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Synthesis and structure–activity relationships of andrographolide analogues as novel cytotoxic agents☆

Srinivas Nanduri,[a,*] Vijay Kumar Nyavanandi,[a] Siva Sanjeeva Rao Thunuguntla,[a] Sridevi Kasu,[a] Mahesh Kumar Pallerla,[a] P. Sai Ram,[a] Sriram Rajagopal,[b] R. Ajaya Kumar,[b] Rajagopalan Ramanujam,[b] J. Moses Babu,[c] Krishnamurthi Vyas,[c] A. Sivalakshmi Devi,[c] G. Om Reddy[c] and Venkateswarlu Akella[a]

[a]*Discovery Chemistry, Dr. Reddy's Laboratories Ltd, Discovery Research, Bollaram Road, Miyapur, Hyderabad 500 049, India*
[b]*Discovery Biology, Dr. Reddy's Laboratories Ltd, Discovery Research, Bollaram Road, Miyapur, Hyderabad 500 049, India*
[c]*Analytical Research, Dr. Reddy's Laboratories Ltd, Discovery Research, Bollaram Road, Miyapur, Hyderabad 500 049, India*

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Abstract—Andrographolide **1**, the cytotoxic agent of the plant *Andrographis paniculata* was subjected to semi-synthetic studies leading to the preparation of a number of potent and novel analogues. Of the analogues synthesized, while 8,17-epoxy andrographolide **6** retained the cytotoxic activity of **1**, ester derivatives of **6** exhibited considerable improvement in activity. Lower activity was observed when the epoxy moiety in the triacetate **9**, derived from **6** was modified. Synthesis and structure–activity relationships are discussed.

1. Introduction

Andrographolide **1** (Fig. 1), is the major labdane diterpenoidal constituent of the plant *Andrographis paniculata* (family Acanthaceae),[1] which is used extensively in the traditional systems of Indian and Chinese medicine.[2,3] Extracts of the plant and their constituents are reported to exhibit a wide spectrum of biological activities of therapeutic importance including antibacterial,[4] antiinflammatory,[5] antimalarial,[6,7] immuno stimulant,[8] hepatoprotective,[9] and antithrombotic[10] properties. Potent antiHIV activity of the succinoyl derivatives of **1** are described.[11,12] The interesting cytotoxic and antitumor activities of the extracts and the constituents of the plant have been explored by several researchers in recent years. Potent cell differentiation inducing activity on mouse myeloid leukemia (M1) cells by the methanolic extract of *A. paniculata* and its constituents is reported.[13] Furthermore, an alcoholic extract of the plant

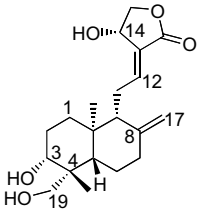

Figure 1. Andrographolide (**1**).

and its major constituent **1** have been shown to affect the cell cycle progression in prostate and breast cancer cell lines.[14] Identification of **1** as a cytotoxic agent against mouse[15] and human cancer cell lines[16] through bioactivity-guided chromatographic fractionation are reported. A thorough study on the anticancer and immunomodulatory potential of **1** is recently reported.[17] However, no systematic attempt has been made to improve the cytotoxic activity of **1** and to synthesize novel and potent agents, except for a report on the antitumorigenic properties of alkanoyl derivatives[18] of **1**. In this paper, the results on the design, synthesis, and biological evaluation of new analogues of **1** as potent cytotoxic agents are described.

Keywords: Andrographolide; *Andrographis paniculata*; Cytotoxic agents.
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*Corresponding author. Tel.: +91-40-23045439; fax: +91-40-23045438; e-mail: nandurisrinivas@drreddys.com

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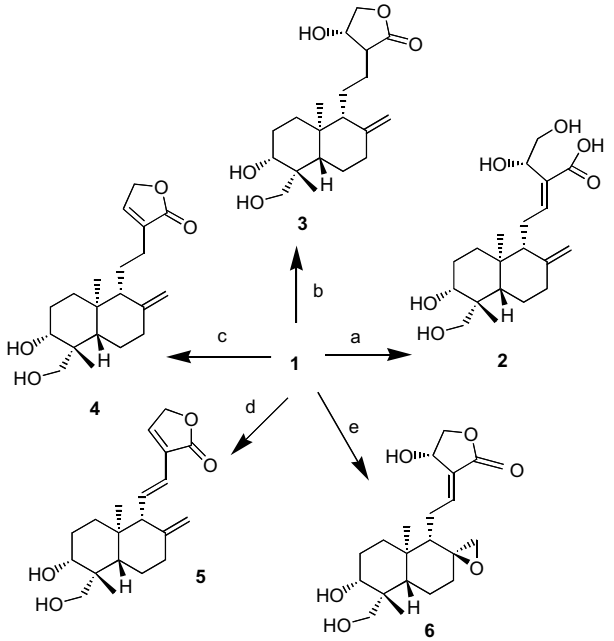
2. Chemistry

The structure of andrographolide contains (1) an α-alkylidene γ-butyrolactone moiety, (2) two olefin bonds $\Delta^{8(17)}$ and $\Delta^{12(13)}$, and (3) three hydroxyls at C-3, C-19, and C-14. Of the three hydroxyl groups, the one at C-14 is allylic in nature, and the others at C-3 and C-19 are secondary and primary, respectively. The derivatives of andrographolide were synthesized by modifying the above structural features.

In order to determine the importance of lactone moiety of andrographolide **1** for cytotoxic activity, it was opened to yield andrographolic acid **2** by reported procedure.[19] To understand the role of the exocyclic double bond ($\Delta^{12(13)}$) in **1**, **3** was prepared by selectively reducing the conjugated double bond. To further study, the role of allylic hydroxyl at C-14 and the conjugated double bond, **4** and **5** were prepared, in which the exocyclic double bond ($\Delta^{12(13)}$) isomerized to the endocyclic double bond ($\Delta^{13(14)}$), with the simultaneous removal of C-14 hydroxyl. Compounds **4** and **5** were synthesized from the triacetyl derivative of **1** by reported procedures.[20] On the other hand, selective epoxidation of the exocyclic double bond ($\Delta^{8(17)}$) with *m*-CPBA led to **6** (Scheme 1).

In vitro cytotoxic activity screening of the above analogues showed that while **2**–**5** demonstrated loss of activity, **6** did not exhibit any significant difference in its activity compared to **1** (discussed in biological results).



Scheme 1. Synthesis of compounds **1**–**6**. Reagents and conditions: (a) (i) Aq 1 N NaOH/reflux, 1 h, (ii) 6 N HCl, 70%[19]; (b) (i) 2,2-dimethoxy propane/benzene and DMSO, PPTS/reflux, 2 h, 95%, (ii) $NaBH_4$, MeOH/rt, 1 h, 85%, (iii) AcOH and H_2O (7:3)/rt, 15 min, 90%; (c) (i) $Ac_2O/ZnCl_2$/rt, 1 h, 97%, (ii) $NaBH_4$, MeOH/rt, 1 h, 85%, (iii) 10% methanolic HCl/rt, 8 h, 80%[20]; (d) (i) $Ac_2O/ZnCl_2$/rt, 1 h, 97%, (ii) dry pyridine/reflux, 4 h, 80%, (iii) 10% methanolic HCl/rt, 8 h, 80%[20]; (e) m-CPBA/$CHCl_3$/MeOH, rt, 6 h, 90% (isomeric mixture).

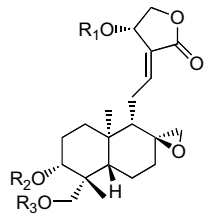
As **6** retained the cytotoxic activity of **1** and possesses a C-8 epoxy moiety as structural novelty, it was taken as a lead structure for further modifications aimed at understanding the contribution of the three hydroxyl groups. A number of novel ester derivatives of **6** were designed and synthesized to achieve this objective. Table 1 includes the novel ester derivatives synthesized and their syntheses are given in Schemes 2–4.

Thus, the three hydroxyls of **6** at C-14, C-3, and C-19 were acylated simultaneously or selectively. The triacetyl derivative **9** was synthesized by heating **6** in acetic anhydride. The structure of **9** was elucidated by spectral studies and finally confirmed by single crystal X-ray diffraction studies.[21] The perspective view of the molecule is shown in Figure 2. Both the six-membered rings adopt the chair conformation, whereas the five-membered ring is in an envelope conformation. Van der Waals contacts stabilize the molecules in the lattice. The absolute stereochemistry at C-4, C-9, and C-10 was assumed to be the same as that observed in the *ent*-labdane structure.[22,23] (Crystallographic data for **9** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number 222220.) Based on this information, the stereochemistry at C-8 and C-14 have been determined to be *S*. To examine the role of the carbon chain length in the ester functionality of **9**, the corresponding tripropionyl derivative **10** was synthesized by heating **6** in propionic anhydride. Compounds **9** and **10** can also be synthesized by selectively epoxidizing the $\Delta^{8(17)}$ double bond of the corresponding ester derivative of **1**. The 3,19-diacetyl derivative **14** was synthesized by this method (Scheme 2).

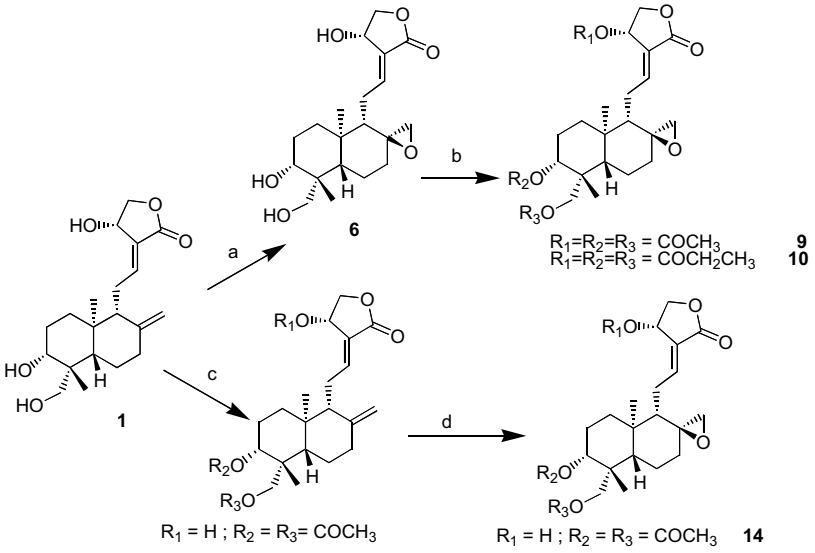
Selective esterification of the allylic alcohol at C-14 was achieved by protecting the C-3, C-19 hydroxyls on the decalin system with an isopropylidene moiety and subsequently modifying the C-14 alcohol (Scheme 3). Thus, the 14-*O*-acetyl derivative was obtained by heating 8,17-epoxy-3,19-isopropylidene andrographolide **7** in the corresponding anhydride. 14-*N*-Boc-glycinyl and cinnamoyl esters were prepared by treating **7** with the appropriate acyl carbonates (generated by the addition of ethyl chloroformate to the corresponding acids). Selective removal of C-3, C-19 isopropylidene protection in the above derivatives resulted in the formation of compounds having ester groups at C-14 and hydroxyls at C-3 and C-19. Thus, derivatives **11**, **16**, and **19** were synthesized. Synthesis of derivatives **17**, **18**, and **20**–**23** with novel ester linkages at C-14 and alkyl esters at C-3 and C-19 was carried out by deprotection of the C-3, C-19-isopropylidene group followed by transformation of the C-3 and C-19 hydroxyls into the alkyl esters by heating in the corresponding anhydrides.

Synthesis of derivatives **12**, **13**, and **15** (Scheme 4) having either acyl or hydroxyl groups at C-14, C-3, and C-19 was achieved by routine selective acylations of the hydroxy groups present in **6**. Thus, the acetyl derivatives were prepared by heating 8,17-epoxy-19-trityl andrographolide **8** with acetic anhydride. The 3-mono-acetyl and 3,14-diacetyl derivatives formed were separated by chromatography. The 3,14-dipropionyl derivative was pre-

Table 1. Novel ester derivatives of epoxy andrographolide **6**



S. No.	R_1	R_2	R_3
6	H	H	H
9	$COCH_3$	$COCH_3$	$COCH_3$
10	$COCH_2CH_3$	$COCH_2CH_3$	$COCH_2CH_3$
11	$COCH_3$	H	H
12	H	$COCH_3$	H
13	$COCH_3$	$COCH_3$	H
14	H	$COCH_3$	$COCH_3$
15	$COCH_2CH_3$	$COCH_2CH_3$	H
16	$COCH_2NHBoc$	H	H
17	$COCH_2NHBoc$	$COCH_3$	$COCH_3$
18	$COCH_2NHBoc$	$COCH_2CH_3$	$COCH_2CH_3$
19	$COCH{=}CH{-}C_6H_5$	H	H
20	$COCH{=}CH{-}C_6H_5$	$COCH_2CH_3$	$COCH_2CH_3$
21	$COCH{=}CH{-}(4\text{-}OCH_3)C_6H_4$	$COCH_2CH_3$	$COCH_2CH_3$
22	$COCH{=}CH\text{-}(3,4\text{-di } OCH_3)C_6H_3$	$COCH_2CH_3$	$COCH_2CH_3$
23	$COCH{=}CH\text{-}(3,4\text{-}OCH_2O\text{-})C_6H_3$	$COCH_2CH_3$	$COCH_2CH_3$



Scheme 2. Synthesis of compounds **9**, **10**, and **15**. Reagents and conditions: (a) m-CPBA/DCM/MeOH/rt, 8 h, 90% (isomeric mixture); (b) acetic or propionic anhydride/reflux, 30 min, 70% (required isomer); (c) acetic acid/70 °C, 6 h, 40%[18]; (d) m-CPBA/DCM/rt, 4 h, 70% (required isomer).

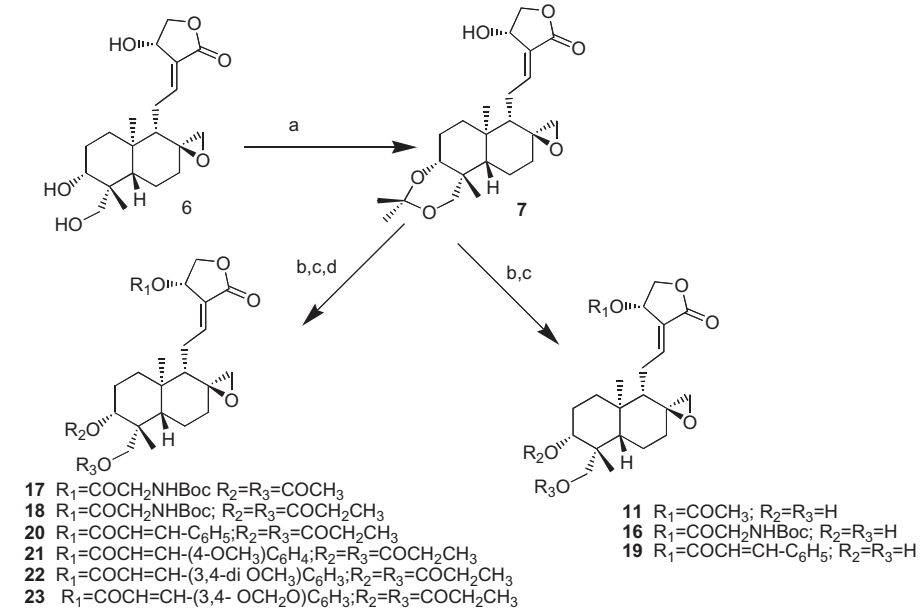
pared by heating **8** in propionic anhydride. The 19-trityl group in the above derivatives was selectively deprotected with formic acid in dichloromethane, thus yielding derivatives **12**, **13**, and **15**.

In addition to the above syntheses, in order to examine the role of the C-8, C-17 epoxy moiety of the new derivatives in exhibiting cytotoxic activity, modifications on the epoxy moiety in the above series was performed. These changes were made in **9** (Scheme 5).

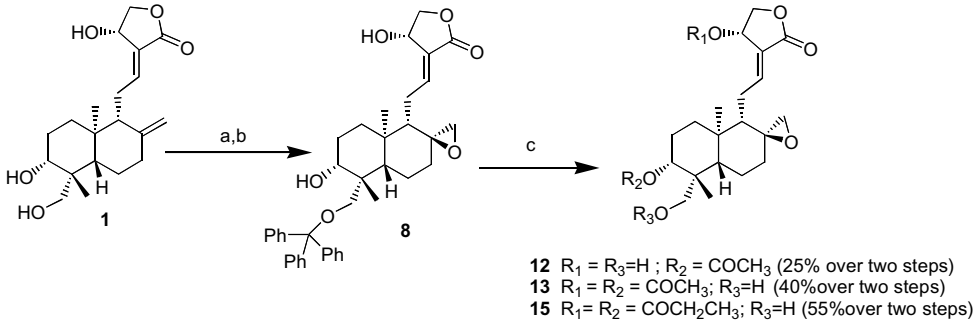
Accordingly, the epoxide in **9** was transformed into the corresponding aldehyde **24** by treatment with $BF_3{\cdot}Et_2O$. The aldehyde was also accompanied by the formation of a tetracyclic compound **25**, presumably as a by product derived from the aldehyde. Further, the aldehyde **24** was converted to the corresponding alcohol **26** by $NaBH_4$ reduction.

3. Biological results and discussion

The in vitro cytotoxic activity of andrographolide **1** and its analogues **2**–**26** were evaluated against breast (MCF-7/ADR), CNS (U251), colon (SW620), lung (H522), ovarian (SKOV3), prostate (DU145), and renal (A498)



Scheme 3. Synthesis of compounds **11**, **16**–**23**. Reagents and conditions: (a) 2,2-dimethoxy propane/benzene and DMSO/PPTS/rt, 8 h, 70% (required isomer); (b) for **11** acetic anhydride/reflux, 1 h, 80%; for **16**–**23** $R_1COOH/ClCOOEt/NEt_3/DCM$/rt, 1 h, 60–85%; (c) AcOH and H_2O (7:3)/rt, 15 min, 85%; (d) acetic or propionic anhydride/reflux, 30 min, 65–75%.



Scheme 4. Synthesis of compounds **12**, **13**, **15**. Reagents and conditions: (a) tritylchloride/pyridine/60 °C, 6 h, 80%; (b) m-CPBA/CH_2Cl_2/rt, 2 h, 70% (required isomer); (c) (i) acetic or propionic anhydride/reflux, 30 min, (ii) formic acid/CH_2Cl_2/rt, 30 min.

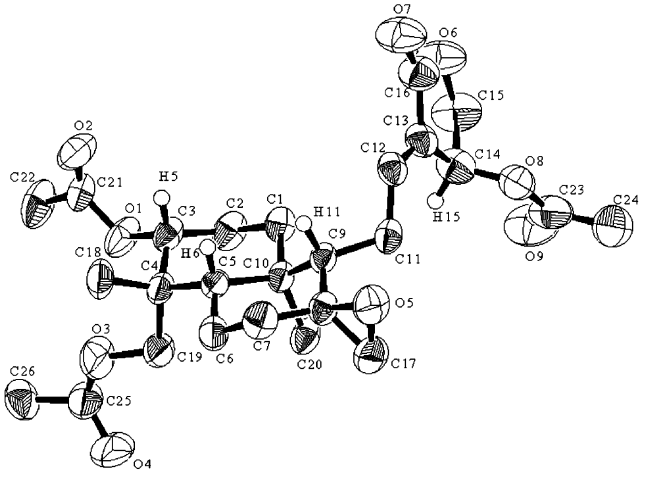
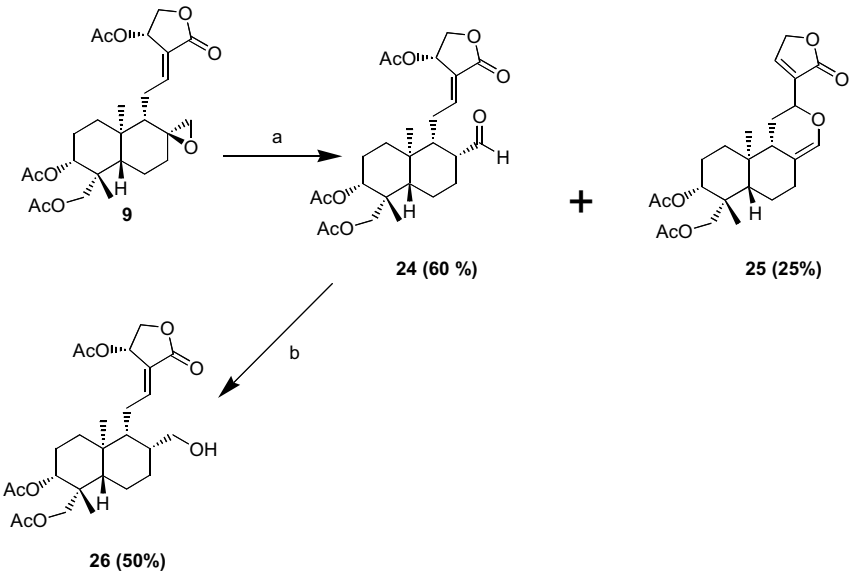


Figure 2. Single-crystal X-ray structure of **9**.

cancer cell lines using the NCI standard protocol for screening anticancer molecules.[24] The concentration that causes 50% inhibition of cancer cell growth against various cell lines are expressed as GI_{50} values and are given in Table 2. The selected active analogues were further evaluated for their in vivo antitumor activity using modified hollow fiber assay.

In the above study, andrographolide **1** was found to possess moderate activity against all the cell lines with GI_{50} values ranging from 3 to 30 μM. Loss of activity was observed for **2** and **3** namely andrographolic acid and 12,13-dihydro andrographolide, respectively, clearly demonstrating that the intact lactone moiety of andrographolide and the conjugated $\Delta^{12(13)}$ double bond play an important role in the observed cytotoxic activity of **1**. Further confirmation of the importance of the conjugated $\Delta^{12(13)}$ double bond and the hydroxyl at C-14 was evident from the similar loss of activity observed for **4** and **5**. However, **4** exhibited potent activity against ovarian cell line in nanomolar concentrations. Compound **6** exhibited moderate activity against all the cell lines similar to **1** (GI_{50} values ranging from 4 to



Scheme 5. Synthesis of compounds **24**–**26**. Reagents and conditions: (a) $BF_3{\cdot}Et_2O/CH_2Cl_2/0\,^{\circ}C$, 10 min; (b) $NaBH_4$/MeOH/0 °C, 30 s.

Table 2. In vitro cytotoxic activities of andrographolides (**1**–**26**)

Compound	Cytotoxicity ($GI_{50}\,\mu M$)[a]						
	Breast MCF-7/ADR	CNS U251	Colon SW 620	Lung H 522	Ovarian SK OV3	Prostate DU145	Renal A 498
1	15	3	10	17	15	20	30
2	30	>100	>100	80	>100	>100	4
3	nd[b]	85	>100	>100	>100	2	nd
4	nd	>100	54	>100	0.18	5	nd
5	>100	81	>100	>100	nd	2	nd
6	20	16	30	30	4	20	30
9	5	2.5	1	15	2.5	15	0.25
10	nd	3	2	3	1.36	3	nd
11	20	50	20	25	5	25	7
12	20	45	>100	60	50	50	20
13	20	30	55	30	2.5	6.5	6
14	30	40	20	nd	>100	nd	20
15	30	35	7	40	3	20	40
16	7	40	40	>100	6	20	5
17	4	4	3	4	8	7	20
18	15	20	30	4	15	20	4
19	2	6	0.5	6	4	5.5	9
20	0.08	6	2.5	20	10	4	3
21	3	3	0.6	4.8	2	2	3
22	1.5	2	0.4	7.5	2.5	2.5	3.5
23	20	35	65	3	0.3	4	6
24	nd	62	32	34	3	12	nd
25	40	30	40	50	40	40	nd
26	nd	19	7	19	3	2	nd

[a] Cytotoxicity GI_{50} values are the concentrations corresponding to 50% growth inhibition.
[b] nd—not done.

30 μM), indicating that the exocyclic $\Delta^{8(17)}$ double bond may not play any important role and can be replaced with an epoxy moiety.

Significant improvement in the activity of **6** was observed when it was converted to its triacetyl **9** and tripropionyl **10** derivatives (GI_{50} values ranging from 0.25 to 5 μM against most of the cell lines). To understand further, the number of hydroxyls on **6**, which need to be converted to esters for exhibiting potent activity, two monoacetyl (**11** and **12**) and two diacetyl (**13** and **14**) derivatives were evaluated and their activities compared with **9**. In general, all the mono and diacetyl derivatives were found to be less active compared to **9**, indicating that all the three hydroxyls need to be converted into their ester functionalities for potent activity. Interestingly, **11** and **13**, which contained an acetyl group at C-14 were found to possess potent activity against

ovarian and renal cell lines (GI_{50} values less than 10 μM) compared to **12** and **14**, indicating that the presence of an ester functionality at C-14, and possibly also at C-3 and C-19, is essential for improving the activity of **6**. In view of this observation, a number of compounds having ester side chains at C-14 and hydroxyls or alkyl esters at C-3 and C-19 were synthesized and screened.

Thus, **16**–**18** having a *N*-Boc-glycinyl group, and **19**–**23** with substituted or unsubstituted cinnamoyl side chains at C-14 with either hydroxyls or alkyl ester groups at C-3 and C-19 were screened.

Their cytotoxic activity results indicated that

1. The activity of **16** with C-14 *N*-Boc-glycinyl moiety and hydroxyls at C-3 and C-19 improved when the free hydroxyls were converted to their corresponding alkyl esters **17** and **18**. Further, **17** having acetyl moieties at C-3 and C-19 showed better activity compared to its corresponding propionyl derivative **18**.
2. When conjugated esters were added as side chains at C-14 (derivatives **19**–**23**), the activity improved considerably compared to the parent molecule **6** against all the cell lines. While these compounds possessed GI_{50} values less than 10 μM against all the cell lines, **20** exhibited potent activity against breast cancer cell line (GI_{50} 0.08 M) and **19**, **21**, and **22** showed superior activity against colon cancer cell line (GI_{50} 0.5, 0.6, and 0.4 μM, respectively). Compound **23** was found to be less active compared to the above derivatives.

To further understand the role of the epoxy moiety present in the above ester derivatives of **6** for their potent activity, the epoxy modified analogues of **9** (**24**–**26**) were studied. All the three compounds exhibited lower activity compared to **9** suggesting that the epoxide is necessary for retaining activity.

In summary, most of the ester derivatives of **6**, exhibited good in vitro cytotoxic activity possessing GI_{50} values ranging from 1 to 10 μM. Compounds **9**, **17**–**20** along with the parent compounds **1** and **6**, were further evaluated for their in vivo antitumor activity using modified hollow fiber animal assay.[25,26] The cell lines needed for this assay were selected from in vitro studies. Poly vinylidine fluoride hollow fibers containing different types of human cancer cell lines were implanted intraperitoneally (IP) and subcutaneously (SC) into Swiss Albino Mice (SAM). The compounds were administered by IP route at two different doses for 4 days. The doses were determined based on the MTD values of the compounds. The in vivo efficacy of the compounds was evaluated by determining the percentage growth of the cells using MTT assay. Compounds inhibiting 50% or greater growth compared to the vehicle control were considered active and a score of 2 was assigned at the different doses tested. The results obtained were tabulated in Table 3. The results indicated that Andrographolide **1**, possessed moderate activity at 50 mg/kg and good activity at 100 mg/kg doses. However, the corresponding 8,17-epoxy derivative **6** showed moderate activity at 300 mg/kg. Improvement in the in vivo activity of **6** was observed with the acylation of the hydroxyls as in compounds **9**, **17**–**20**. Of these ester derivatives, compound **19** was found to be the most potent compound exhibiting superior activity compared to both the parent compounds **1** and **6**. Further in vivo studies of **19** in various xenograft models are in progress.

Table 3. In vivo hollow fiber assay results of andrographolide derivatives

Compound	Dose (mg/kg)	SC score	IP score	Total score
1[b]	50	6	10	16
	100	14	14	28
6[a]	300	6	6	12
9[a]	100	2	0	2
	150	8	8	16
17[b]	100	8	6	14
	200	14	14	28
18[b]	100	2	0	2
	200	8	4	12
19[b]	25	2	6	8
	50	14	14	28
20[b]	100	8	12	20
	200	14	14	28

[a] Compound tested against 4 cell lines.
[b] Compounds tested against 7 cell lines.

4. Conclusion

In conclusion, in our endeavor to develop promising anticancer molecules based on the naturally occurring andrographolide **1**, we have designed and synthesized **19** and a number of related analogues possessing potent in vitro and in vivo anticancer activity.[27] An analysis of the in vitro results indicated that (a) the intact α-alkylidene γ-butyrolactone moiety of andrographolide, (b) the $\Delta^{12(13)}$ double bond, (c) the C-14 hydroxyl or its ester moiety, and (d) the $\Delta^{8(17)}$ double bond or epoxy moiety are responsible for the cytotoxic activity exhibited by andrographolide and its analogues.

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21. X-ray crystallographic analysis of **9**: A colorless crystal of **9** $C_{26}H_{36}O_9$ [0.50 mm×0.40 mm×0.15 mm], was mounted on a glass fiber in a random orientation. The data was collected on a Rigaku AFC7S diffractometer with Cu K_α ($\lambda = 1.5418$ Å) radiation. The cell parameters and the orientation matrix for data collection were obtained from least squares refinement, using the setting angles of 25 reflections in the range of 25–33° (2θ). The data were collected at a temperature of 298 K. A total of 2861 unique reflections were collected. The structure was solved by direct methods using SIR92. Hydrogen atoms were included in the refinement but restrained to ride on the atom to which they are bonded. The structure was refined in full matrix least squares where the functioned minimized was $\sum w(|Fo|^2 - |Fc|^2)^2$. Refinement was performed on a Silicon Graphics Indy workstation using teXsan software. Crystallographic drawings were done using the program ORTEP.
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