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Dihydroindenoisoquinolines function as prodrugs of indenoisoquinolines

Xiangshu Xiao,[a] Ze-Hong Miao,[b] Smitha Antony,[b] Yves Pommier[b] and Mark Cushman[a,*]

[a]*Department of Medicinal Chemistry and Molecular Pharmacology and the Purdue Cancer Center, School of Pharmacy and Pharmaceutical Sciences, Purdue University, West Lafayette, IN 47907, USA*
[b]*Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892-4255, USA*

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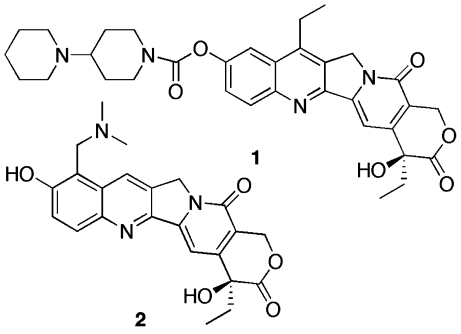
Abstract—Dihydroindenoisoquinolines are analogs of cytotoxic indenoisoquinoline topoisomerase I (Top1) inhibitors, exhibiting potent cytotoxicity but weak inhibitory activity toward Top1. Through COMPARE analysis, cytotoxicity studies in Top1-deficient cells, chemical synthesis and biological evaluation of methylated dihydroindenoisoquinoline **5**, we demonstrated that dihydroindenoisoquinolines function as prodrugs of indenoisoquinolines in cancer cells.

Two camptothecin (CPT) derivatives, irinotecan (**1**, Camptosar®) and topotecan (**2**, Hycamptin®), are the only topoisomerase I (Top1) inhibitors approved by the FDA as anticancer drugs. The antitumor activity observed in clinical trials validated this class of Top1 inhibitors as effective chemotherapeutic agents.[1] On the other hand, two major problems associated with CPTs limit their clinical utility. First, the lactone moiety present in the CPTs hydrolyzes to the hydroxy carboxylate form, which has a high affinity to human serum albumin protein.[2] Second, the cleavage complexes stabilized by the CPTs reverse rapidly after drug removal, necessitating long continuous infusions to achieve maximal antitumor effect.[3] These two problems warrant searching for other non-camptothecin Top1 inhibitors with better pharmacokinetic profiles.[4]

Indenoisoquinoline **3a** (NSC 314622), initially synthesized in 1978,[5] was found to be a novel Top1 inhibitor with better pharmacokinetic features than CPT.[6] The moderate biological activity prompted us to investigate its structure–activity relationships and a number of the analogs have demonstrated potent cytotoxicity.[7–16] During these studies, the dihydroindenoisoquinolines **4** were usually found to be more cytotoxic, albeit exhibiting weak or no activity in poisoning Top1, than the corresponding indenoisoquinolines **3** (Fig. 1).[7,15]



As a method for elucidating the mechanism of action of these dihydroindenoisoquinolines, a COMPARE analysis[17,18] was done in the National Cancer Institute (NCI) database using compound **4a** (referred to NSC 344505) as a seed.[6] The top 50 hits with the highest Pearson correlation coefficients are shown in Table 1, among which are a large number of Top1 inhibitors. Indeed, the top 31 compounds are all Top1 inhibitors consisting of 29 CPTs and 2 epipodophyllotoxins. This COMPARE analysis strongly suggests that NSC 344505 acts as a Top1 inhibitor in cancer cells. However, this indication was not supported by the fact that NSC 344505 showed

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* Corresponding author. Tel.: +1 765 494 1465; fax: +1 765 494 6790; e-mail: cushman@pharmacy.purdue.edu

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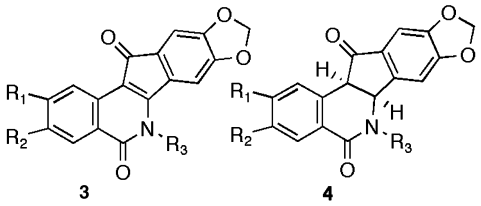


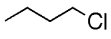
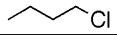
compd	R_1	R_2	R_3	top1	MGM (μM)
3a	OMe	OMe	Me	++	20
3b	OMe	OMe	allyl	+	4.16
3c	H	NO_2		+++	0.83
4a	OMe	OMe	Me	±	5.0
4b	OMe	OMe	allyl	0	0.98
4c	H	NO_2		+	0.018

Figure 1. Chemical structures of indenoisoquinolines **3**, dihydroindenoisoquinolines **4** and their biological activities.

Table 1. COMPARE analysis of NSC 344505 (seed) using GI_{50} data obtained in the 2-day assays from the National Cancer Institute in Vitro Anticancer Drug Screen

Rank	NSC[a]	Max X[b]	CORR[c]	*N*[d]	Mechanism/class
1	344,505	1	1.00	60	Top1
2–32			0.820–0.748		29 CPTs[e]
33	627,687	2	0.749	49	Unknown
34	9706	9	0.746	60	Alkylating agent
35	246,131	3	0.745	58	Top2
36–40			0.740–0.735		5 CPTs
41	376,254	1	0.735	60	Analog 344505
42–44		2	0.732	60	3 CPTs
45	314,622	5	0.730	60	Top1
46	6396	9	0.729	60	Alkylating agent
47	68,3416	2	0.729	57	Top1
48	670,656	1	0.729	54	Enediyne
49	692,758	3	0.727	60	Unknown
50	670,656	1	0.727	58	Enediyne

[a] Compound number in the NCI database.
[b] Number of the tests that were averaged for this compound.
[c] Pearson correlation coefficient for the seed and the database compound.
[d] Number of cell lines common to both the seed and the database compound.
[e] The other two database compounds are epipodophyllotoxins.

little activity in poisoning Top1 in a cell-free system.[7] This apparent discrepancy suggests that NSC 344505 (**4a**) may function as a prodrug of NSC 314622 (**3a**), undergoing a two-electron oxidation (dehydrogenation) in the cancer cells before poisoning Top1. The increased cytotoxicity of **4a** relative to **3a** could possibly be due to enhanced cellular uptake.

To demonstrate specific targeting of Top1 in cells by NSC 344505, antiproliferative assays were performed in P388 and its Top1-deficient subclone P388/CPT45.[19]

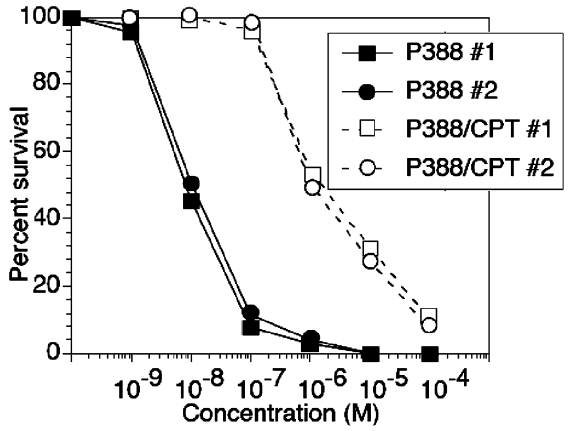


Figure 2. Resistance of Top1-deficient P388/CPT45 cells to NSC 344505. #1 and #2 refer to two independent experiment runs. The assay was done using the MTT method.

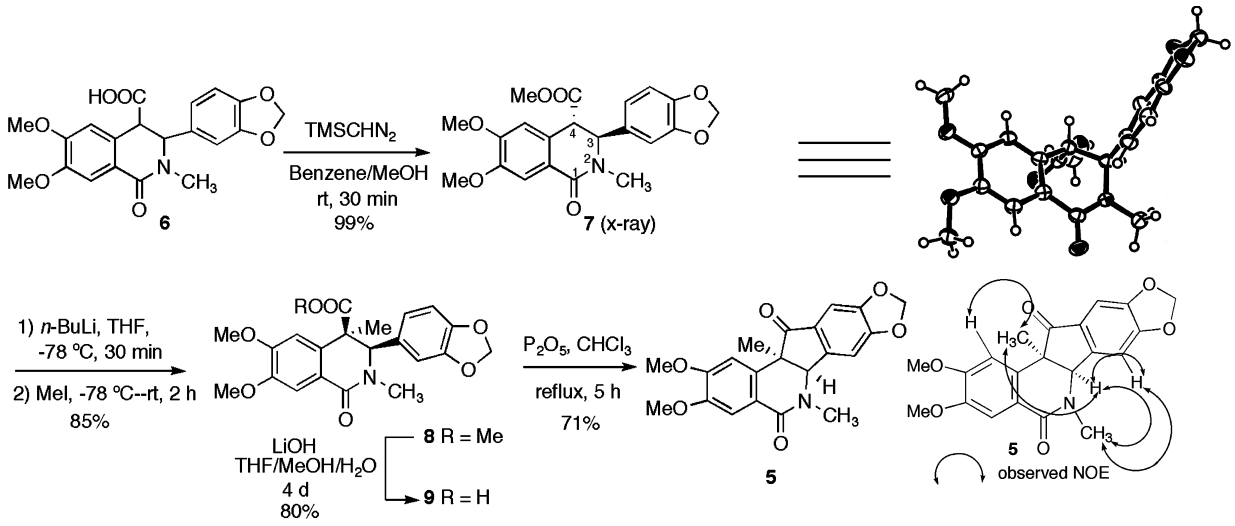
NSC 344505 shows potent antiproliferative activity in P388 leukemia cells, while considerable resistance (128-fold) to P388/CPT45 cells was observed (Fig. 2, Table 2). Top1 targeting was further investigated in a separate human colon carcinoma cell line with 8–10-fold reduction of Top1 by RNA interference.[20] Approximate 35-fold resistance was seen in the Top1-defective HCT116 cells (HCT116-*Top1*). Taken together, these experiments demonstrate selective targeting of Top1 by NSC 344505 in cells.

To further test the prodrug hypothesis, a methylated dihydroindenoisoquinoline **5** was designed. If this hypothesis were true, then compound **5** would be expected to not be cytotoxic since it will not be able to undergo oxidation in cancer cells to give the corresponding planar indenoisoquinoline as the Top1 inhibitor. The synthesis of **5** is presented in Scheme 1. Methylation of either *cis* or *trans* acid **6** with $TMSCHN_2$ in MeOH/benzene[21] afforded only the thermodynamically more stable *trans* ester **7**, whose relative configuration was confirmed by both ^1H NMR (H3 and H4 appeared as broad singlets)[22] and single crystal X-ray analysis. The observed conformation of **7** by X-ray confirmed the previous prediction of the pseudoaxial disposition of the 3-phenyl ring in **7** and other related compounds due to the A-strain.[23] Deprotonation at the α-position to the ester **7** followed by addition of MeI provided the ester **8**, which

Table 2. Cytotoxicity of compound **4a** in Top1-deficient and parental tumor cells

Cell line	P388	P388/CPT45	HCT116	HCT116-V	HCT116-*Top1*
GI50 (nM)[a]	9.88	1264.20	11.99	59.92	417.67
RF[b]	1	128	1	5	35(7[c])

[a] GI_{50} values were calculated with the program Prism 4.0a from two independent experiments.
[b] RF (resistance factor) is the ratio of the GI_{50} for resistant cells to that for the corresponding parental cells.
[c] This value is the ratio of the GI_{50} for Top1 siRNA cells (HCT116-*Top1*) to that for the vector-control cells (HCT116-V).



Scheme 1. Synthesis of compound **5**.

Table 3. Cytotoxicities and Top1 inhibitory activities of indenoisoquinoline analogs

Compd	Cytotoxicity (GI_{50}, μM)[a]								MGM[b]	Top1 cleavage[c]
	Lung HOP-62	Colon HCT-116	CNS SF-539	Melanoma UACC-62	Ovarian OVCAR-3	Renal SN12C	Prostate DU-145	Breast MDA-MB-435		
3a	1.3	35	41	4.2	73	68	37	96	20	++
4a	9.4	2.0	3.1	0.42	6.7	2.1	4.1	17	5.0	±
5	>100	43.5	24.0	26.0	96.2	82.4	58.1	>100	51.3	++[d]

[a] The cytotoxicity GI_{50} values are the concentrations corresponding to 50% growth inhibition.
[b] Mean graph midpoint for growth inhibition of all human cancer cell lines successfully tested.
[c] The compounds were tested at concentrations ranging up to 100 μM. The activity of the compounds to produce Top1-mediated DNA cleavage was expressed semi-quantitatively as follows: 0: no activity; +: weak activity; ++: similar activity as the parent compound **3a**.
[d] Activity was only seen at 100 μM concentration.

was then saponified to give acid **9**. Dehydration of **9** with P_2O_5 in refluxing chloroform resulted in cyclization product **5**,[24] whose relative stereochemistry was confirmed by the observed NOE (2.85%) between the C-3 methine and the C-4 methyl group (Scheme 1).

Compound **5** was examined for antiproliferative activity against the human cancer cell lines in the NCI screen, in which the activity of the compound was evaluated with approximately 55 different cancer cell lines of diverse tumor origins. The GI_{50} values obtained with selected cell lines, along with the mean graph midpoint (MGM) values, are summarized in Table 3. The MGM is based on a calculation of the average GI_{50} for all of the cell lines tested (approximately 55) in which GI_{50} values below and above the test range (10^{-8}–10^{-4} M) are taken as the minimum (10^{-8} M) and maximum (10^{-4} M) drug concentrations used in the screening test. Therefore, the MGM value represents an overall assessment of toxicity of the compound across numerous cell lines. For comparison, the activities of the previously reported compounds **3a**[7] and **4a**[7] are also included in the table. The relative potencies of the compounds in the production of topoisomerase I-mediated DNA cleavage are also listed in the table.

As expected from the prodrug hypothesis for the dihydroindenoisoquinolines, the methylated analog **5** is not very cytotoxic overall with an MGM value of 51.3 μM. It should be noted that the actual cytotoxicity GI_{50} of **5** is far higher than 51.3 μM, since 11 out of the 56 successfully tested cell lines show cytotoxicities higher than 100 μM. However, they were treated as 100 μM during the calculation of the MGM. Interestingly, compound **5** shows moderate Top1 inhibitory activity at 100 μM. However, this inhibition is definitely not due to intercalation between the base pairs in the cleavage site as proposed for other indenoisoquinolines, since its geometry would prevent this process.

In conclusion, the mechanism of action of dihydroindenoisoquinoline **4a** was studied by COMPARE analysis, indicating that it is a Top1 inhibitor though its Top1 inhibitory activity is weak in a cell-free system. The relevance of Top1 to the observed cytotoxicity for **4a** was confirmed by its resistance to P388/CPT45 and HCT116-*Top1* cells. These results suggested that **4a** functions as a prodrug of **3a** in cancer cells to poison Top1. Chemical synthesis and biological evaluation of methylated analog **5** further support this prodrug hypothesis.

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24. Selected physical data for compound **5**: mp >225 °C (dec.). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.54 (s, 1H), 7.04 (s, 1H), 6.99 (s, 1H), 6.94 (s, 1H), 6.05 (d, J = 1.2 Hz, 1H), 6.00 (d, J = 1.2 Hz, 1H), 4.68 (s, 1H), 3.89 (s, 3H), 3.84 (s, 3H), 3.56 (s, 3H), 1.63 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.4, 162.6, 154.8, 152.3, 149.4, 148.7, 148.5, 132.6, 128.8, 119.4, 110.2, 108.1, 103.9, 102.8, 102.6, 67.3, 56.1, 56.0, 53.9, 37.6, 22.8; ESIMS (rel intensity) m/z 382 (MH^+, 100). Anal. Calcd for $C_{21}H_{19}NO_6 \cdot 0.5H_2O$: C, 64.61; H, 5.16; N, 3.59. Found: C, 64.64; H, 5.25; N, 3.85.