−1.92 (5H, m), 1.90 (3H, s, $COCH_3$), 1.84−1.39 (5H, m), 1.31 −1.17 (2H, m), 1.06 (9H, s, $SiC(CH_3)_3$), 1.04 (3H, s, H-18), 0.80 (3H, s, H-20); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.61 ($COCH_3$), 170.51 ($COCH_3$), 169.01 (C-16), 150.92 (C-12), 135.82 (2xPh), 135.74 (2xPh), 133.44 (Ph), 133.39 (Ph), 129.67 (Ph), 129.64 (Ph), 127.62 (2xPh), 127.59 (2xPh), 123.19 (C-13), 79.63 (C-3), 71.57 (C-15), 67.57 (C-14), 63.83 (C-19), 58.20 (C-8), 54.99 (C-5), 54.13 (C-5), 49.55 (C-17), 42.82 (C-4), 39.98 (C-10), 37.57 (C-1), 36.18 (C-7), 26.92 ($SiC(CH_3)_3$), 23.58 (C-2), 23.01 (C-11), 22.81 (C-6, $COCH_3$), 21.06 ($COCH_3$), 20.86 (C-18), 19.25 ($SiC(CH_3)_3$), 14.66 (C-20); HRMS (ESI) *m/z* calcd for $C_{26}H_{44}O_5SiNa$ $[M+Na]^+$ 487.2856, found 487.2858.

Cell culture and in vitro anti-proliferation assay

Four mammalian cell lines were used; HepG2 (Hepatocellular carcinoma), Hela (Cervical carcinoma), CHO (Chinese hamster ovary) and UISO-BCA1 (Human breast carcinoma). HepG2, Hela, and CHO cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA). UISO-BCA1 was a kind gift from Dr. John M Pezzuto (Department of Phamacognosy and Pharmacy, College of Pharmacy, University of Illinois at the Chicago, Illinois, USA). All cell lines were grown in MEM with 10 % fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5 % CO_2. The final concentration of DMSO for dissolving the test compounds was less than 0.1 %.

The sulphorhodamine (SRB) assay which is a colorimetric assay was used for evaluation of cell growth. Total cell number is indirectly estimated by staining total cellular protein with the dye SRB [15]. Cells were plated onto 96-well plates at densities of 4×10^3 cells/well and were incubated at 37 °C for 24 h before experimentation. Stock solutions of analogues were prepared in DMSO and subsequently diluted to the indicated final concentrations containing DMSO less than 0.1 %. Cells were treated with andrographolide analogues at different concentrations for 72 h. Each treatment was performed in triplicates. DMSO 0.1 % was used as a vehicle control. After incubation, cells were fixed in trichloroacetic acid (TCA), washed and stained with SRB. The bound stain was solubilized with Tris -base, pH 10.5. The absorbance was measured at 515 nm. The IC_{50} values (concentration of the tested compound that caused 50 % inhibition of cell growth) were estimated using GraphPad Prism version 5.01(GraphPad Software Inc, CA, USA).

Topo II α mediated supercoiled pBR322 DNA relaxation assay

The activity of human DNA topoisomerase II α (p170 form) (hTopoII α) (TopoGEN, Florida, USA) on the relaxation of supercoiled plasmid DNA pBR322 (Fermentas, Maryland, USA) was determined by measuring the conversion of supercoiled pBR322 plasmid to relaxed form [16]. The reaction mixture contained 4 μL of Buffer A (0.5 M Tris-HCl (pH 8.0); 1.5 M NaCl; 0.1 M $MgCl_2$; 5 mM dithiothreitol (DTT) and 300 μg/mL BSA), 4 μL of Buffer B (20 mM ATP), 0.5 μg plasmid pBR322, and 1U topoisomerase II α enzyme. To evaluate the inhibitory action of the andrographolide analogues, the compounds were dissolved in DMSO at different concentrations in a total volume 20 μL (final DMSO concentration was less than 0.1 %). The reaction mixture was incubated for 30 min at 37 °C, and terminated by adding 0.1 % SDS and 1 μL of 1 mg/mL proteinase K. The mixture was further incubated at 50 °C for 30 min. The products were subjected to electrophoresis using 1 % agarose gel

electrophoresis at 1.5 V/cm in TBE (Tris Borate EDTA) buffer. The gel was stained with 0.5 μg/mL ethidium bromide for 30 min and destained in distilled water for 30 min. The gel was visualized and quantified by Gel documentation (Chem-Genius, Syngene, UK). Inhibition of hTopo II α was estimated from the density of supercoiled plasmid in agarose gel.

Detection of apoptosis

The apoptotic cell death in HepG2 cells was first investigated by examining their morphological alterations. HepG2 cells (3×10^4 cells/mL) were seeded on sterile slides and exposed to andrographolide analogues for the indicated period. After incubation, the slides were gently washed with phosphate buffer saline (PBS) and stained with DAPI for 20 min at 37 °C. Cell morphologies were examined under a fluorescent microscope (HB-10101AF, Nikon, Japan) at magnification of 400X, and photographed. The apoptotic cells were characterized by nuclear condensation and fragmentation.

DNA fragmentation, a hallmark of apoptosis, was detected by using APO-BrdU™ TUNEL assay kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. Briefly, the HepG2 cells, plated in a 6-well plate (5.0×10^5 cells/well), were incubated at 37 °C for 24 h. Cells were then treated with andrographolide analogues for 24 and 48 h. After incubation, cells were trypsinized, re-suspended and incubated

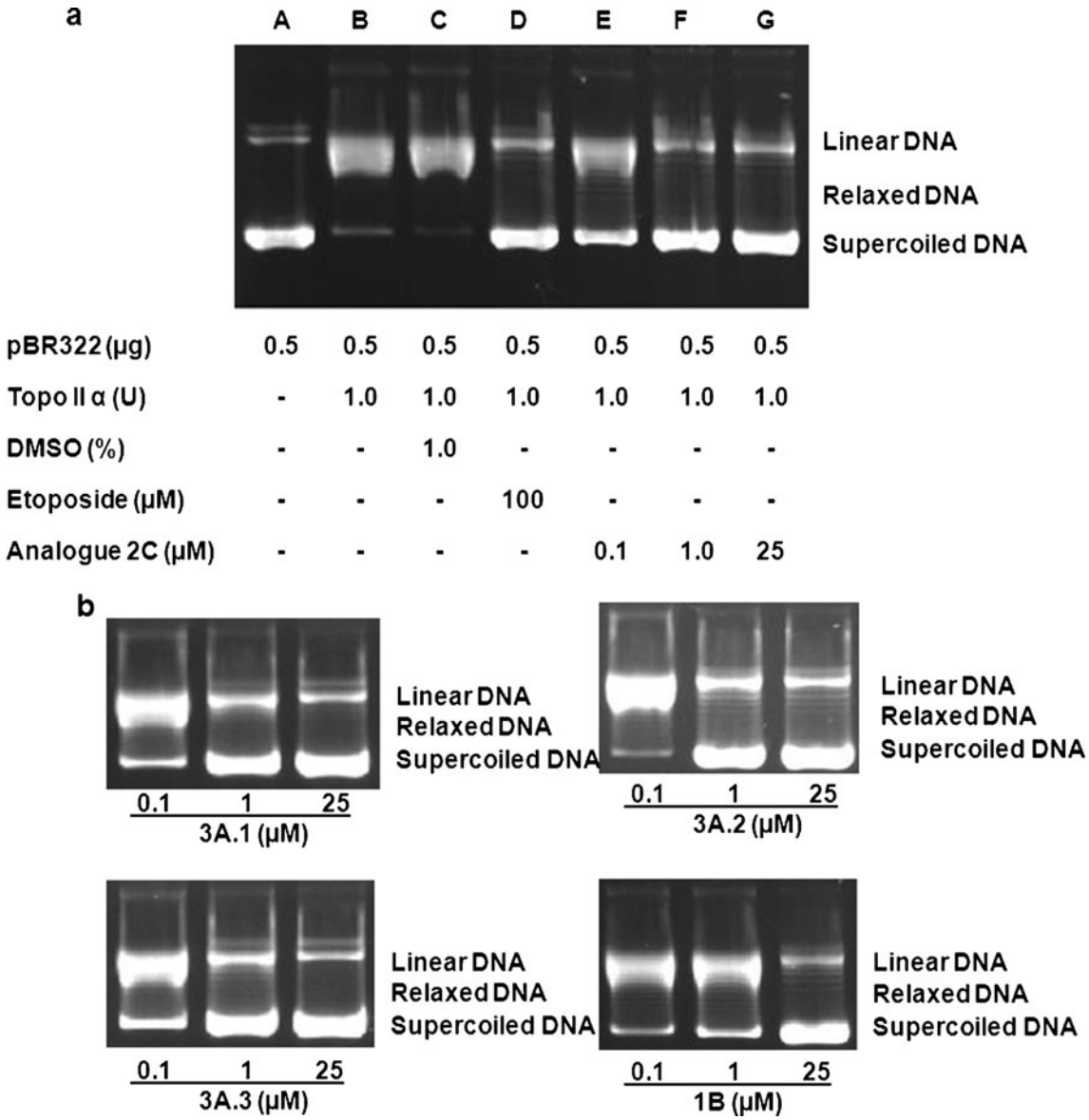

Fig. 2 **a.** Inhibitory effect of andrographolide analogue 2C on hTopo II α activity by the pBR322 DNA cleavage assay in cell free system**.** In lane C, DMSO (1 %) was used as a vehicle control. In lane D, Etoposide, a hTopo II inhibitor, at 100 μM was used as a positive control. Lanes E, F, G were andrographolide analogue 2C at various concentrations (0.1, 1, and 25 μM). **b.** Inhibitory effects of andrographolide analogues 3A.1, 3A.2, 3A.3 and 1B at 0.1, 1 and 25 μM on hTopo II α activity in cell free system

in 1 % paraformaldehyde on ice. The incubated cells were fixed in 70 % cold ethanol overnight. The fixed cells were incubated with DNA labeling solution (0.75 μL of Terminal-deoxynucleotidyl Transferase (TdT) enzyme in 10 μL TdT reaction buffer, 8 μL of BrdUTP, and 31.25 μl of H_2O) for 120 min at 37 °C. At the end of incubation, cells were re-suspended in antibody solution (5 μl of the Alexa Fluor® 488 dye-labeled anti-BrdU antibody and 95 μl of rinse buffer) and incubated for 30 min at room temperature. Propidium iodide/RNaseA mixture was added into cell suspension and further incubated for 30 min at room temperature. Solution was mixed gently every 5 min in dark. Twenty thousand events in all treatments sets were analyzed using BD FACS Canto TM flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis

Data were presented as means±SEM. Each value was obtained from at least three independent experiments.

Results

Synthesis of semi-synthetic andrographolide analogues

Figure 1 shows the semi-synthetic andrographolide analogues used in the experiment. The analogues were obtained after the modifications of both the core structure and functional groups of andrographolide. The analogue structure-1 contains the core structure of andrographolide. In the structure-2, the core structure was modified to contain double bond at C-14 and hydroxyl group (-OH) at C-12 whereas the structure 3 was modified to contain epoxide at C-17 position. At C-19 position, all analogues contained one of the following silicon based molecules including triisopropylsilyl (TIPS), *tert*-butyldiphenylsilyl (TBDPS), or *tert*-butyldimethylsilyl (TBS).

Inhibition of recombinant human topoisomerase II α activity by andrographolide analogues

The ability of andrographolide analogues to inhibit recombinant human topoisomerase II α activity was evaluated by measuring the extent of conversions of supercoiled pBR322 DNA plasmid to relaxed form (Fig. 2, Table 1). Andrographolide, the parent compound did not affect topoisomerase II α enzyme activity. Etoposide, a topoisomerase poison, was used as a positive control since, at 100 μM, it stabilizes the DNA cleavable complex as well as completely inhibits recombinant human topoisomerase II α activity. As shown in Fig. 2 and Table 1, andrographolide analogues 3A.1, 3A.2, 3A.3, 1B and 2C at concentration of 100 μM fully inhibited activities of topoisomerase II α enzyme.

Table 1 Inhibition of human topoisomerase II α activity by Andrographolide Analogues

Compounds	R_1	R_2	R_3	Topoisomerase II α Inhibition (%)
Androrapholide	H	H	H	None
Analogues				
1A	H	H	TBDPS	None
2A	H	H	TBDPS	18.2±0.10
3A.1	H	H	TBDPS	100.0±0.00
3A.2	H	Ac	TBDPS	100.0±0.00
3A.3	Ac	Ac	TBDPS	100.0±0.00
1B	H	H	TIPS	100.0±0.00
2B	H	H	TIPS	67.5±0.10
1C	Ac	Ac	TBS	None
2C	Ac	Ac	TBS	100.0±0.00
Etoposide				100.0±0.00

Data are means±SEM percent inhibition by andrographolide analogues at 100 μM on the activity of recombinant human topoisomerase II α. Etoposide was used as positive control and andrographolide for a negative control. TBDPS: *tert*-butyldiphenylsilyl, TIPS: triisopropylsilyl, TBS: *tert*-butyldimethylsilyl. Percent inhibition presented in μM was obtained from 3 independent experiments

To determine the potency of the active andrographolide analogues, percent inhibition of hTopo II α was tested at 0.1, 1 and 25 μM of various analogues. As shown in Table 2, analogues 3A.1, 3A.2, 3A.3 and 2C strongly inhibited the topoisomerase II α activity at concentration of 1 μM.

Anti-proliferative effect of andrographolide analogues

The analogues with potent topoisomerase II α inhibitory activity including analogues 3A.1, 3A.2, 3A.3, 1B, and 2C were further investigated for their anti-proliferative activities in four mammalian cell lines ($HepG_2$, Hela, CHO and UISO-BCA-1) using SRB assay. IC_{50} values of these analogues in four cell lines were estimated to be 4 to 7 μM (Table 3).

Table 2 Inhibition of human topoisomerase II α activity by andrographolide analogues

Compounds	Percent Inhibition		
	0.1 μM	1 μM	25 μM
3A.1	40.11±0.02	100±0.00	100±0.00
3A.2	37.11±0.02	100±0.00	100±0.00
3A.3	37.08±0.02	100±0.00	100±0.00
1B	15.4±0.03	15.6±0.02	100±0.00
2C	17.11±0.01	100±0.00	100±0.00

Data are means±SEM percent inhibition by andrographolide analogues at 0.1 μM, 1 μM and 25 μM. Percent inhibition presented in μM obtained from 3 independent experiments

Table 3 Cytotoxicity of Andrographolide analogues (IC_{50} values) in various mammalian cancer cell lines

Compounds	IC_{50} (μM)			
	HepG2	Hela	CHO	UISO-BCA1
Etoposide	2.61±0.28	0.92±0.06	46.77±1.46	6.17±0.46
Andrographolide	45.65±1.30	24.62±0.03	100±0.36	27.46±0.25
3A.1	5.95±0.19	4.74±0.06	6.30±0.06	4.91±0.04
3A.2	5.53±0.05	4.94±0.04	6.14±0.01	4.49±0.05
3A.3	5.50±0.04	4.85±0.04	6.00±0.03	4.44±0.01
1B	5.79±0.01	5.15±0.10	5.86±0.08	5.38±0.08
2C	5.57±0.04	5.06±0.13	6.03±0.01	5.24±0.03

IC_{50} value presented in μM was obtained from 3 independent experiments which were conducted in triplicates. Data show means±SEM

Interestingly, IC50 values of all these five andrographolide analogues on CHO cells were approximately 6 μM, which was much lower than that of etoposide (46 μM).

Apoptosis induced by andrographolide analogues

Andrographolide analogue 2C, which was one of the highest inhibitor on hTopo II α, was further investigated for its ability to induce cell death in HepG2 cells by using DAPI staining for DNA. As illustrated in Fig. 3, the analogue 2C effectively induced DNA fragmentation and condensation at 24 h. Induction of apoptosis in HepG2 cells was further confirmed by using flow-cytometric APO-BrdU™ TUNEL assay, which detected the fragmentation of DNA. Analog 2C (10 μM) markedly induced apoptosis of HepG2 cells by 24 h (Fig. 4). Percent cell death was approximately 78±0.02 %, which was much higher (about 15 folds) than those induced by the parent andrographolide and the positive control etoposide.

Discussion

The present study demonstrated that five out of nine andrographolide analogues exhibited potent inhibitory activities on topoisomerase II α in vitro. The active analogues were also cytotoxic to a number of mammalian cell lines including Hela, CHO, BCA1 and HepG2. In addition, these compounds induced apoptosis in HepG2 cells. This is the first report that andrographolide analogues inhibit recombinant human topoisomerase II α enzymes.

Topoisomerase inhibitors have been suggested as interesting anti-cancer drugs [1, 4]. In this study, we showed that andrographolide analogues 3A.1, 3A.2, 3A.3, 1B and 2C at 100 μM concentration effectively inhibited the activity of topoisomerase II α. Of note, these analogues are relatively more potent than etoposide, a specific topoisomerase II α inhibitor [3]. To our knowledge, there has been no report on the inhibitory action of andrographolide (diterpenoid lactone) as well as andrographolide analogues on topoisomerse IIα enzymes. In 2006, Miyata and coworkers [17] reported inhibitory action of twelve diterpene compounds derived from the roots of *Euphorbia kansui* on topoisomerase II enzymes. However, the structure of diterpene is different from diterpenoid lactone of andrographolide. In the present study, our analogues were obtained from the modifications on both the core structure and functional groups of andrographolide. The analogue structure-1 contains the core structure of andrographolide whereas the analogue structure-2 possesses double bond at C-14 and hydroxyl group (-OH) at C-12. On the other hand, the double bond at C-17 of the core structure was changed to epoxide in the analogue structure-3. At C-19 position, all analogues contained one of the following silicon based molecules including triisopropylsilyl (TIPS), tert-butyldiphenylsilyl (TBDPS), and

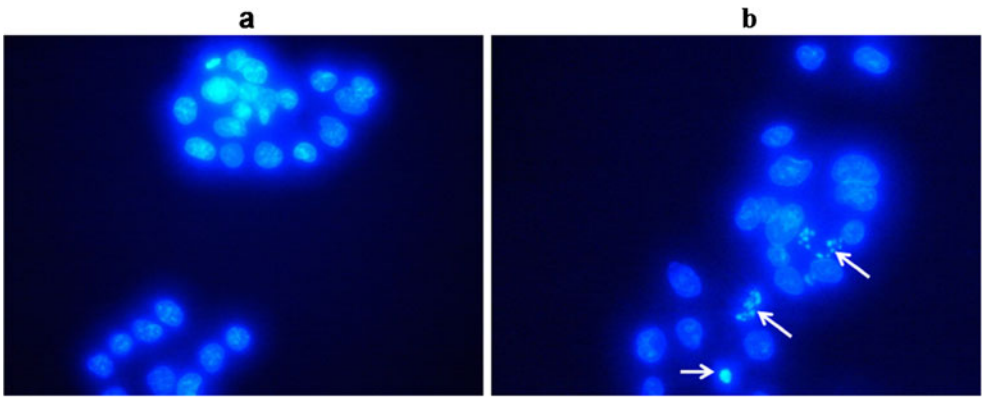

Fig. 3 Effect of andrographolide analogue 2C on condensation and fragmentation of DNA in $HepG_2$ cells which were treated with **a** vehicle (0.1 % DMSO) and **b** andrographolide analogue 2C (10 μM) for 24 h. Apoptotic cells were stained with DAPI and seen under fluorescence microscopy at magnification 400X. The arrow (→) represents apoptosis cells with nuclear condensation as well as fragmentation of DNA

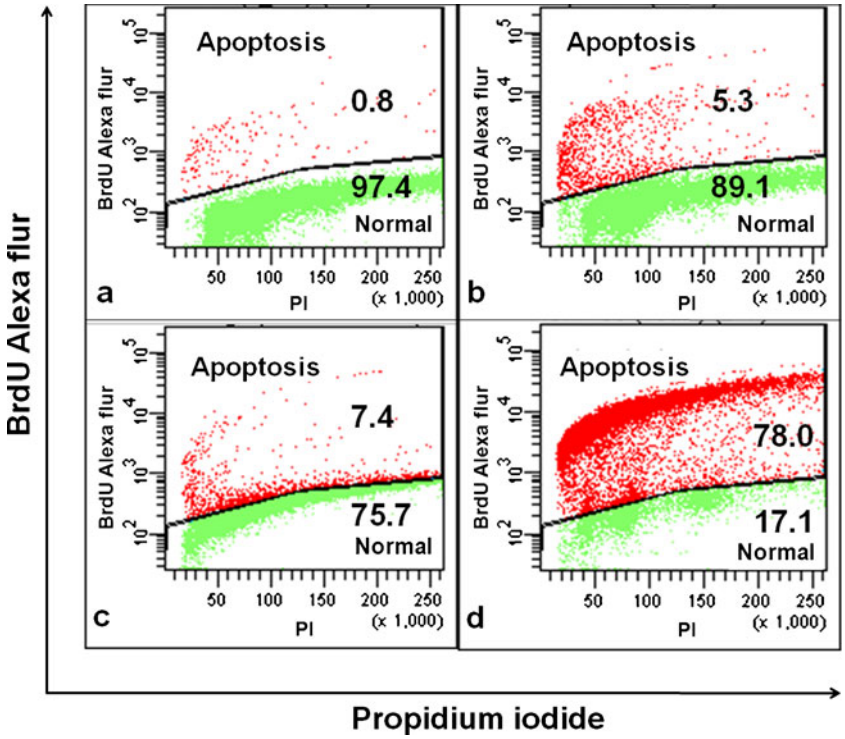

Fig. 4 FACS analysis showing apoptosis in HepG2 cells induced by andrographolide analogues. Andrographolide analogue 2C at 10 μM induced apoptotic cell death after treatment for 24 h, detected by TUNEL assay. Detection of apoptotic nuclei was done by using Flow-cytometric APO-BrdU™ TUNEL assay kit in 20,000 events. **a**: 0.1 % DMSO, **b**: 10 μM etoposide, **c**: 10 μM andrographolide and **d**: 10 μM andrographolide analogue 2C

tert-butyldimethylsilyl (TBS). The silicon base compounds have been widely used in synthetic organic chemistry to protect hydroxyl group. The inhibition of hTopo II α activity by our andrographolide analogues depends on both core structure and functional group at C-19 position. The presence of TBDPS group at C-19 on the compounds having different core structures (1A, 2A, 3A.1) caused different inhibitory activity on hTopo II α. The effect of core structure was also observed in the analogues 1C and 2C with the same TBS group at C-19. Thus, analogue 1C at 100 μM had no inhibitory activity whereas analogue 2C completely inhibited hTopo II α activity. Among the three core structures, the core structure 3 seems to be the most active moiety. Addition of acetyl group on the core structure (3A.2 and 3A.3) did not alter the inhibitory activity. These results indicate that the core structure of andrographolide in the analogues is essential for the inhibitory activity on hTopo II α. However, when the functional group of andrographolide at C-19 was changed to TIPS, the compounds with core structure 1 (1B) and core structure 2 (2B) showed increases in the inhibitory activities. The non-active analogue 1A became fully active when its functional group at C-19 was replaced by TIPS, i.e. analogue 1B. Likewise, the activity of 2A was substantially increased in 2B. When the activities among the compounds with core structure 1 were compared, only that containing TIPS (1B) inhibited the activity of hTopo II α. For the compounds with structure 2 (2A, 2B and 2C), all analogues inhibited hTopo II α enzyme at varying degree. Of these, analogue 2C containing TBS gave the highest inhibitory potency compared to 2A and 2B. These results indicated the importance of functional group at C-19 position of andrographolide analogues in providing the inhibitory activity against hTopo II α.

Topoisomerase II inhibitors have been used for treatment of a variety of cancers including lung cancer, leukemia, sarcoma, breast cancer and ovarian cancer [3]. In the present study, the anti-cancer potential of the andrographolide analogues were also observed. These compounds effectively inhibited proliferation of HepG2, Hela and CHO cell lines (Table 3), which have previously been reported to response to topoisomerase II inhibitors [12, 13, 19, 20]. Andrographolide analogues containing one of silicon based molecules (TIPS, TBDPS and TBS) at C-19 position in all 3 structures possess an anti-proliferative activity. The inhibition concentration 50 (IC_{50}) of the active andrographolide analogues ranged from 4–7 μM, which were much lower than that of the parent andrographolide, suggesting that the functional group at C-19 position is essential for the anti-proliferative properties on four mammalian cell lines. Recently, our groups have reported the importance of functional group containing silicon at C-19 position of the andrographolide analogue structure 1 in the anti-proliferation effect on a panel of cancer cells [9]. By using silicon, the increases in lipophilicity and stability of the compounds have been reported [21].

Etoposide is widely used for treatment of cancers by inhibiting hTopo II α activity [3]. However, there are a number of cancers that resist to etoposide treatment. In the present study, CHO was used since it is not sensitive to etoposide [18] and the parent form of andrographolide.

Interestingly, this cell line is sensitive to our andrographolide analogues. The mechanism involved in the resistance to anticancer drugs may, at least, be related to drug transport, drug-target interaction and drug detoxification [22]. It is possible that our analogues may have different inhibitory mechanism from that of etoposide. Currently, several etoposide analogues have been developed by modifying its structure to make a prodrug to improve its inhibitory activity in the resistant cells [23]. For the cells that are resistant to etoposide, our modified analogues may be useful as a prodrug.

Topoisomerase II inhibitors including doxorubicin, etoposide and teniposide have been widely used for treatment of leukemia [3]. The newly developed topoisomerase II inhibitors have been aimed for treatment of leukemia especially the most intensely investigated P388 (murine leukemia) and K562 (human chronic myelocytic leukemia) [24, 25]. P388 was also sensitive to our andrographolide analogue structure 1 as previously reported [9]. Since andrographolide analogue 2C strongly inhibited hTopo II α in vitro, the anti-proliferative effects of different analogues were examined in various types of cell lines. Results showed that the inhibitory potency of various analogues on proliferation of four cell lines used was not different. Since andrographolide has been reported to induce apoptosis in HepG2 cells [12, 13], this cell was chosen for further evaluation on the induction of apoptosis by our andrographolide analogues. Cells undergoing apoptosis demonstrate unique morphological and molecular markers including DNA fragmentation, chromatin condensation, and formation of apoptotic bodies. In the present study, apoptotic induction has been observed in HepG2 cells treated with compound 2C. This was evidenced by nuclear fragmentation and formation of granular apoptotic bodies (Fig. 3). Interestingly, a significant number of cells (78 %) containing DNA strand breaks were observed at 10 μM concentration of analogue 2C. The underlying mechanism of analogue 2C-induced HepG2 apoptotic cell death is under investigation.

In conclusion, we have semi-synthesized a new family of andrographolide analogues which exhibited potent inhibitory activity toward recombinant human topoisomerase II α, caused cytotoxicity in various types of mammalian cell lines, and induced apoptosis in HepG2 cells. The structure-activity relationship studies on the inhibition of recombinant human topoisomerase II α activity revealed that both core structure and silicon based functional group at C-19 position of andrographolide are important for the inhibition and anti-proliferation of four types of mammalian cell lines. The andrographolide analogue 2C is, therefore, a promising compound for further development as an anticancer drug.

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Conflict of interest None.

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