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Highly Cytotoxic Benzo[*c*]pyrido[2,3,4-*kl*]acridines

Sarah Chackal,[a] Michael Facompré,[b] Raymond Houssin,[a] Jean-François Goossens,[a] Nicole Pommery,[a] Jean-Pierre Hénichart[a] and Christian Bailly[b,*]

[a]*Institut de Chimie Pharmaceutique Albert Lespagnol, EA 2692, Université de Lille 2, BP 83, 59006 Lille, France*
[b]*Inserm U-524 and Laboratoire de Pharmacologie Antitumorale du Centre Oscar Lambret, IRCL, Place de Verdun, 59045 Lille, France*

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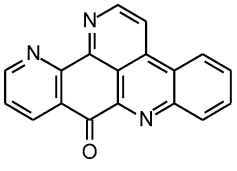
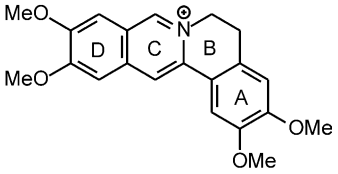
Abstract—Several benzo[*c*]pyrido[2,3,4-*kl*]acridines bearing different substituents on the A and E rings were synthesized and evaluated for their capacity to bind to DNA and to inhibit DNA topoisomerases. Potent cytotoxic compounds were discovered but no strict correlation with their DNA binding affinity and effects on topoisomerases were observed. DNA is one but not the unique target of these compounds.

The search for new pharmaceuticals from the marine environment has produced a large number of alkaloids containing a pyrido[2,3,4-*kl*]acridine skeleton.[1] Among them, ascididemin (ASC) is a planar pentacyclic aromatic DNA-intercalating agent isolated from *Cystodytes dellechiajei*[2] and exerts highly cytotoxic properties toward human colon (HCT 116) and breast (MCF 7) cancer cell lines[3] as well as human leukemia cells.[2] ASC is a weak inhibitor of topoisomerases,[4] but its precise mechanism of action remains poorly understood. Moreover, despite its interesting therapeutic profile, the toxicity toward non-tumor cells limits the therapeutic potential of this acridine derivative.

On the other hand, plants have also proved to be a rich source of alkaloids such as the protoberberines, analogues of berberine which is an isoquinoline widely used in chinese and indian folk medicine for many years.[5]



Ascididemin



5,6-Dihydro-8-desmethylcoralyne

Berberine-related isoquinolines include several naturally occurring and synthetic agents possessing a significant and specific pharmacological activity.[6,7] In particular, 5,6-dihydro-8-desmethylcoralyne (DHDMC) is a potent inducer of topoisomerase I-mediated DNA breaks[8] with atypical DNA binding properties. Indeed, a portion of the molecule intercalates into the double helix while the other moiety protrudes into the minor groove of DNA.[8]

These considerations prompted us to design a new series of potential DNA-binding and cytotoxic agents. We developed the benzo[*c*]pyrido[2,3,4-*kl*]acridine skeleton which combines the structural features of ASC and DHDMC: the isoquinoline skeleton of DHDMC was replaced by the quinoline heterocycle found in ASC and one ring was substituted with either a methoxy group, as in DHDMC, or with more extended side chains.

Here we report the synthesis of benzopyridoacridines **1**–**3** (Table 1) together with their capacity to interfere with topoisomerases and to bind DNA, along with their cytotoxicity activity toward human prostatic PC-3 cells.

Chemistry

The synthesis of **4**, **5** was accomplished as recently described,[9] in seven and eight steps respectively, starting from 2,3-dimethoxybenzoic acid. The Friedländer condensation of **4**, **5** with **6a**–**e** (azeotropic distillation of

*Corresponding author. Tel.: +33-320-16-92-18; fax: +33-320-16-92-29; e-mail: christianbailly@lille.inserm.fr

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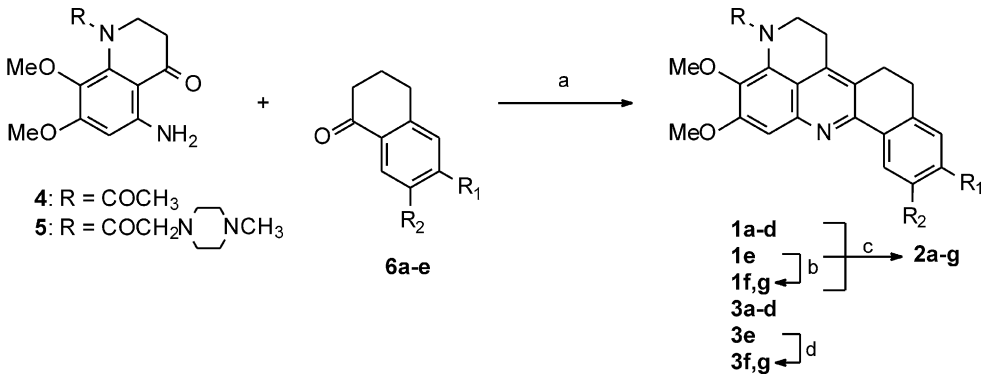
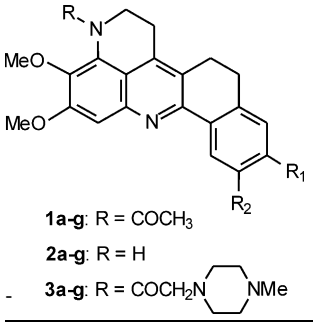


Scheme 1. Conditions and reagents: (a) PPTS, butan-1-ol, reflux (48–85%); (b) (i) $Cl(CH_2)_nNHCb_2NHCbz$, K_2CO_3, DMF, 80 °C (71–75%); (ii) Pd on C, $HCOONH_4$, MeOH, reflux (30–55%); (c) HCl 6N, reflux (35–54%); (d) (i) $Cl(CH_2)_nNHBoc$, K_2CO_3 DMF, 80 °C (82–87%); (ii) MeOH, HCl, rt (64–75%).

Table 1. Compounds **1–3**



	R_1	R_2
a	H	H
b	OMe	H
c	H	OMe
d	$O(CH_2)_3N$ NMe	H
e	OH	H
f	$O(CH_2)_2NH_2$	H
g	$O(CH_2)_3NH_2$	H

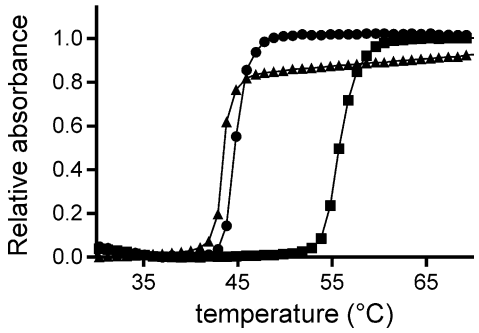


Figure 1. Thermal denaturation curves for poly(dAT)$_2$ in the absence (▲) and presence of (■) **3b** and (●) **3g** at a drug-DNA(nucleotide) ratio of 0.1. T_m measurements were performed in BPE buffer pH 7.1 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA).

water with butan-1-ol, in presence of pyridinium *p*-toluene sulfonate) gave the tetrahydrobenzo[*c*]pyrido[2,3,4-*kl*]acridines **1a–e** and **3a–e**, respectively. Acid hydrolysis of amides **1a–e** produced the secondary amines **2a–e** (Scheme 1) and *O*-alkylation of **1e** with *N*-benzylcarbamoyl-ε-chloroalkylamines followed by catalytic cleavage of Cbz group (Pd on C, ammonium formate) gave **1f**, **g** whose acidic hydrolysis provided **2f**, **g**. Benzopyridoacridines **3f**, **g** were obtained using the same strategy: in this case *O*-alkylation involved *N*-Boc protected ε-chloroalkylamines.

DNA-Binding

Melting temperature (T_m) and fluorescence measurements were carried out to evaluate the relative DNA binding affinities of the different compounds. The T_m analyses were performed with the polynucleotide poly(dAT)$_2$ which melted at a low temperature (42 °C) under our experimental conditions (Fig. 1).[10] Calf thymus (CT) DNA, used for the fluorescence assay, melted at a higher temperature (66 °C) and is therefore less convenient to use for T_m analyses. Moreover, poly (dAT)$_2$ gave monophasic melting curves, as illustrated in Figure 1, whereas biphasic melting profiles were observed with some compounds when using CT DNA.

Table 2. DNA binding and cytotoxicity

Compd	ΔT_m (°C)[a]	K_{app} (10^5 M^{-1})[b]	IC_{50} (nM)[c]
1a	nd	nd	$>10^3$
1b	nd	nd	$>10^3$
1c	nd	nd	$>10^3$
1d[d]	0.9	0.028	11.2
1e[d]	nd	nd	$>10^3$
1f[d]	2.1	0.042	$>10^3$
1g[d]	3.1	0.063	$>10^3$
2a[d]	0	0.036	$>10^3$
2b[d]	0	0.03	$>10^3$
2c[d]	0	0.024	$>10^3$
2d[d]	7.5	49.8	19.9
2e[d]	1.1	0.016	$>10^3$
2f[d]	12.1	20.1	$>10^3$
2g[d]	11.9	68.6	$>10^3$
3a[d]	0	<0.5	$>10^3$
3b[d]	0.9	<0.5	$>10^3$
3c[d]	0.9	<0.5	$>10^3$
3d[d]	9.9	30.4	$>10^3$
3e[d]	nd	nd	nd
3f[d]	11.9	45.1	$>10^3$
3g[d]	11.9	66.6	5.1

[a]Variation of the ΔT_m (T_m drug-DNA complex−T_m DNA alone) of the complexes between DNA and the test compounds.
[b]Apparent binding constant measured by fluorescence.
[c]Drug concentration that inhibits PC-3 cell growth by 50% after incubation for 72 h.
[d]Hydrochloride.

The ΔT_m values are collated in Table 2 along with the apparent binding constant obtained by using a competitive displacement fluorometric assay.[11] The results obtained by the two methods are consistent. In general, the compounds which gave high T_m values showed a strong capacity to displace ethidium bromide from its intercalation sites in DNA (Fig. 2). DNA binding affinities vary significantly from one compound to another but a direct SAR can be observed. The compounds equipped with a cationic side chain generally bind to DNA much more strongly than the corresponding unsubstituted analogues. For example, **3d** and **3g** with a piperazino- or amino- propyloxy chain at position R_1 form stable complexes with DNA whereas the analogues **3a**, **b** with no cationic chain at R_1 have little or no affinity for DNA.

The difference between the drugs is also clearly visible from the absorption spectral changes induced by DNA. Addition of DNA induces a marked hyperchromic effect at 420 nm with **3g** but not with **3b** (Fig. 3). In general, the agreement between the UV, T_m and fluorescence measurements is satisfactory and also consistent with additional analyses performed by surface plasmon resonance (SPR).[12] The biosensor analysis (not shown) indicated that **3g** binds to the sequences AATT and $[CG]_4$ with a binding constant of 2.87 10^4 and 4.88 10^4 M^{-1}, respectively.

Biological Evaluation

Compounds **1–3** were evaluated for in vitro cytotoxicity against the human prostate carcinoma PC-3 cell line using a conventional MTT assay.[13] IC_{50} values are collated in Table 2. Compounds **1d**, **2d** and **3g** proved to be extremely cytotoxic with IC_{50} values in the 5–20 nM range. A priori, no direct correlation can be established with their anti-topoisomerase activity (see below) and DNA binding affinity. The most cytotoxic compound **3g** shows a high affinity for DNA but this is the case also with **2g** which is inactive. DNA is certainly one but not the unique target for these cytotoxic agents.

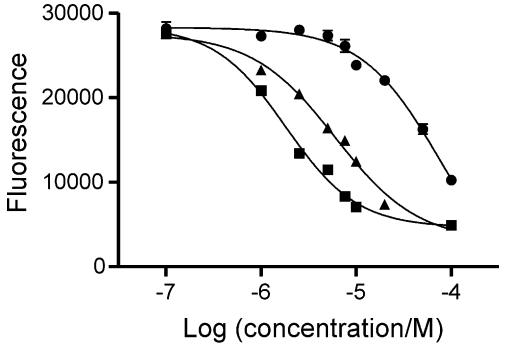


Figure 2. Fluorescence quenching of ethidium bromide bound to DNA by compounds (▲) **2f**, (■) **2e** and (●) **2g**. The fluorescence emission was measured at 595 nm with an excitation of 515 nm.

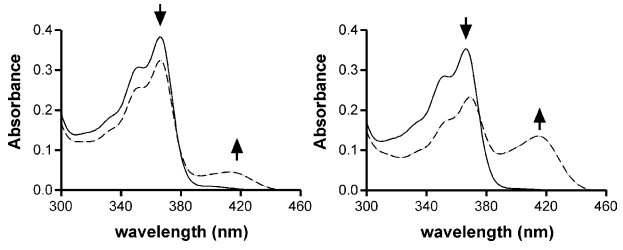


Figure 3. Absorption spectra of **3b** (left) and **3g** (right) (20 μM each) in the absence (solid line) and presence (dashed line) of CT DNA (20 μM), in 1 mM Na cacodylate, pH 7.0.

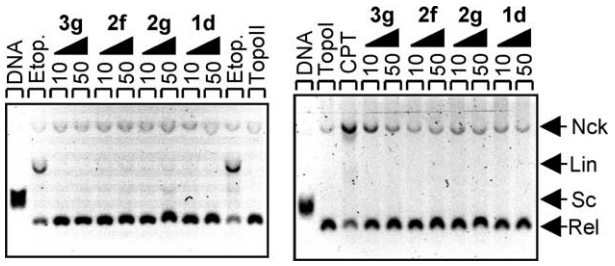


Figure 4. Effect of the drugs on *topoisomerases I* (right) and *II* (left). The drugs were tested at 10–50 μM. Camptothecin (CPT) and Etoposide (Etop.) were used as positive controls (50 μM each) for the poisoning of *topoisomerases I* and *II*, respectively. Nck, nicked; Lin, linear; Sc, supercoiled; Rel, relaxed DNA.

Effects on Topoisomerases

A relaxation assay[14] using supercoiled plasmid DNA was used to evaluate the effects of the compounds on the catalytic activity of human topoisomerases I and II. A set of data for selected compounds is presented in Figure 4. The drugs do not promote DNA cleavage by topoisomerase II, in contrast to etoposide and ASC. But, an inhibition of topoisomerase I, typified by a slight increase of the nicked DNA band, was observed with some compounds, such as **3g**. However, the amount of nicked DNA species remains weak compared to the reference drug camptothecin which produces a high level of single strand breaks. The weak inhibition of topoisomerase I observed with **3g**, but not **2g**, is not insufficient to explain the high cytotoxicity of these drugs. Targets other than topoisomerase I are likely implicated in mediating their cytotoxic activity.

Conclusion

A novel series of potent cytotoxic agents has been designed. The benzo[*c*]pyrido[2,3,4-*kl*]acridine chromophore, combining the molecular architecture of the alkaloids ASC and DHDMC, represents a promising molecular support for the discovery of novel antitumor agents and may be also useful to the discovery of topoisomerase I poisons.

Acknowledgements

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11. Binding studies were carried out by a competitive displacement fluorometric assay with DNA-bound ethidium. Fluorescence data were recorded at room temperature with a SPEX Fluorolog fluorometer. Excitation was set at 515 nm and fluorescence emission was monitored over the range 550–700 nm. Experiments were performed with an [ethidium]:[DNA] molar ratio of 12.6:10 and a drug concentration range of 0.01–100 μM in a BPE buffer pH 7.1. C_{50} values for ethidium displacement were calculated using a fitting function incorporated into Prism 3.0 and the apparent equilibrium binding constants (K_{app}) were calculated as follows: $K_{app} = (1.26\ \mu M/C_{50}) \times K_{ethidium}$, with $K_{ethidium} = 10^7\ M^{-1}$. Pavlov, V.; Kong Thoo Lin, P.; Rodilla, V. *Chem. Biol. Interact.* **2001**, *137*, 15. Baguley, B. C.; Denny, W. A.; Atwell, G. J.; Cain, B. F. *J. Med. Chem.* **1981**, *24*, 170.
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13. Cell culture and growth assays: human prostate cancer PC-3 cells were maintained in RPMI 1640 culture medium supplemented with 10% FCS. For growth assays, the cells were seeded onto 96-well plates at a density of approximately 3 10^4 cells/well. After 3 days, the cell medium was changed to serum-free medium and the cells were starved for 24 h for the culture synchronisation. The stimulation of the growth of quiescent cells was then performed by 10 ng/mL EGF plus TSe (50 ng/mL transferrin and 50 pg/mL selenium) and the tested compounds were added to culture medium. After an additional 72 h, the cell growth was estimated by the colorimetric MTT test. Hirst, S. J.; Barnes, P. J.; Twort, C. H. C. *Am. J. Resp. Cell Mol. Biol.* **1992**, *7*, 574.
14. For the DNA relaxation experiments. Supercoiled pKMp27 DNA (0.5 μg) was incubated with 4 units human topoisomerase I or II (TopoGen Inc.) at 37 °C for 1 h in relaxation buffer (50 mM Tris pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA) in the presence of varying concentrations of the drug under study. Reactions were terminated by adding SDS to 0.25% and proteinase K to 250 μg/mL. DNA samples were then added to the electrophoresis dye mixture (3 μL) and electrophoresed in 1% agarose gels containing ethidium bromide (1 μg/mL) at room temperature for 2 h at 120 V. Gels were washed and photographed under UV light. Bailly, C. *Methods Enzymol.* **2002**, *340*, 610.