Inhibition of Topoisomerase II Catalytic Activity by Pyridoacridine Alkaloids from a *Cystodytes* sp. Ascidian: A Mechanism for the Apparent Intercalator-Induced Inhibition of Topoisomerase II

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Received May 2, 1994[⊗]

Several new pyridoacridine alkaloids, dehydrokuanoniamine B (**1**), shermilamine C (**2**), and cystodytin J (**3**), in addition to the known compounds cystodytin A (**4**), kuanoniamine D (**5**), shermilamine B (**6**), and eilatin (**7**), were isolated from a Fijian *Cystodytes* sp. ascidian. Their structures were determined by analyses of spectroscopic data. These compounds along with a previously reported pyridoacridine, diplamine (**8**), showed dose-dependent inhibition of proliferation in human colon tumor (HCT) cells *in vitro*. All compounds inhibited the topoisomerase (TOPO) II-mediated decatenation of kinetoplast DNA (kDNA) in a dose-dependent manner. The pyridoacridines' ability to inhibit TOPO II-mediated decatenation of kDNA correlated with their cytotoxic potencies and their ability to intercalate into calf thymus DNA. These results suggest that disruption of the function of TOPO II, subsequent to intercalation, is a probable mechanism by which pyridoacridines inhibit the proliferation of HCT cells. Incorporation studies show that pyridoacridines disrupt DNA and RNA synthesis with little effect on protein synthesis. It appears that DNA is the primary cellular target of the pyridoacridine alkaloids. These results are consistent with those for known DNA intercalators.

Introduction

A common target for many compounds that ultimately affect DNA replication is the enzyme topoisomerase (TOPO) II.[1-4] DNA topological transformations such as catenation, unwinding, and the reverse of these functions are controlled by topoisomerases.[5] The three-dimensional structure of DNA regulates many of its functions within the cell. Topoisomerases are ultimately involved in regulating the tertiary structure of DNA and are instrumental in maintaining chromosome structure and separating daughter chromatids during mitosis.[5,6] TOPO II acts by transiently breaking both strands of double-helix DNA, thereby allowing the topological transformations that are necessary for DNA replication.[6]

The high levels of TOPO II that exist in proliferating cells versus the lower levels present in nonreplicating cells result in some TOPO II inhibitors preferentially killing cancer cells,[4] presumably because of the greater need for cell replication machinery in the former. Drugs that interact with nucleic acids are therefore among the most useful cancer chemotherapeutic agents.[7] Intercalating compounds such as acridines, ellipticines, anthracyclines, actinomycins, and anthracenediones are known to induce TOPO II-mediated double-strand (ds) breaks in DNA.[1-4] Many intercalators are thought to stabilize the covalent enzyme–DNA "cleavable" complex by interfering with the DNA rejoining step catalyzed by TOPO II.[8,9] There is, however, a class of compounds that inhibits the catalytic activity of TOPO II without stabilizing cleavable complexes.[10,11] These compounds also inhibit the replication of nucleic acids. We report here that members of the pyridoacridine class of alkaloids isolated from marine ascidians inhibit the activity of TOPO II without affecting cleavable complex formation.

Our search for compounds from marine organisms with potential pharmacological utility has led to the isolation of a series of pyridoacridine alkaloids from a Fijian *Cystodytes* sp. ascidian: dehydrokuanoniamine B (**1**), shermilamine C (**2**), cystodytin J (**3**), and the known compounds cystodytin A[12] (**4**), kuanoniamine D[13] (**5**), shermilamine B[14] (**6**), and eilatin[15] (**7**). Initial interest in this organism stemmed from activity noted in the biochemical induction assay (BIA)[16] and the observed cytotoxicity of the crude extract against the human colon tumor cell line HCT116. Pyridoacridines derived from ascidians and sponges are reported to exhibit a variety of interesting properties including DNA intercalation,[11,17] TOPO II inhibition,[10] calcium release,[12] anti-HIV,[18] and *in vivo* antitumor[11] activities. Our pyridoacridines exhibited varying degrees of cytotoxicity toward human colon tumor (HCT) cells and were subsequently shown to both inhibit the function of TOPO II and intercalate into DNA. The ability of these compounds to inhibit TOPO II correlated with their ability to intercalate and inhibit the growth of HCT cells. Their capacity to intercalate into DNA, inhibit TOPO II, disrupt DNA and RNA synthesis, and inhibit replication of HCT cells is consistent with a cytotoxicity mechanism involving intercalation and disruption of TOPO II function. In this study, we examined this proposed mechanism for pyridoacridine-induced inhibition of HCT cell replication.

Chemistry

Isolation and Structure. Shipboard screening with the BIA[16] showed that the MeOH extract from a purple fleshy *Cystodytes* sp. ascidian[19,20] was capable of interacting with DNA.[21] Subsequent bioassay-guided frac-

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[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1994.

0022-2623/94/1837-3819$04.50/0

Table 1. NMR Data[a] for the TFA Salt of 1 in DMSO-d_6

atom no.	δ ^{13}C	δ ^{1}H	(mult, J(Hz))	HMBC[b] correlations
2	142.72	8.46	(d, 6.5)	C3, C3a, C12b
3	107.57	7.87	(d, 6.5)	C2, C3b, C12c
3a	148.50			
3b	114.13			
4	125.53	8.27	(d, 8.2)	C3a, C6, C7a
5	122.98	7.27	(ddd, 8.2, 5.3, 2.1)	C3b, C6, C7
6	135.30	7.72	(m)	C4, C7a
7	117.51	7.72	(m)	C3b, C5
7a	140.48			
8	–	11.71	(bs)	C3b, C12c
8a	132.80[c]			
9	108.44			
9a	143.15			
11	153.83	9.43	(s)	C9a,[d] C12a
12a	132.74[c]			
12b	131.80			
12c	118.25			
13	30.98	3.12	(t, 7.3)	C8a, C9, C9a, C14
14	36.19	3.31	(td, 7.3, 5.3)	C9, C16
15	–	8.35	(t, 5.3)	C14, C16
16	167.84			
17	118.08	5.63	(septet, 1.1)	C16, C18, C19, C20
18	150.30			
19	19.50	2.15	(d, 1.1)	C17, C18, C20
20	26.84	1.79	(d, 1.1)	C17, C18, C19

[a] Proton and carbon data were acquired at 500 and 125 MHz, respectively. [b] The HMBC experiment was optimized to observe $^{n}J_{CH}$ couplings of 8.5 Hz. [c] Interchangeable. [d] Observed only when the HMBC experiment was optimized to observe 5 Hz $^{n}J_{CH}$ couplings.

Table 2. NMR Data[a] for the TFA Salt of **2** in DMSO-d_6

atom no.	δ ^{13}C	δ ^{1}H	(mult, J(Hz))	HMBC[b] correlations
2	146.06	8.35	(d, 5.9)	C3, C3a, C13b
3	105.92	7.45	(d, 5.9)	C2, C3b, C13c
3a	144.48			
3b	114.74			
4	124.78	8.04	(d, 7.9)	C3a, C6, C7a
5	122.22	7.11	(dd, 7.9, 7.9)	C3b, C7
6	133.78	7.55	(dd, 7.9, 7.9)	C4, C7a
7	116.97	7.51	(d, 7.9)	C3b, C5
7a	140.09			
8	–	11.37	(bs)	
8a	131.45			
9	111.46			
9a	127.97			
11	29.49	3.58	(s)	C9a, C12
12	164.27			
13	–	9.64	(bs)	C9a, C11
13a	117.86[c]			
13b	130.56			
13c	117.14			
14	27.97	2.89	(t, 7.2)	C8a, C9, C9a, C15
15	36.61	3.08	(m)	C17
16	–	8.56	(t, 5.7)	C15, C17
17	168.31			
18	117.92[c]	5.70	(t, 1.1)	C17, C20, C21
19	150.81			
20	19.60	2.22	(s)	C18, C19, C21
21	26.95	1.84	(s)	C18, C19, C20

[a] Proton and carbon data were acquired at 500 and 125 MHz, respectively. [b] The HMBC experiment was optimized to observe $^{n}J_{CH}$ couplings of 8.5 Hz. [c] Interchangeable.

tionation of the MeOH extract of the frozen ascidian led to the isolation of a bright yellow alkaloid which was solely responsible for the BIA activity of the crude extract. This compound exhibited spectroscopic properties identical with those reported for eilatin (**7**), a dibenzotetraazaperylene pyridoacridine, previously isolated from a Red Sea *Eudistoma* sp. ascidian.[15] Further examination of the extract led to the isolation of several additional pyridoacridines (**1**–**6**) from this organism. These compounds were inactive in the BIA assay but showed significant cytotoxicity toward HCT cells *in vitro*.

Compound **1** was obtained as an orange amorphous solid. The molecular formula, $C_{23}H_{21}N_4OS$, was obtained by high-resolution FAB mass measurement. Due to its limited solubility, **1** was converted to its TFA salt for subsequent NMR studies. The extensive conjugation and heteroaromatic nature of **1** were evident from its UV spectrum and blue shift upon addition of acid. Analysis of the NMR data (Table 1) revealed four isolated spin systems, two exchangeable signals, and one downfield aromatic singlet. The signals at δ 8.27 (H4, d, J = 8.2 Hz), 7.27 (H5, ddd, J = 8.2, 5.3, 2.1 Hz), and 7.72 (H6 & H7, m) in the ^{1}H NMR spectrum (DMSO-d_6) were assigned to a 1,2-disubstituted benzene ring. Another spin system consisting of signals at δ 8.46 (H2, d, J = 6.5 Hz) and 7.87 (H3, d, J = 6.5 Hz) were indicative of a trisubstituted pyridine ring. A very strong nuclear Overhauser enhancement (NOE) correlation between δ 7.87 (H3) and 8.27 (H4) in the ROESY[22,23] spectrum of **1** facilitated assignment of these aromatic signals to adjacent rings. The proton-detected heteronuclear multiple bond correlation (HMBC)[24] experiment showed correlations between the quaternary carbon signal at δ 114.13 (C3b) and the exchangeable proton signal at δ 11.71 (H8) and also to the aromatic signals at δ 7.27 (H5) and 7.87 (H3). Additional HMBC correlations between the δ 118.25 (C12c) quaternary carbon and H8 as well as H3 were indicative of a pyridoacridine. Signals at δ 3.31 (H14, dt, J = 5.3, 7.3 Hz) and 3.12 (H13, t, J = 7.3 Hz) indicated a pair of coupled methylene groups, while those at δ 5.63 (H17, septet, J = 1.1 Hz), 2.15 (H19, d, J = 1.1 Hz), and 1.79 (H20, d, J = 1.1 Hz) suggested an isobutenyl group. In addition to the downfield shift of C20,[12] the presence of a significant ROESY correlation between H17 and H20 established that they were *cis* relative to each other. These data plus a singlet at δ 9.43 (H11) suggested a compound belonging the kuanoniamine class.[13,17] Assignments of C9a and C12a were based on HMBC correlations to H11 and chemical shift comparisons with literature values for these carbons.[13] The regiochemistry of the nitrogen and the sulfur of the thiazole ring and the correct assignments of the adjacent carbons have been firmly established for the kuanoniamine class.[13,17,25]

The NMR data for **2** (Table 2) were almost identical to those reported for shermilamine B (**6**),[14,26] with the greatest differences occurring in the regions of the spectrum corresponding to the side chain. Thorough examination of our data revealed that **2** possessed the same isobutenyl side chain found in **1**.

Similarly, the spectral data for **3** were virtually identical to those reported for other cystodytins (e.g., **4**).[12,27] The NMR data (Table 3) revealed that the differences lie in the side chain of the molecule with **3** possessing an acetyl side chain.

The additional pyridoacridines (**4**–**7**) showed spectroscopic properties matching those reported in the literature.[12–15] Pyridoacridine **8**, isolated from a *Diplosoma* sp. ascidian, was previously reported by our group.[28] All the pyridoacridine structures are presented in Figure 1.

Table 3. NMR Data[a] for **3** in $CDCl_3$

atom no.	δ ^{13}C	δ ^{1}H	(mult, J(Hz))	HMBC[b] correlations
1	131.87	8.29	(d, 8.1)	C3, C4a, C11a
2	131.87	7.94	(ddd, 8.1, 7.1, 1.4)	C4, C11a
3	129.83	7.83	(ddd, 8.1, 7.1, 1.4)	C1/C2, C4a
4	122.84	8.42	(d, 8.1)	C2, C4b, C11a
4a	121.78			
4b	136.92			
5	118.99	8.18	(d, 5.5)	C4a, C10b
6	149.76	8.94	(d, 5.5)	C4b, C5, C7a
7a	146.48			
8	183.31			
9	132.82	6.85	(s)	C7a, C10a, C12
10	152.17			
10a	150.33			
10b	117.84			
11a	145.32			
12	31.72	3.25	(t, 6.4)	C9, C10, C10a, C13
13	39.28	3.79	(dt, 5.9, 6.4)	C10, C12, C15
14	–	6.59	(bs)	C13, C15
15	170.43			
16	23.30	2.02	(s)	C15

[a] Proton and carbon data were acquired at 500 and 125 MHz, respectively. [b] The HMBC experiment was optimized to observe $^{n}J_{CH}$ couplings of 8.5 Hz.

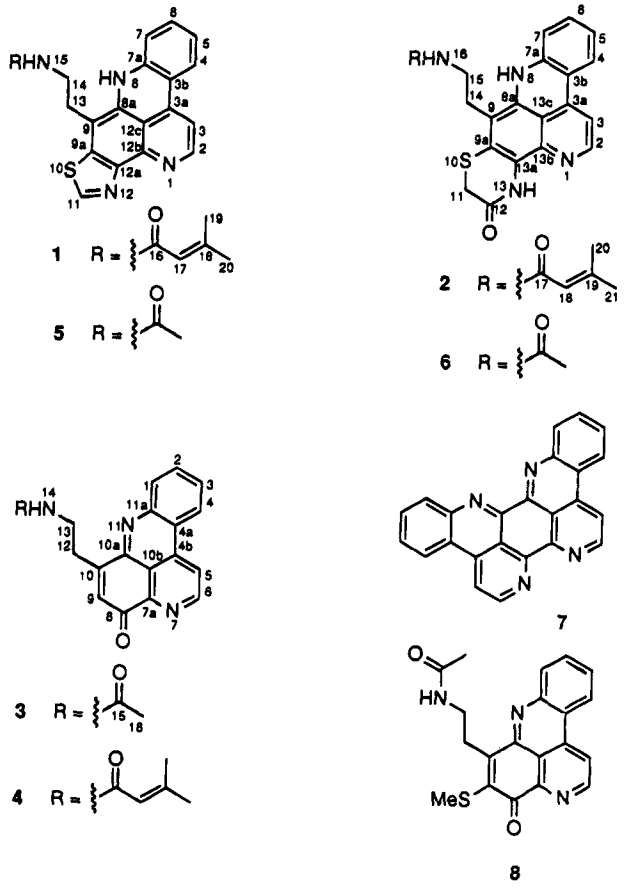

Figure 1. Structures of the pyridoacridine alkaloids. Numbering schemes for compounds 1–**3** parallel those published for compounds **5**, **6**, and **4**, respectively.

DNA Intercalation Studies. An ethidium bromide displacement assay was used to evaluate the relative ability of the pyridoacridines to interact with DNA.[29] When intercalated into DNA, ethidium bromide exhibits dramatically enhanced fluorescence along with a shift of its emission maximum.[30] When a competing intercalator displaces ethidium bromide from DNA, the fluorescence of ethidium bromide decreases. The fluorescence of ethidium bromide, determined in the presence of DNA as a function of pyridoacridine concentration, provides a measure of the relative intercalative ability of the compound.

Biology

HCT Cytotoxicity. To determine the relative ability of pyridoacridines to inhibit replication, they were evaluated using the MTT assay[31–33] for cytotoxicity against the human colon tumor cell line HCT116.

Biochemical Induction Assay. The BIA uses a λ-*lacZ* lysogenic *Escherichia coli* strain (BR513) that is sensitive to DNA-damaging agents.[16] β-Galactosidase, the product of the *lacZ* gene, is produced upon induction of the λ prophage by such agents. The level of β-galactosidase produced by a test compound is a direct measure of SOS[34] induction. Detection of β-galactosidase is accomplished by monitoring the production of a highly colored azo dye resulting from reaction of a bromonaphthol with a diazonium salt. The bromonaphthol is a product of β-galactosidase-catalyzed cleavage of the 6-bromo-2-naphthyl-β-D-galactopyranoside (BNG) substrate.

Decatenation Inhibition Assay. The relative ability of the pyridoacridines to inhibit TOPO II was measured *in vitro* using the decatenation inhibition assay. This assay measures a compound's ability to inhibit the TOPO II-catalyzed release of monomer-length DNA from high molecular weight kDNA.[35] The released monomer-length DNA can be resolved and quantitated on a 1% agarose gel, while the fully catenated kDNA is too large to penetrate into the gel. Enzyme inhibition is measured by monitoring the disappearance of the monomer-length DNA bands as a function of increasing compound concentration.

Differential Cytotoxicity. Double-strand break repair-deficient *xrs* Chinese hamster ovary (CHO) cell lines are sensitive to compounds that inhibit TOPO II by stabilizing cleavable complexes and inhibiting the rejoining reaction catalyzed by the enzyme.[36,37] The pyridoacridines were tested for their ability to exert enhanced toxicity toward the ds break repair-deficient xrs-6[38] CHO mutant line relative to the repair-proficient BR1[39] cell line. Toxicity is measured using a modification of the MTT tetrazolium salt colorimetric assay used to determine HCT cytotoxicity.[31–33]

DNA, RNA, and Protein Synthesis. Compounds **3** and **8**, which were the best intercalators, TOPO II inhibitors, and the most cytotoxic pyridoacridines of the series, were tested for their effects on the rates of DNA, RNA, and protein synthesis. The inhibition of incorporation of the radiolabeled precursors [^{3}H]leucine (protein), [^{3}H]uridine (RNA), and [^{3}H]thymidine (DNA) into HCT cells was used to access the effects of the pyridoacridines on macromolecular synthesis.

Results and Discussion

Inhibition of HCT Replication. The cytotoxicity assay provided a relative measure of the pyridoacridines toxic potencies against HCT cells *in vitro*. The results in Table 4 show that all compounds are active to varying degrees. Compounds **3** and **8** were the most potent; both inhibit HCT replication with IC_{50} values of less than 2 μM.

Biochemical Induction. Evaluation of the pyridoacridines in the BIA assay indicated that **7** showed a

Table 4. Cytotoxicity, Differential Toxicity, Topoisomerase Inhibition, and Intercalation of the Pyridoacridine Alkaloids and Control Compounds

compound	inhibition IC_{50}, μM HCT	inhibition IC_{50}, μM xrs-6	DC ratio[a]	topoisomerase II inhibition IC_{90},[b] μM	intercalation K,[c] μM
1	8.3	80.0	1	115	>100
2	16.3	8.1	1	138	>100
3	1.6	135.6	1	8.4	54
5	7.8	88.9	2	127	62
6	13.8	14.9	1	118	>100
7	5.3	ND[d]	ND[d]	ND[d]	>100
8	<1.4	71.2	1	9.2	21
etoposide	2.5	0.14	7	68	ND[d]
m-AMSA	6.3	0.24	4	33	ND[d]
mitoxantrone	ND[d]	0.001	9	1.1	ND[d]

[a] Differential cytotoxicity (DC) ratio = BR1 IC_{50}/xrs-6 IC_{50}. [b] Concentration at which 90% of monomer-length kDNA production is apparently inhibited. [c] Concentration of compound required to reduce ethidium bromide fluorescence to 50% of control (see Results and Discussion). [d] ND = not determined.

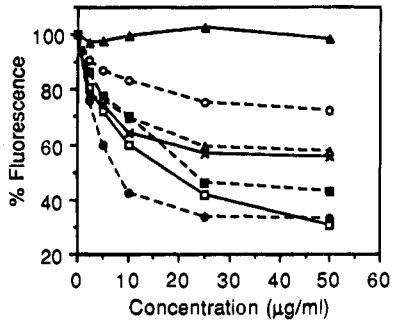

Figure 2. Normalized fluorescence for ethidium bromide in PBS (0.5 μg/mL) with calf thymus DNA and increasing concentrations of **1** (– –△– –), **2** (–▲–), **3** (–□–), **5** (– –■– –), **6** (– –○– –), **7** (–×–), and **8** (– –●– –). Excitation was at 530 nm with emission at 600 nm. The fluorescence in the presence of DNA and absence of compound represents 100%.

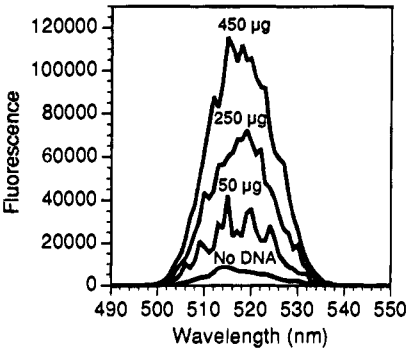

Figure 3. Fluorescence spectra of eilatin (**7**) in PBS solution (2.8 μM) with 50, 250, and 450 μg of calf thymus DNA. Excitation was at 520 nm.

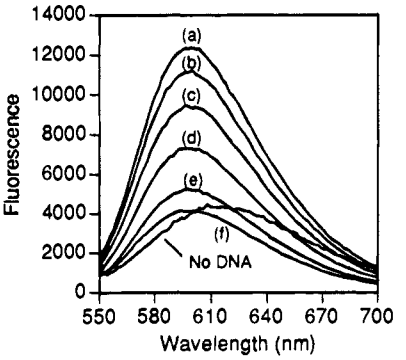

Figure 4. Fluorescence spectra of ethidium bromide in PBS solution (2.5 μM) with 50 μg of calf thymus DNA and 0.0 (a), 3.4 (b), 6.8 (c), 13.7 (d), 27.4 (e), and 68.5 (f) μM diplamine (**8**). Excitation was at 530 nm.

positive response at greater than 2 μg/disk, suggesting that it was capable of inducing an SOS response in *E. coli* cells.[16] The remaining compounds were inactive in this assay. Compound **7** is a 1,10-phenanthroline derivative and has two sets of nitrogen atoms capable of chelating Ni(II) ions.[15] 1,10-Phenanthrolines with the nitrogen atoms correctly situated for bidentate coordination are excellent ligands for chelating metal ions.[40] 1,10-Phenanthroline metal complexes have been reported to cleave ds DNA in an oxygen-dependent reaction, yielding products which are inhibitors of DNA polymerase I.[41] It has been shown that metal complexes of 1,10-phenanthroline bind to DNA by intercalation.[42] It is therefore not surprising that **7** showed activity in the BIA assay since the compound also intercalates into DNA (Figures 2 and 3). However, these data suggest that intercalation alone is not sufficient for the induction of an SOS response since other pyridoacridines which are inactive in the BIA are better DNA intercalators than **7** (Figure 2).

Intercalation. Figure 4 shows a series of fluorescence emission spectra for ethidium bromide measured in the presence of calf thymus (CT) DNA and various concentrations of the best intercalator, **8**. It is apparent that, upon increasing the concentration of **8**, there is a decrease in the fluorescence of ethidium bromide due to its displacement from DNA. For example, 69 μM **8** was sufficient to displace nearly all ethidium bromide from DNA, bringing its fluorescence to a level approaching that of the nonintercalated molecule.

The ethidium bromide displacement constants (K, Table 4) are measures of intercalation. Figure 2 and Table 4 show the trend in the ability of the compounds to intercalate into DNA with **3** and **8** being the most efficient intercalators. These compounds are also the most cytotoxic of the series. Equally important is that the less active compounds do not intercalate well into DNA. For example, **2** is the poorest intercalator and the least cytotoxic pyridoacridine followed by **6** which is the second to last in terms of cytotoxicity and intercalative ability.

Compound **7** fluoresces and produces an emission peak at 514 nm when irradiated at 520 nm. Figure 3 shows the increase in the fluorescence intensity of **7** at the emission maximum upon addition of CT DNA. Addition of 50 μg of CT DNA causes a 484% increase in the fluorescence of **7**. The fluorescence of **7** continues to increase as more DNA is added, similar to that of the intercalator ethidium bromide.[30] The increase in

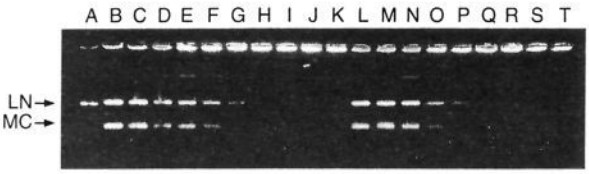

Figure 5. Agarose gel showing inhibitory effects of etoposide and **8** on TOPO II-catalyzed decatenation of kDNA. Lane A, linear monomer-length 2.5 kb DNA marker; lane B, control reaction (kDNA + TOPO II); lanes C–K, 8.5, 28.3, 42.5, 56.6, 84.9, 283.2, 424.7, 566.3, and 849.5 μM etoposide, respectively; lanes L–T, 1.4, 4.6, 6.8, 9.1, 13.8, 45.8, 68.5, 91.3, and 137.0 μM **8**, respectively. LN, linear DNA; MC, monomer circle DNA.

fluorescence intensity upon the addition of DNA provides additional evidence for intercalation.

On the basis of these data, it appears that for the pyridoacridines intercalation ability is essential for TOPO II inhibition and HCT cytotoxicity.

Inhibition of TOPO II Catalytic Activity. Figure 5 shows the abilities of etoposide and **8** to effect dose-dependent inhibition of TOPO II-catalyzed kDNA decatenation *in vitro*. In this assay, **8** (IC_{90} = 9.2 μM)[43] is a more potent inhibitor of TOPO II catalytic activity than the classic cleavable complex-stabilizing etoposide (IC_{90} = 68 μM). A relative ranking of the pyridoacridines' ability to inhibit TOPO II-catalyzed decatenation is provided in Table 4. The table gives the concentrations at which TOPO II inhibition was apparent (approximately 90% inhibition). While all compounds are capable of inhibiting decatenation, **3** and **8** are the most potent (IC_{90} = 8.4 and 9.2 μM, respectively) inhibitors while **2** and **6** are among the least potent (IC_{90} = 138 and 118 μM, respectively). This is consistent with their relative cytotoxicity and intercalative abilities.

The concentrations of compound required for enzyme inhibition are greater than those needed to inhibit HCT cell growth. This may be due to the large amount of DNA required for visualization and quantification and consequently the large amount of enzyme used for DNA cleavage.

Differential Cytotoxicity. Since many clinically useful compounds damage DNA or interfere with its replication, two cell lines with differing DNA repair capacities were used to evaluate the pyridoacridines. Enhanced toxicity toward the DNA ds break repair-deficient CHO xrs-6 cell line versus the repair-competent BR1 line indicates "cleavable complex"-mediated cytotoxicity; BR1/xrs-6 IC_{50} ratios greater than 3 are considered significant in this assay.[44–46] None of the pyridoacridines showed significant BR1/xrs-6 differential toxicity (Table 4), suggesting that no cleavable complex formation has occurred and that the compounds do not cause ds breaks in DNA.[36,37]

Cleavable Complex Formation. The involvement of cleavable complex formation in cell death is not supported by the results from the BR1/xrs-6 assay. However, the K^+-SDS assay was carried out to definitively determine if inhibition of TOPO II-mediated kDNA decatenation was associated with cleavable complex formation. Compound **8** produced no detectable protein–DNA complex above 1.4 μM, the concentration at which the compound inhibited HCT replication (data not shown). Lower concentrations of **8** were used to exclude the possibility of self inhibition[8] due to disruption of the enzyme–substrate interaction; however, cleavable complex formation could not be demonstrated at any level. Although it is not clear exactly how these compounds kill cells, protein-DNA covalent links or lesions resulting from disintegration of "cleavable complexes" are evidently not responsible for cytotoxicity. This and other results suggest that DNA, not the protein–DNA complex, is the target of the pyridoacridines.

Drug Effects on DNA, RNA, and Protein Synthesis. Assays to determine the effects of compounds **3** and **8** on cellular macromolecule synthesis were performed using 90–95% lethal drug concentrations (10 μg/mL). Dramatic effects on RNA and DNA synthesis were observed. RNA synthesis fell to approximately 50% of control levels in the first hour and continued to fall to approximately 10% of control levels by 9 h, while DNA synthesis fell from 100% of control levels in the first hour to below 50% by the third hour and to approximately 90% inhibition by hours 6 and 9 of drug treatment. No effect was observed on protein synthesis during the 9 h exposure period. These effects are similar to those observed in our laboratory for other DNA- or topoisomerase-targeting drugs (e.g., actinomycin D, mitoxantrone, and camptothecin) and are consistent with effects expected of topoisomerase inhibition.

Conclusions

The therapeutic utility of intercalating agents against a wide variety of tumors is well established, and there is continued interest in the antitumor and biochemical activities of these DNA active agents.[47] On the basis of the observed correlations between HCT cytotoxicity, TOPO II inhibition, and DNA intercalation, it was hypothesized that the pyridoacridines brought about cell death by inhibiting DNA interactive proteins (e.g., TOPO II) following intercalation. Our studies indicate that, as a result of intercalation, the pyridoacridines inhibit proliferation of HCT cells by interfering with nucleic acid structure and function. This interference is brought about partly by disruption of TOPO II enzyme function. We hypothesize that, by intercalating into DNA, the pyridoacridines disrupt the interaction between TOPO II and DNA, and consequently, the enzyme cannot carry out its normal functions during replication. The strong correlation between DNA intercalation, TOPO II inhibition, and cytotoxicity, in addition to the ability of these compounds to disrupt DNA synthesis, strongly supports this hypothesis.

The hypothesis suggests one possible cytotoxicity mechanism for these compounds and provides a reasonable explanation for our observations and those of others,[10] that pyridoacridines inhibit the enzyme topoisomerase II. Our findings are consistent with the report that dercitin, a member of the pyridoacridine class, does not significantly stabilize cleavable complexes.[25] The fact that these compounds showed neither enhanced toxicity toward DNA ds break repair-deficient CHO cell lines nor produced cleavable complexes suggests that they inhibit TOPO II catalytic activity not by producing a cleavable complex but by interacting with DNA itself. Since the pyridoacridines intercalate into DNA with high affinity and may change the topology of the molecule, it is likely that they inhibit other DNA-binding enzymes necessary for replication. Therefore, other enzymes such as polymerases or TOPO I may be

unable to bind or function properly due to the presence of intercalator molecules in the DNA.[48]

The relative cytotoxic, intercalative, and TOPO II inhibitory activities of the pyridoacridines give some indication of the effects of structural variation within this class. Inspection of the structures in Figure 1 reveals that **3** and **8** have only four rings and are iminoquinones. They are the best intercalators, and TOPO II inhibitors and the most potent cytotoxins of the series. The diminished potencies of the other pyridoacridines may result in part from steric effects of the additional ring(s), although the electronic effect of the iminoquinone may also be important. Since intercalation appears to be crucial for cytotoxicity, it understandable why **6** and **2** are among the least active in all the assays.

Although the TOPO II–DNA complex is considered the primary target of a number of DNA intercalators,[4] the results of our studies revealed that the pyridoacridines do not act by stabilizing cleavable complexes. Thus, intercalator-induced cytotoxicity involving TOPO II inhibition does not always involve cleavable complex formation. Therefore, in evaluating cytotoxic compounds that act as TOPO II poisons, neither inhibition of enzymatic activity, cytotoxic potency, nor intercalative ability alone should be taken as suitable criteria for predicting mechanism of action or chemotherapeutic potential.

In view of its activity in the BIA assay, **7** is one of the more interesting pyridoacridines. Compound **7** intercalates efficiently into DNA as evidenced by its fluorescent properties in the absence and presence of DNA and by its ability to displace ethidium bromide from DNA. (See Figures 2 and 3.) Compound **7** was also reported to chelate a metal ion.[15] Compound **7**, like other phenanthroline compounds, may cleave DNA by producing DNA-destructive hydroxyl radicals following intercalative binding to DNA.[42,49,50] Other pyridoacridines may have less hydroxyl radical-generating ability due in part to a diminished ability to chelate metal ions and are thus less able to nick DNA in a manner similar to **7**.

It is likely that the pyridoacridines as a class do not act by a single mechanism in causing cell death. By inhibiting TOPO II, the pyridoacridines can impair DNA synthesis, gene expression, chromosome segregation, and ultimately cell proliferation. We have provided evidence that TOPO II inhibition plays a part in pyridoacridine cytotoxicity. Despite the fact that many clinically useful anticancer drugs are intercalators, the mechanisms by which many of these compounds act are not well understood. It is known, for example, that not all intercalators give rise to TOPO II-mediated DNA breaks, despite binding to the same macromolecule (i.e., DNA). We can use the pyridoacridines, which have a reasonably well-defined mode of action, in conjunction with other compounds, as tools for probing the mechanism of cell death in aberrant eukaryotic cells. With this enhanced understanding, we may be able to design better anticancer compounds.

Experimental Section

Drugs, Chemicals, Reagents, Nucleic Acids, and Enzymes. The isolation and chemical characterization of the pyridoacridines are described below. Their purity was assessed by chromatographic and NMR analyses. Drug standards (bleomycin sulfate and etoposide), ethidium bromide, Fast Blue RR salt (4-(benzoylamino)-2,5-dimethoxybenzenediazonium chloride hemi[zinc chloride]), 6-bromo-2-naphthyl-β-D-galactopyranoside (BNG), tris(hydroxymethyl)aminomethane (trizma base), CT DNA, and MTT were purchased from Sigma Chemical Co., St. Louis, MO. Bacteriological agar was purchased from SCOTT Laboratories, Inc., Fiskerville, RI. Bacteriological media (peptone, yeast extract, glucose, and tryptone, etc.) were purchased from Difco Laboratories, Detroit, MI. All inorganic salts were purchased from Aldrich Chemical Co., Inc., Milwaukee, WI. Partially purified human (ovarian carcinoma) TOPO IIα was supplied to us by Dr. J. A. Holden of the University of Utah.

General Procedures. The ^1H and ^{13}C NMR spectra were obtained at 500 and 125 MHz, respectively, on a Varian Unity 500 spectrometer. Proton chemical shifts are reported in parts per million relative to residual undeuterated solvent. IR spectra were recorded on a Perkin-Elmer 1600 FT spectrophotometer. UV spectra were obtained in MeOH on a Beckman DU-8 spectrophotometer. High- and low-resolution FAB mass spectral measurements were made on a Finnigan MAT 95 or VG 7050E spectrometer. Fluorescence measurements were made on an PC1 spectrofluorometer equipped with a thermostated cuvette compartment and interfaced with an IBM computer. For ethidium bromide displacement studies, excitation wavelength was 530 nm while the emission spectra were recorded at a fixed wavelength of 600 nm or from 550 to 700 nm with resolution of 8 nm. For **7** fluorescence, excitation was at 520 nm with emission spectra recorded from 480 to 580 nm with resolution of 8 nm. All spectra were corrected for background fluorescence.

Cell Culture. Human colon carcinoma HCT116 cells were obtained from American Type Culture Collection (Rockville, MD). The HCT116 cells were grown as a monolayer in McCoy's medium containing 10% fetal bovine serum (BioFluids, Rockville, MD), 100 units/mL penicillin, 100 μg/mL streptomycin, and 50 units/mL nystatin. The DNA repair-proficient CHO line, BR1, was developed by our group[39] and expresses O^6-alkylguanine-DNA alkyltransferase in addition to normal CHO DNA repair enzymes. The CHO DNA ds break repair-deficient line, xrs-6, was a generous gift from Dr. P. Jeggo.[36] Both of these CHO cell lines were grown as monolayers in αMEM containing serum and antibiotics as above. All cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Before drug treatment, cells grown as monolayers were detached with trypsin treatment. Medium and antibiotics were obtained from Gibco Laboratories, Grand Island, NY.

Biochemical Induction Assay. *E. coli* strain BR513–84 was a generous gift from Lederle Laboratories, Pearl River, NY. The BIA spot test was a slight modification of that reported by Elespuru and White.[16] Briefly, an overnight culture of BR513-84 was diluted to an A_{600} of 5.7 and used to inoculate agar which was then placed on a bioassay plate prepared with a nutrient-containing basal layer. Test compounds applied to paper disks (Difco; 1/4 in. sterile) were placed on the bioassay plate which was then incubated for 3 h at 37 °C. β-Galactosidase production is a measure of the induction taking place during the incubation period. After removal of the disks, BNG substrate and Fast Blue RR salt were applied to the plate in an agar overlay. The BNG substrate was cleaved by β-galactosidase into a bromonaphthol product which couples with the diazonium salt, Fast Blue RR, to give a red/purple azo dye.

Cytotoxicity Assays. All the cell lines were adapted by us for use in the MTT microtiter plate tetrazolium cytotoxicity assay.[31–33] Test compounds were dissolved in 100% DMSO at 10 mg/mL and serially diluted. The final DMSO concentration in the cell culture wells was 1%.

HCT116, BR1, and xrs-6 cells (30 000, 30 000, and 40 000 cells/well, respectively) were seeded in 200 μL of growth medium in Corning 96-well microtiter plates and allowed to attach for 4 h. Each dose of drug was run in four replicate wells on the plate. Each plate also contained eight replicate control wells treated with DMSO only. The cell growth in the DMSO wells was used to determine the zero inhibition growth level for each microtiter plate in an experiment. Four hours

after seeding, 2 μL of DMSO or DMSO-containing drug was added to the cells. Drug exposure lasted for 18 h, after which the medium was removed and replaced with 200 μL of drug-free medium. The cells were refed on the third day of the assay with 100 μL of fresh complete McCoy's medium. This was followed by the addition of 11 μL of MTT solution (5 mg/mL in phosphate-buffered saline, pH 7.4) and 4 h further cell incubation. The MTT was reduced by viable cells to a purple formazan product.[32] The formazan product was solubilized by the addition, to each well, of 100 μL of 0.04 N HCl in 2-propanol. The absorbance at 540 nm for each well was measured using a BIO RAD MP450 plate reader. The average absorbance for each set of quadruplicate drug-treated wells was compared to the average absorbance of the control wells to determine the percent growth inhibition (fractional survival) at any particular drug dose.

K^+-SDS Protein–DNA Coprecipitation Assay. The K^+-SDS precipitation assay was adapted from methods previously reported.[51,52] BR1 cells were seeded into 25 cm^2 at a density of 40 000 cells/flask in complete αMEM. The next day, the cells were refed with 5 mL of complete McCoy's medium to which was added 0.5 μCi of [*methyl*-^{3}H]thymidine (76.3 Ci/mmol, obtained from New England Nuclear) and complete αMEM followed 1 h later by the addition of various concentrations of drug in DMSO or an equivalent DMSO concentration without drug (1%, v/v) for 1 h. Following drug treatment, the culture medium was removed and 1 mL of prewarmed (65 °C) lysis solution (1.25% SDS, 5 mM EDTA, pH 8.0, and 0.4 mg/mL salmon testis DNA) was added directly to the cells. The lysate was transferred to a prewarmed 1.5 mL microcentrifuge tube containing 250 μL of 325 mM KCl. The lysate was then sheered by vortex at the highest setting of a vortex mixer for 20 s and then cooled on ice for 10 min. The cooled lysate was centrifuged for 10 min in a microcentrifuge, the supernatant discarded, and the pellet redissolved in 0.8 mL of warm wash buffer (10 mM Tris buffer, pH 8.0, 100 mM KCl, 1 mM EDTA, and 0.1 mg/mL salmon testis DNA) and incubated for 10 min at 65 °C. The lysate was cooled, pelleted, and redissolved again before a final cooling, pelleting, and resuspension in 0.8 mL of wash buffer for counting by standard liquid scintillation counting techniques.

Assay for DNA TOPO II Catalytic Activity. The *in vitro* inhibition of TOPO II decatenation of kDNA from *Crithidia fasciculata* was carried out as described by Muller and co-workers.[35] Nuclear extracts of TOPO IIα were prepared from ovarian carcinoma tissue by the method of Holden and co-workers.[53] The pyridoacridines and standards were tested against the partially purified enzyme as described previously.[45] Briefly, 1 unit (~50 000 cell nuclei) of purified enzyme (enough to decatenate 0.25 μg of kDNA in 30 min at 34 °C) was used for each reaction. The reaction was carried out in a 0.5 mL microcentrifuge tube containing 19.5 μL of H_2O, 2.5 μL of 10× buffer (1× buffer contains 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, and 30 μg of BSA/mL), 1 μL of kDNA (0.2 μg), and 1 μL of DMSO-containing compound. This combination was mixed thoroughly and kept on ice. One unit of TOPO II was added immediately before incubation in a water bath at 34 °C for 30 min. Following incubation, the decatenation assay was stopped by the addition of 5 μL of stop buffer (5% sarkosyl, 0.0025% bromophenol blue, and 25% glycerol) and placed on ice. Electrophoresis of DNA was carried out on a 1% agarose gel in TAE buffer containing ethidium bromide (0.25 μg/mL). Ultraviolet illumination at a 310 nm wavelength allowed the visualization of DNA. The gels were photographed using a Polaroid Land camera.

Macromolecule Synthesis Inhibition Assay. The ability of test compounds to inhibit DNA, RNA, and protein synthesis was determined by pulse labeling drug-treated cell cultures with radioactive precursors. Human colon tumor cells (HCT116) were seeded into 96-well microtiter plates at a density of 8000 cells/200 μL well. The next day, cultures were treated with a concentration of drug estimated to be between 90% and 95% toxic (t = 0). Control wells were treated with an equivalent volume of DMSO (0.5% or 1%). DNA, RNA, and protein synthesis was measured by the addition of 0.5 μCi of [*methyl*-^{3}H]thymidine (76.3 Ci/mmol), 5,6-[^{3}H]uridine (38.5 Ci/mmol), or 4,5-[^{3}H]leucine (60.0 Ci/mmol; obtained from New England Nuclear), respectively, to four quadruplicate wells during the drug exposure. Precursors were added to the cells at t = 0, 3, 6, and 9 h. Two sets of controls were run with every experiment; these were pulse labeled at t = 0 and 3 h. Following a 1 h incubation with precursor, the cells were washed, supplied with fresh medium, and incubated for an additional 30 min. The medium from each well was then aspirated and cells were lysed by the addition of 100 μL of 0.1% SDS. The lysates from the four replicate wells were pooled in a 1.5 mL centrifuge tube. The culture wells were washed with another 100 μL of 0.1% SDS which were pooled with their respective lysates; 200 μL of 50% perchloric acid was then added to each microcentrifuge tube with mixing, and the samples were refrigerated overnight. The following day, the acid precipitates were vacuum filtered through Whatman GF/A glass microfiber filters. The filters were then washed with 0.8 mL of 10% perchloric acid and counted for radioactivity using standard liquid scintillation techniques.

Collection, Extraction, and Isolation Procedures. The brown, fleshy, encrusting ascidian, *Cystodytes* sp.,[20] was collected by SCUBA (−10 m) near Waya Island, Fiji, and kept frozen until extracted. The freeze-dried animal (182 g) was homogenized in a blender and extracted with MeOH–$CHCl_3$ (1:1, 5 × 900 mL) to give 2.8 g of crude solid after evaporation of the solvent. A portion of the crude extract was partitioned according to the Kupchan scheme.[54] The carbon tetrachloride soluble partition fraction was chromatographed on silica gel using a step gradient from 25% EtOAc in hexane to 100% EtOAc and then continuing to 50% MeOH in EtOAc. The mixture of compounds eluting from the silica column with 100% EtOAc was separated by HPLC (DYNAMAX-60 Å 8 μm amino, 10 mm i.d. × 250 mm column; 2.5% MeOH in CH_2Cl_2; 3.0 mL/min) to yield shermilamines C (**2**; 12.6 mg) and B[14] (**6**; 8.4 mg). Fractions eluting from the silica gel column with 10–30% MeOH in EtOAc contained a mixture of compounds which was resolved into dehydrokuanoniamine B (1; 3.7 mg), kuanoniamine D[13] (**5**; 10.5 mg), and eilatin[15] (**7**; 4 mg) by HPLC on amino support using 4% MeOH in CH_2Cl_2. BIA bioautography[16] on silica gel was used to guide the isolation of **7**. The $CHCl_3$ soluble Kupchan partition fraction was chromatographed on silica gel using the step gradient discussed above. The fraction eluting with 100% EtOAc containing crude cystodytin A was further purified by HPLC (DYNAMAX-100 Å 5 μm silica, 4.6 mm × 250 mm column; 5% MeOH in $CHCl_3$; 1.0 mL/min) to yield cystodytin A[12] (**4**; 3.5 mg). The fraction eluting from the silica gel column with 10% MeOH in EtOAc was further purified by silica gel HPLC as described above to yield cystodytin J (**3**; 4.2 mg).

Physical Properties. Dehydrokuanoniamine B (1): orange solid; IR (film) 3284, 3186, 3151, 3129, 3071, 3058, 3036, 3026, 2921, 2851, 1641, 1632, 1567, 1452 cm^{-1}; UV(MeOH) λ_{max} (ϵ) 237 (50 967), 261 (30 120), 344 (16 413), 359 (16 953), 452 (6047); UV(MeOH, TFA) λ_{max} (ϵ) 277 (43 640), 267 (20 720), 306 (40 333), 359 (11 447), 527 (5940); MS (FAB, glycerol) m/z 401 $(M + H)^+$, 302, 288; HR FAB MS (glycerol) m/z 401.1429 $(M + H)^+$, $C_{23}H_{21}N_4OS$ requires 401.1436; ^{1}H and ^{13}C NMR, Table 1.

Shermilamine C (2): orange solid; IR (film) 3285, 3203, 3052, 2925, 2854, 1634, 1433 cm^{-1}; UV(MeOH) λ_{max} (ϵ) 231 (9185), 279 (6669), 296 (5693), 350 (2683), 390 (1896), 463 (1664); UV(MeOH, TFA) λ_{max} (ϵ) 218 (12 414), 277 (6033), 300 (7491), 318 (8123), 359 (1703), 382 (1716), 538 (1423); MS (FAB, glycerol) m/z 431 $(M + H)^+$, 332, 318; HR FAB MS (glycerol) m/z 431.1529 $(M + H)^+$, $C_{24}H_{23}N_4O_2S$ requires 431.1542; ^{1}H and ^{13}C NMR; Table 2.

Cystodytin J (3): yellow solid; MS (FAB, 3-nitrobenzyl alcohol) m/z 318 $(M + H)^+$, 247; MS (FAB, glycerol) m/z 320 $((M + 2) + H)^+$, 247; HR FAB MS (glycerol) m/z 320.1390 $((M + 2) + H)^+$, $C_{19}H_{18}N_3O_2$ requires 320.1399; ^{1}H and ^{13}C NMR; Table 3.

Preparation of TFA Salts of 1 and 2. The compounds were dissolved in MeOH containing 0.05% TFA. Removal of the solvent under reduced pressure yielded magenta solids, the trifluoroacetate salts.

Acknowledgment. This work was supported by NIH Grant CA 36622. Partial funding for the Varian Unity 500 spectrometer was provided by NIH Grant S10 RR06262. L.A.M. acknowledges support from an NIH predoctoral fellowship, supported by Grant CA 36622. We thank the Ministry of Home Affairs, Fiji Islands, and the crew of the Mollie Dean for assistance in collecting the ascidian and Dr. F. Monniot, Muséum National d'Histoire Naturelle, Paris, France, for identification of the specimen. We thank Lederle Laboratories, Pearl River, NY, for providing the BIA cell line. We are grateful to Dr. Donald Blumenthal for assistance in acquiring the fluorescence data and Dr. Elliot Rachlin for acquiring mass spectrometric data.

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