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Antitumor agents. Part 230: $C_{4'}$-esters of GL-331 as cytotoxic agents and DNA topoisomerase II inhibitors ☆

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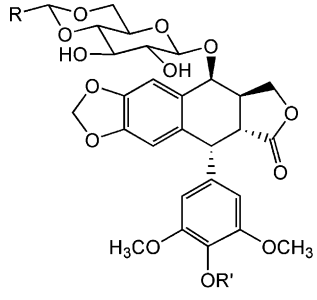
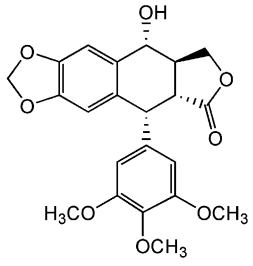
Abstract—Three $C_{4'}$-acyl derivatives (**5**–**7**) of GL-331 (**4**) were synthesized and evaluated for cytotoxic and DNA topoisomerase II (topo II) inhibitory activity. All three compounds were cytotoxic against KB and KB-7d cells. Compounds **5** and **7**, but not **6**, were potent inhibitors of the DNA topoisomerase II in vitro and this relative activity ranking was maintained for cytotoxicity, in vitro cell growth inhibition, and ability to induce cellular double-strand DNA breaks. These results provided new information on the structure–activity relationships of the $C_{4'}$ molecular area of **4**-analogues.

Etoposide (**1**) and teniposide (**2**) are semisynthetic derivatives of the natural podophyllotoxin (**3**) and are currently used clinically against various cancers, including small-cell lung cancer, testicular carcinoma, lymphoma, and Kaposi's sarcoma.[2,3] Interestingly, podophyllotoxin is known as an antimicrotubule agent, while etoposide and teniposide inhibit the catalytic activity of DNA topoisomerase II (topo II).[4,5] Although it is still unclear how podophyllotoxin and etoposide analogues interact with either tubulin or topo II, the structural preferences for topo II inhibitors over antimicrotubule agents have been roughly identified as: (1) demethylation at $C_{4'}$, (2) β-configuration at C_4, and (3) bulky substitution at C_4. Among these structural parameters, a free $C_{4'}$-hydroxyl resulting from demethylation was considered as a structural determinant for topo II inhibitors.[6,7] A number of 4′-acyl derivatives of **1** were previously prepared by the Bristol Myers Company.[8] Most of them retained significant antitumor activity in vivo against P388 leukemia and some were even more active than **1**.[9] These results stimulated a re-examination of the structure–activity relationships (SAR) at the $C_{4'}$ molecular area of **1**-analogues. GL-331 (**4**) is a 4β-arylamino **1**-derivative currently under Phase II clinical evaluation. In this paper, the evaluation of three $C_{4'}$-acyl derivatives (**5**–**7**) of **4** is reported. Our goal was to determine whether these esters retain the activity profiles and molecular target of **1**, and if they act as prodrugs.



1. R = CH_3; R' = H

2. R =  ; R' = H



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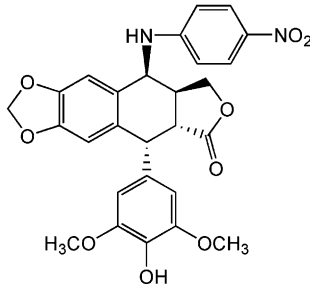

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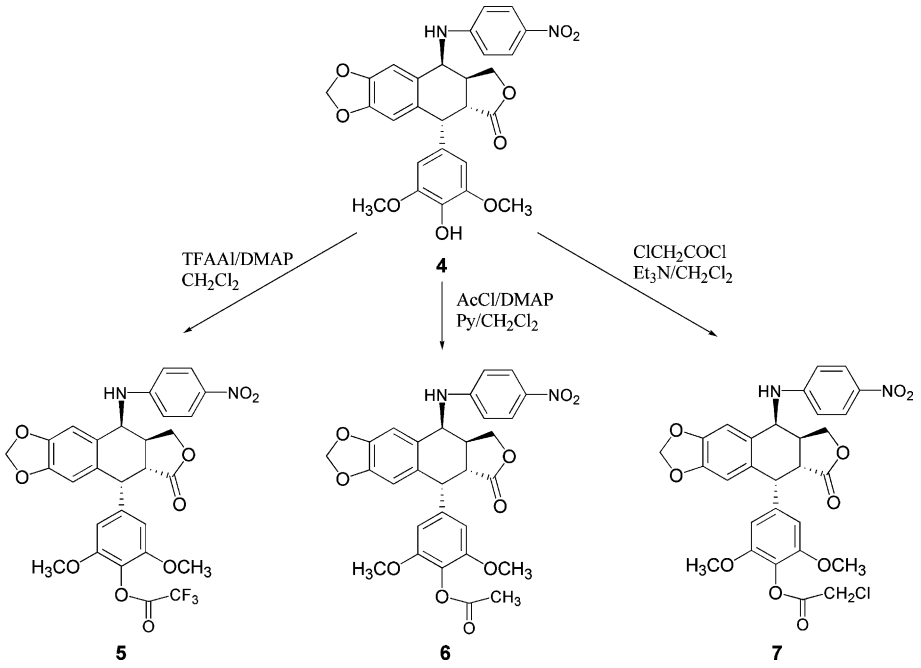

Scheme 1. Synthesis of **5**–**7**.

Compounds **5**–**7**[10] were synthesized from GL-331 (**4**) (Scheme 1). Briefly, the corresponding acyl chlorides or acid (for **5** only) were condensed with **4** under base catalysis to give compounds **5**–**7**.

Compounds **5**–**7** were evaluated for their cell growth inhibition,[11] cytotoxicity,[12] in vitro topo II inhibition,[13] and induction of double-stranded cellular DNA breaks[14] using both etoposide (**1**) and GL-331 (**4**) as standards.

Preliminary screening indicated that all three compounds were active against **1**-resistant KB-7d cell replication with potency similar to that of GL-331 (**4**) (Table 1). These results suggested that the esters **5**–**7** retain the superior cell inhibition and drug-resistance profiles of the parent compound **4**.

Compounds **5**–**7** were subsequently tested for cytotoxicity at a concentration of 10 μM using a clonogenic protocol[12] (Table 2). Like GL-331 (**4**), these ester derivatives were more cytotoxic against the **1**-resistant KB-7d cells than the wild-type KB cells. All compounds showed significant cytotoxicity against both KB and KB-7d cells. Compound **5** was equally active as **4**.

Compounds **5**–**7** were also examined for topo II (p170) inhibition at a concentration of 50 μM in a standard in vitro assay using the pYRG plasmid substrate (Fig. 1).[13]

Table 1. Inhibition of KB-7d cell replication by **5**–**7**

Compound	**1**	**4**	**5**	**6**	**7**
ED_{50} (μM)[a]	11.0	1.5	1.5	2.9	2.3

[a] ED_{50} is the concentration that inhibits 50% cell replication after 3 days of continuous treatment.

Table 2. Cytotoxicity testing of **5**–**7**

Compound	KB cell cytotoxicity (%)[a,b]	KB-7d cell cytotoxicity (%)[a,b]	LD_{50}[c] KB (μM)	LD_{50}[c] KB-7d (μM)
1	52	NMD	50	NMD
4	66	94	<10	<10
5	62	90	<10	<10
6	28	60	>20	<10
7	43	46	>10	>10

NMD = not markedly different from control.

[a] All compounds were tested at a concentration of 10 μM.

[b] The percentage values represented the inhibition of colony formation and were calculated by comparing the number of colonies surviving drug treatment with that in the control.

[c] LD_{50} is the concentration that kills 50% of the cells after 30 min of treatment.

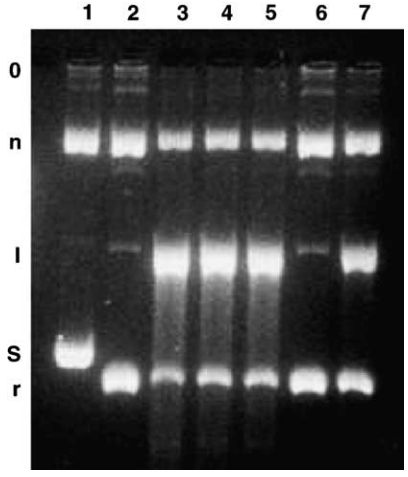

Figure 1. In vitro topoisomerase II inhibition by **5**–**7**. Lane 1: pYRG DNA, lane 2: enzyme control, lane 3: GL-331 (**4**), lane 4: etoposide (**1**), lane 5: compound **5**, lane 6: compound **6**, and lane 7: compound **7**. o: origin, n: nicked, l: linear, s: supercoiled, and r: relaxed.

Compounds **5** and **7** induced levels of linear DNA comparable to **1** and **4**, and therefore, were potent poisons of topo II in vitro. Although compound **6** did induce DNA breaks, the activity was rather weak. Compounds **1**, **4**, and **5**–**7** showed a similar rank order potency using a pBR322 substrate (data not shown).

The ester derivatives were further tested for their ability to induce double-stranded DNA breaks in KB cells using a gel lysis assay[14] (Fig. 2). The results in this cellular assay were consistent with the prior in vitro findings (Fig. 1). Compounds **5** and **7** showed comparable in vitro topo II inhibitory activity to **4**, and while, compound **6** also induced detectable breaks (cf. lanes 3 and 5), its activity was much weaker. This experiment was coupled with a cytotoxicity assay[12] (cf. Table 2), and the results obtained for esters **5**–**7** were consistent with their rank order activity as in vitro topo II inhibitors (Fig. 2).

In general, compound **5** was equipotent to GL-331 in all assays, and **7** was less potent. In addition, although **6** was active in cell growth inhibition and cell killing assays, it was only weakly active in the mechanistic assays. The compounds showed consistent potency rankings (**5** > **7** > **6**) for cytotoxicity (Table 2) and topo II inhibition [both in vitro (Fig. 1) and in KB cells (Fig. 2)], which suggested that topo II inhibition might be the primary mechanism underlying the cytotoxicity. These data also suggested that electron-withdrawing substituents on the β-carbon of the ester moiety might enhance topo II inhibition. Because electron-withdrawing substitution could also contribute to the instability of the compounds (e.g., **5** was susceptible to hydrolysis), the correlation between the activities, especially cell-based activity, and chemical stability implied that these $C_{4'}$-acyl derivatives might act as prodrugs and exert their cytotoxicity by hydrolyzing to the parent compound **4**. However, the results of the mechanistic studies with the ester analogues seemed to challenge the above postulation because enzyme inhibition in vitro and cellular DNA break-induction activity were in good agreement. We thought it unlikely that the compounds would be hydrolyzed under the in vitro experimental conditions, especially **6** and **7**. To support this assumption, **6** and **7** were incubated in the Tris–HCl buffer under the same conditions used for the enzymatic assay (37 °C for 1 h), and no significant amount of **4** was detected using TLC (data not shown).

In summary, we synthesized and evaluated three $C_{4'}$-acyl derivatives of GL-331. All three compounds showed significant cell growth inhibition and cytotoxic activity. Compounds **5** and **7** showed potent inhibition of topo II in vitro and induced DNA breaks in KB cells. However, compound **6** was less active in inducing cellular DNA breaks and was a less potent enzyme inhibitor in vitro. These results, especially the in vitro topoisomerase II inhibition data, conflicted with the widely accepted SAR that a free 4′-hydroxyl is essential for the topo II inhibitory activity of **1**-related analogues. Further exploration of $C_{4'}$-modified **1**-analogues is in progress.

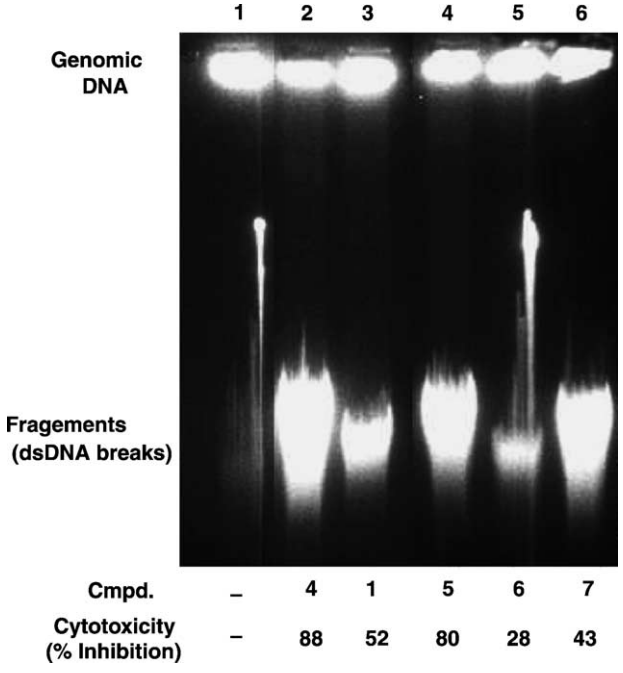

Figure 2. Cellular DNA break-induction and cytotoxicity of GL-331 esters in KB cells. Lane 1: control with no drug, lane 2: 20 μM GL-331 (**4**), lane 3: 50 M etoposide (**1**), lane 4: 20 μM compound **5**, lane 5: 20 μM compound **6**, and lane 6: 20 μM compound **7**. †The percentage values represented the inhibition of colony formation and were calculated by comparing the number of colonies surviving drug treatment with that in the control.

Acknowledgements

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10. The structures of compounds **5**–**7** were confirmed by the following data:
Compound **5**: yield 71%; ^{1}H NMR ($CDCl_3$) δ 8.15 (d, $J = 9.0$ Hz, 2H, 3″, 5″-H), 6.78 (s, 1H, 5-H), 6.59 (s, 1H, 8-H), 6.56 (d, $J = 9.0$ Hz, 2H, 2″, 6″-H), 6.38 (s, 2H, 2′, 6′-H), 6.02, 5.99 (AB, $J = 1.5$ Hz, 2H, –OCH_2O–), 4.85

(m, 1H, –NH), 4.68 (d, $J = 4.5$ Hz, 1H, 4-H), 4.63 (d, $J = 6.9$ Hz, 1-H), 4.43 (t, $J = 7.8$ Hz, 1H, 11-H), 3.85 (t, $J = 7.8$ Hz, 1H, 11-H), 3.73 (s, 6H, 3′, 5′-OCH_3), 3.14 (m, 2H, 2, 3-H). ESI-MS m/e 616 $[M]^+$.

Compound **6**: yield 79%; ^{1}H NMR ($CDCl_3$) δ 8.15 (d, $J = 9.0$ Hz, 2H, 3″, 5″-H), 6.76 (s, 1H, 5-H), 6.58 (s, 1H, 8-H), 6.57 (d, $J = 9.0$ Hz, 2H, 2″, 6″-H), 6.34 (s, 2H, 2′, 6′-H), 6.00, 5.99 (AB, $J = 1.0$ Hz, 2H, –OCH_2O–), 4.83 (m, 1H, –NH), 4.67 (d, $J = 4.5$ Hz, 1H, 4-H), 4.60 (d, $J = 6.9$ Hz, 1-H), 4.43 (m, 1H, 11-H), 3.90 (m, 1H, 11-H), 3.71 (s, 6H, 3′, 5′ –OCH_3), 3.09 (m, 2H, 2, 3-H), 2.32 (s, 3H, $COCH_3$). ESI-MS m/e 561 $[M-H]^+$.

Compound **7**: yield 97%; ^{1}H NMR ($CDCl_3$) δ 8.12 (d, $J = 9.3$ Hz, 2H, 3″, 5″-H), 6.79 (s, 1H, 5-H), 6.60 (d, $J = 9.3$ Hz, 2H, 2″, 6″-H), 6.58 (s, 1H, 8-H), 6.39 (s, 2H, 2″-H), 5.99 (s, 2H, –OCH_2O–), 4.86 (d, $J = 7.3$ Hz, 1H, –NH), 4.77 (d, $J = 7.5$ Hz, 1H, 4-H), 4.69 (d, $J = 4.5$ Hz, 1-H), 4.41 (t, $J = 7.8$ Hz, 1H, 11-H), 4.37 (s, 2H, CH_2Cl), 3.88 (t, $J = 9.3$ Hz, 1H, 11-H), 3.71 (s, 6H, 3′, 5′-OCH_3), 3.10 (m, 2H, 2, 3-H). ESI-MS m/e 595 $[M-H]^+$.

11. The cell growth inhibition assay using sulforhodamine B (SRB) was carried out according to standard procedure described in Rubinstein, L. V.; Shoemaker, R. H.; Paull, K. D.; Simon, R. M.; Tosini, S.; Skehan, P.; Scudiero, D. A.; Monks, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1113.
12. The protocol for clonogenic assay was described in Krishnan, P.; Bastow, K. F. *Cancer Chemother. Pharmacol.* **2001**, *47*, 187, Briefly, 250 freshly trypsinized KB cells were exposed to drug for 30 min, and then cells were immediately plated and left undisturbed for 6 days. Colonies were fixed with formalin, stained with toluidine blue and then scored. The plating efficiency and percentage of total cells forming colonies were calculated.
13. Inhibition of topoisomerase II catalytic activity was examined using a plasmid DNA relaxation assay Krishnan, P.; Bastow, K. F. *Anti-Cancer Drug Des.* **2000**, *15*, 255, using human p170 topo II and pYRG substrate purchased from TopoGen, Inc. (Columbus, OH) (Fig. 1).
14. A gel-based semi-quantitative assay (originally published in Bastow, K. F.; Wang, H. K.; Cheng, Y. C.; Lee, K. H. *Bioorg. Med. Chem.* **1997**, *5*, 1481) was used. Briefly, KB cells were treated with drugs for various times and were then harvested, cast in agarose plugs and de-proteinized. Gel plugs were subjected to agarose-gel, electrophoresis and levels of ethidium-stained broken DNA fragments were determined using a STORM phosphor-Imager (Molecular Dynamics, Sunnyvale, CA), and the supplied Image Quant software. The relative amounts of DNA damage were compared by measuring the fluorescence intensity of the genomic DNA fragments that migrate on the gel (Fig. 2).