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Synthesis, cytotoxicity, and structure–activity relationship (SAR) studies of andrographolide analogues as anti-cancer agent

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ABSTRACT

A series of analogues of andrographolide, prepared through chemo-selective functionalization at C14 hydroxy, have been evaluated for in vitro cytotoxicities against human leukemic cell lines. Two of the analogues (**6a**, **9b**) exhibited significant potency. Preliminary studies on structure–activity relationship (SAR) revealed that the α-alkylidene-γ-butyrolactone moiety of andrographolide played a major role in the activity profile. The structures of the analogues were established through spectroscopic and analytical data

Mankind has benefited enormously through drugs discovered from natural resources. Roughly around 50% of the currently used anti-cancer drugs were discovered from studies on these secondary metabolites.[1] Therefore, the development of novel therapeutic agents relies largely on the library of natural products and/or natural product based molecules. Primarily this involves synthetic transformation of the lead molecule(s) allowing rapid access of complex molecular entities with diversified structures.

The herb *Andrographis paniculata* Nees (Acanthaceae) is popular in India, China and other Asian countries due to its different uses in traditional medicine. The metabolites isolated from this herb are mainly diterpenoids, flavonoids and sterols. The major constituent is andrographolide, a labdane diterpene. Extracts of this herb and its various constituents are reported to have a broad range of biological activities, such as antidiabetic,[2] antimalarial,[3] antibacterial,[4] antiinflammatory,[5] hepatoprotective,[6] and others.[7] Besides these, the aerial parts of *A. paniculata* have been traditionally used as medicine to treat cancer.[8] In recent past, the potent anti-cancer activities of andrographolide have indeed been established.[9] However, despite its impressive biological activities, the major drawback of andrographolide is poor water solubility making it difficult to prepare formulations for clinical use. So, various semi-synthetic analogues are being developed[10] and evaluated in order to find out a better lead. Notably, Jada et al. recently reported[10c] that 14-acetylandrographolide is more potent (in vitro) against leukemia compared to andrographolide. In another report,[10d] DRF 3188, a novel derivative of andrographolide having α,β-unsaturated ester side chain at C14, was shown to have better anti-cancer activity than the parent molecule.

During the course of studies on structural modifications of bioactive natural products for value addition,[11] we have chosen andrographolide for chemo-selective functionalization at C14 hydroxy in order to develop the pharmacophore(s) possessing better apoptotic index than andrographolide. Andrographolide **1** contains (a) two double bonds (Δ^{12} and $\Delta^{8(17)}$), (b) an α-alkylidene-γ-butyrolactone, and (c) three hydroxyl groups at C3, C14, and C19. However, we opted for selective functionalization at C14 hydroxyl group which is allylic in nature. The idea was that transformation of andrographolide into an appropriate ester derivative (at C14) should increase the solubility as well as activity. If esterases cleave

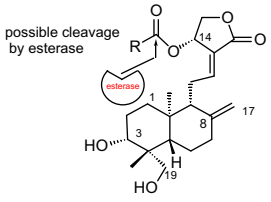

Figure 1. Ester analogue of andrographolide and possible site of cleavage.

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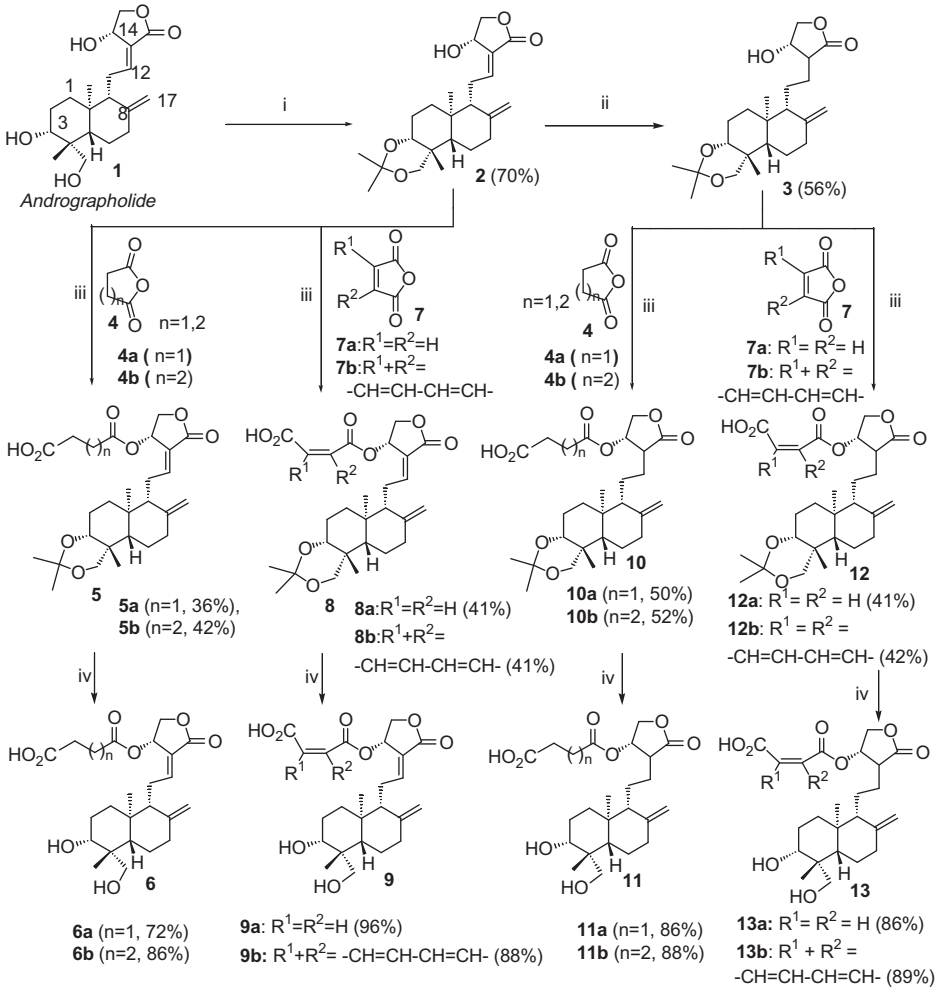
the ester bond under physiological conditions releasing andrographolide in vivo, the ester analogue may be considered as a prodrug[12] (Fig. 1). In pursuance of this goal, we have synthesized a new class of andrographolide analogues having ester linkage at C14 connected through a spacer with a terminal acid moiety and evaluated their cytotoxicities (in vitro) in human leukemic cell lines. The results obtained so far are reported herein.

Andrographolide was isolated in high yield (0.54%) from the leaves of *A. paniculata* and used as starting material for derivatization. The synthetic pathways used in the present study are depicted in Scheme 1. Initially, the hydroxyl groups at C3 and C19 of andrographolide were protected to furnish 3,19-isopropylideneandro- grapholide **2**, which served as the key intermediate in the preparation of analogue library. Synthesis of the desired ester **5a** was smoothly achieved by treatment of intermediate **2** with succinic anhydride **4a** in dry dichloromethane in the presence of catalytic amount of 4-dimethylaminopyridine (DMAP) at room temperature. Encouraged by the result, the higher homologue **5b** was prepared (42%) using glutaric anhydride (**4b**). Removal of the isopropylidene moiety of the products **5a** and **5b** was carried out by exposure to aqueous acetic acid (3:7) affording the targeted analogues **6a** and **6b** in yields of 72% and 86%, respectively. In the next phase, this reaction protocol was applied for the installation of α,β-unsaturated ester at C14 of intermediate **2** using maleic and phthalic anhydride (**7a** and **7b**). The expected products **8a** and **8b** were produced with moderate yields (41% in each case). Subsequent removal of the isopropylidene group from the intermediates **8a** and **8b** furnished (96% and 88%) the targeted products **9a** and **9b**, respectively.

In order to understand the role of the α-alkylidene part attached to the γ-butyrolactone ring of andrographolide for structure–activity relationship (SAR) studies, we planned to prepare the corresponding saturated derivatives. Accordingly, chemo-selective reduction of the double bond (Δ^{12}) of intermediate **2** was accomplished (56% yield) using sodium borohydride in methanol instead of the costly reagent $NiCl_2$ as used earlier.[13] Next, we adopted our earlier strategy for attachment of ester side chain at C14 of the saturated intermediate **3** using succinic, glutaric, maleic and phthalic anhydrides (**4a**, **4b**, **7a**, and 7**b**), which resulted in the formation of the intermediate products **10a**, **10b**, **12a**, and **12b**, respectively. Finally, synthesis of the targeted saturated analogues **11a**–**b** and **13a**–**b** was performed with good yields (86–89%) by deprotection of the isopropylidene group as depicted in scheme 1. Purifications of the final analogues were performed through usual silica gel chromatography followed by semipreparative HPLC.[14] The structures of the products[15] were established based on spectroscopic (IR, NMR and mass spectra) and analytical data.[16]

In vitro screening and structure–activity relationship (SAR) studies: In vitro screening was carried out in selected human leukemic cell lines (U937, K562, and THP1); parallel to this, cytotoxicities against normal cell lines (NIH3T3 and L132) were also checked. The



Scheme 1. Synthesis of C14-ester derivatives of andrographolide. Reagents and conditions: (i) 2,2-dimethoxypropane, *p*-TsOH (cat.), acetone, reflux, 2 h ; (ii) sodium borohydride, MeOH, 0 °C to rt, 1 h ; (iii) succinic or glutaric or maleic or phthalic anhydride (**4a** or **4b** or **7a** or **7b**), 4-dimethylaminopyridine (cat.), DCM, rt, 4–6 h ; (iv) acetic acid/water (7:3), rt, 30 min.

Table 1
Evaluation of in vitro cell growth inhibitory effects of andrographolide analogues in various cell lines[a,b,c]

Entry	Compound	Cell growth inhibition in terms of IC_{50} (μM)				
		U937	THP1	K562	L132	NIH3T3
1	**5a**	19.53	10.01	31.39	21.72	36.69
2	**5b**	15.63	26.15	36.46	15.13	ND
3	**6a**	5.47	5.84	25.30	8.50	10.20
4	**6b**	9.76	7.53	19.55	19.50	22.06
5	**8a**	ND	14.84	ND	31.11	ND
6	**8b**	14.55	12.16	26.92	18.84	16.03
7	**9a**	9.72	7.77	41.95	12.78	11.33
8	**9b**	6.27	5.68	41.12	10.91	8.01
9	**11a**	NA	ND	ND	ND	ND
10	**11b**	NA	NA	NA	NA	NA
11	**13a**	NA	NA	NA	NA	NA
12	**13b**	NA	NA	NA	NA	NA
13	Andrographolide	12.87	6.69	41.85	53.8	44.5

[a] U937, THP1, and K562 are human leukemic cell lines; whereas L132 and NIH3T3 are normal cell lines.
[b] NA means not active and IC_{50} >50 μM.
[c] ND means not detected.

standard MTS-PMS cell viability assay[17] was carried out[18] for determining the antiproliferative activities of the compounds synthesized. The results of the studies are summarized in Table 1. Interestingly, two of the synthesized analogues (**6a** and **9b**) inhibited the proliferation of U937 and THP1 cells by 50% or more at concentration less than 6.5 μM (Table 1, entries 3 and 8), while others displayed the cytotoxicity at higher concentrations. Among the analogues synthesized, the most active agent was **6a**, while **9b** was slightly weaker (Table 1, entry 3 vs 8). Thus, analogue **6a** emerged as the best lead candidate.

It is interesting to note that activities of the unsaturated (Δ^{12}) compounds (**6a–b** and **9a–b**) are lost when the double bond between C12 and C13 (α-alkylidene) is reduced leading to the formation of analogues **11a–b** and **13a–b** (Table 1, entries 9–12). This observation clearly indicates that the presence of the α-alkylidene part attached to the γ-butyrolactone ring is crucial for exhibiting cytotoxicity. Possibly, the activity of andrographolide and their unsaturated analogues (**6a–b** and **9a–b**) is associated with their ability to promote alkylation of biological nucleophiles (e.g., enzymes) through Michael addition[19] to the α-alkylidene-γ-butyrolactone moiety. Furthermore, the results of SAR studies show that increase in lipophilicity by protection of 3,19-hydroxyl groups of andrographolide has detrimental effects on the activity profile (Table 1, entries 1 vs 3, 2 vs 4, 5 vs 7, 6 vs 8). Andrographolide itself was found to be significantly active against THP1 cells but only moderately so against U937 cells (Table 1, entry 13). The IC_{50} values of analogues **6a** and **9b** appear to be lower compared to andrographolide against both cancer and normal cell lines. Thus, the screening studies indicate that analogues **6a** and **9b** are more cytotoxic than andrographolide but have lower specificity (toward normal cell lines) than andrographolide. Further optimization of these structures (**6a** and **9b**) is being carried out to remove this lacuna.

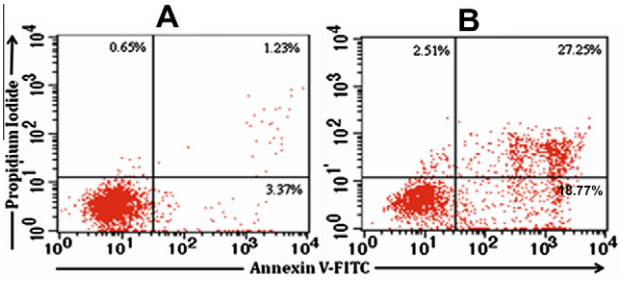


Figure 2. Induction of apoptosis by analogue **6a**: Untreated U937 cells (A) following treatment with **6a** (5.47 μM) for 24 h (B) was co-stained with Annexin V-FITC and propidium iodide, and analyzed by flow cytometry.

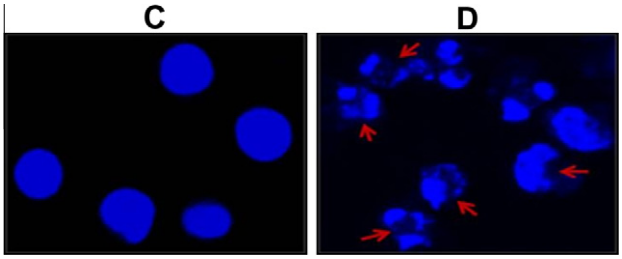


Figure 3. Morphological and nuclear changes of U937 cells upon treatment with analogue **6a**; untreated U937 cells (C) following treatment with **6a** (5.47 μM) for 24 h (D) stained with Hoechst 33258 and observed under a Leica confocal microscope (100×).

In addition, evaluation of the induction of apoptosis by analogue **6a** was carried out using flow cytometry[20] and confocal microscopy.[21] Cells (U937) treated with an IC_{50} concentration (5.47 μM) of **6a** for 24 h showed Annexin-V positivity that was ten fold higher than untreated cells (46.02% vs 4.6%, Fig. 2). The confocal images of cells similarly treated with **6a** and stained with Hoechst 33258, showed formation of apoptotic bodies along with membrane blebbing, whereas untreated cells had intact nuclei (Fig. 3).

In conclusion, in our endeavor to develop promising anti-cancer agent(s) based on andrographolide, we have synthesized a new family of andrographolide analogues and evaluated their in vitro activities against different leukemic cell lines. The structure–activity relationship (SAR) studies indicated that the major role is played by the α-alkylidene-γ-butyrolactone moiety present in andrographolide. Two of the analogues (**6a** and **9b**) were found to be promising for further structural modifications guided by the information as obtained in the SAR studies. Studies in this direction are in progress.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.126.

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14. HPLC was performed on RP C18 column (300 mm × 7.8 mm, 7.0 μm) using methanol/acetonitrile/water/acetic acid (49:10:40:1 or 34:10:55:1) as mobile phase; flow rate = 3 ml/min; t_R = 6.8, 8.9, 16.9, 17.2, 17.8 and 18.4 min (for compounds **6a**, **6b**, **9a**, **9b**, **13a** and **13b**, respectively).
15. All the synthesized final analogues were generally preserved at 0 to −4 °C and remained stable for months. Even allowing them to stand at rt for several weeks did not lead to any decomposition (HPLC analysis). Besides, few of the final analogues (**6a**, **9a**, **11a**–**b**, **13a**) were found to be soluble in chloroform but all of them were fairly soluble in methanol and dimethyl sulfoxide (DMSO) as well.
16. *General procedure for the synthesis of acid **5**/**8**/**10**/**12** (step iii)*: To a well stirred solution of compound **2**/**3** (0.512 mmol) in dry dichloromethane (8 mL) were added acid anhydride **4**/**7** (0.615 mmol) and a catalytic amount (4 mg) of 4-dimethylamino-pyridine (DMAP). The whole mixture was stirred under argon atmosphere at room temperature for 4–6 h. The solvent was evaporated under reduced pressure and the residue was purified through silica gel (100–200 mesh) column chromatography (ethyl acetate–petroleum ether) to afford the desired product **5**/**8**/**10**/**12**.
3,19-Isopropylideneandrographolide-14-α-O-succinate **5a**: gum, 36% yield; IR (neat): 3421, 2936, 1738, 1676 cm^{-1}; ^1H NMR (CD_3OD, 300 MHz): δ 6.98 (1H, t, J = 6.3 Hz), 6.04 (1H, d, J = 5.1 Hz), 4.93 (1H, s), 4.63–4.57 (2H, m), 4.30 (1H, d, J = 11.1 Hz), 4.04 (1H, d, J = 11.7 Hz), 3.54–3.51 (1H, m), 3.20 (1H, d, J = 11.7 Hz), 2.64 (4H, s), 2.57–2.52 (2H, m), 2.47–2.43 (1H, m), 2.07–1.97 (3H, m), 1.83–1.78 (3H, m), 1.41 (3H, s), 1.36–1.28 (6H, m), 1.22 (3H, s), 0.98 (3H, s); ^{13}C NMR (CD_3OD, 75 MHz): δ 175.5, 173.6, 171.4, 151.7, 149.0, 125.6, 109.3, 100.3, 78.0, 73.1, 69.5, 64.9, 57.1, 53.4, 39.5, 38.9, 38.7, 35.5, 29.9, 29.6, 27.5, 26.5, 26.2, 25.7, 24.3, 16.5; ESI-MS: m/z 513.01 $[M+Na]^+$. Anal. Calcd for $C_{27}H_{38}O_8$: C, 66.10; H, 7.81. Found: C, 66.04; H, 7.86.
*General procedure for the synthesis of acid **6**/**9**/**11**/**13** (step iv)*: To a well stirred solution of ester intermediate **5**/**8**/**10**/**12** (0.50 mmol) in 1,4-dioxane (3 mL) was added a solution (3 mL) of acetic acid/water (7:3, v/v). The whole reaction mixture was allowed to stir at room temperature for 30 min and the solvents were evaporated under reduced pressure. The residue was mixed with water (10 mL) and extracted with ethyl acetate (3 × 20 mL). The combined organic extracts were washed with brine (10 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified through silica gel (100–200 mesh) column chromatography (ethyl acetate–petroleum ether) followed by HPLC (see Ref. 14 for details).
Andrographolide-14α-O-succinate **6a**: gum, 72% yield; IR (neat): 3425, 2938, 1739 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 7.06 (1H, t, J = 6.4 Hz), 5.94 (1H, d, J = 5.1 Hz), 4.89 (1H, s), 4.56 (1H, dd, J = 11.5, 6.4 Hz), 4.53 (1H, s), 4.25–4.17 (2H, m), 3.93 (2H, br), 3.52–3.49 (1H, m), 3.33 (1H, d, J = 10.8 Hz), 2.74–2.64 (4H, m), 2.50–2.39 (3H, m), 2.00–1.96 (1H, m), 1.82–1.73 (5H, m), 1.30–1.13 (6H, m), 0.67 (3H, s); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 175.7, 172.1, 169.2, 150.9, 146.5, 123.6, 108.7, 80.2, 71.5, 68.0, 64.0, 55.7, 54.9, 42.4, 38.7, 37.5, 36.7, 28.7, 28.6, 27.8, 25.1, 23.6, 22.6, 14.9; ESI-MS: m/z 473.14 $[M+Na]^+$. Anal. Calcd for $C_{24}H_{34}O_8$: C, 63.98; H, 7.61. Found: C, 64.02; H, 7.56.
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18. *Method of MTS-PMS cell viability assay*: The anti-proliferative activity of andrographolide and its analogues were evaluated in U937, THP1, K562, NIH3T3 and L132 cells using MTS-PMS assay (see Ref. 17 for details). Briefly, cells (2.5–5.0 × 10^4/200 μL) were seeded in 96-well tissue culture plates and incubated with compounds (0–50 μM) for 48 h at 37 °C, 5% CO_2. Following treatment, MTS [3-(4,5-dimethylthiazol-2-yl)5-(3-carboxymethoxyphenyl)-2-(4-sulphonyl)-2*H*-tetrazolium, inner salt] (2.0 mg/ml) and PMS (phenazine methosulphate) (0.92 mg/ml) were added in a ratio of 10:1 (20 μL per well). After incubation for 3 h at 37 °C, the resulting absorbances at 490 nm were measured in an ELISA reader. The specific absorbance that represented formazan production was calculated by subtraction of background absorbance from total absorbance. The mean % viability was calculated as follows: *Mean specific absorbance of treated cells* × *100/Mean specific absorbance of untreated cells*
The results were expressed as IC_{50} values, that is, the concentration that inhibited 50% of cell growth, enumerated by graphic extrapolation using Graph pad prism software (version 5).
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20. *Flow cytometric analysis*: Double staining for Annexin V-FITC and propidium iodide (PI) was performed. Briefly, U937 cells were incubated without or with **6a** (IC_{50} = 5.47 μM) for 24 h at 37 °C, 5% CO_2. Cells were then washed twice in phosphate buffered saline (PBS, 0.02 M, pH 7.2) and resuspended in Annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Annexin V-FITC and propidium iodide were then added according to the manufacturer's instructions and incubated for 15 min in the dark at 25 °C. Data was acquired using a FACS Calibur flow cytometer and analyzed with Cell Quest Pro software.
21. *Confocal microscopy*: Apoptotic cells were characterized by nuclear condensation of chromatin and/or nuclear fragmentation. Briefly, U937 cells were incubated with an IC_{50} concentration (5.47 μM) of **6a** (for 24 h), washed with ice cold PBS and stained with Hoechst 33258 (10 μg/ml, 30 min). The cells were mounted on poly L-lysine coated slides and analyzed in a laser scanning confocal microscope (Leica TCS SP2 system, Leica microsystem, Heidelberg, Germany; 100×). At least 20 microscopic fields were observed for each sample.