Biochemical Pharmacology, Vol. 57, pp. 1141–1145, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00018-0

SHORT COMMUNICATION

Topoisomerase II Inhibition by Aporphine Alkaloids

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ABSTRACT. The aporphine alkaloids (+)-dicentrine and (+)-bulbocapnine are non-planar molecules lacking features normally associated with DNA binding by intercalation or minor groove binding. Surprisingly, dicentrine showed significant activity as a topoisomerase II (EC 5.99.1.3) inhibitor and also was active in a DNA unwinding assay. The DNA unwinding suggests DNA intercalation, which could explain the inhibition of topoisomerase II. Bulbocapnine, which differs from dicentrine only by the presence of a hydroxyl group at position 11 and the absence of a methoxyl group at position 9, was inactive in all assays. Molecular modeling showed that dicentrine can attain a relatively planar conformation, whereas bulbocapnine cannot, due to steric interaction between the 11-hydroxyl group and an oxygen of the methylenedioxy ring. These observations suggest that dicentrine is an "adaptive" DNA intercalator, which can bind DNA only by adopting a somewhat strained planar conformation. The requirement of a suboptimal conformation to achieve DNA binding appears to make dicentrine a weaker topoisomerase II inhibitor than the very planar oxoaporphine alkaloid liriodenine. These results suggest that it may be possible to modulate DNA binding and biologic activity of drugs by modifications affecting their ability to adopt planar conformations. BIOCHEM PHARMACOL **57**;10:1141–1145, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. topoisomerase II; topoisomerase inhibitor; aporphine alkaloid; dicentrine; DNA intercalator; intercalation; DNA binding

The recent identification of liriodenine as a strong topoisomerase II catalytic inhibitor and a topoisomerase II poison [1] led us to investigate other aporphine alkaloids as topoisomerase II inhibitors (Fig. 1). Liriodenine, like many strong topoisomerase II inhibitors, is a very planar molecule and a likely DNA intercalator. However, extensive saturation and an *N*-methyl substituent make aporphine alkaloids such as dicentrine and bulbocapnine very non-planar and would seem to make them poor candidates for DNA binding by intercalation. Bulbocapnine was inactive, but dicentrine showed surprising inhibition of topoisomerase II, which was associated with a DNA binding mode causing extensive DNA unwinding. Modeling shows that dicentrine can adopt a flat conformation that might allow DNA intercalation, but steric interaction between the 11-hydroxyl group and the methylenedioxy ring locks bulbocapnine into a non-planar conformation unsuitable for DNA intercalation. The implications of these results for drug design are discussed.

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§ *Abbreviations*: SV40, simian virus 40; Tdr, thymidine; kDNA, kinetoplast DNA; and ICRF-193, meso-2,3-bis(2,6-dioxopiperazine-4-yl)butane.

Received 25 June 1998; accepted 4 November 1998.

MATERIALS AND METHODS

Compounds and Molecular Modeling

(+)-Dicentrine (9,10-dimethoxy-1,2-methylenedioxyaporphine) from *Stephania* spp. was purchased from NADUCS, Inc., and the structure was confirmed by MS, NMR spectral analysis, and comparison with literature data [2]. (+)-Bulbocapnine HCl from *Corydalis cava* (Fumariaceae) was purchased from the Sigma Chemical Co. Bulbocapnine free base was prepared by dissolving in water, adjusting the pH to 9.0 with ammonium hydroxide solution, extracting with dichloromethane, drying with sodium carbonate, and filtering. The filtrate was then concentrated to obtain the free base, and the structure was confirmed by MS and NMR spectral analysis [3]. Molecular modeling was done using Molecules-3D version 2.10D (Molecular Arts), which uses the DREIDING force field method of optimizing molecular structures [4].

Cell Culture, Virus Infection, and In Vivo *Topoisomerase II Inhibition*

Growth of African green monkey cells (CV-1) and infection with SV40§ have been described [1]. SV40 DNA replication intermediates were pulse-labeled with [*methyl*-^{3}H]Tdr, purified, separated by agarose gel electrophoresis, and visualized by gel fluorography [5, 6]. Topoisomerase II

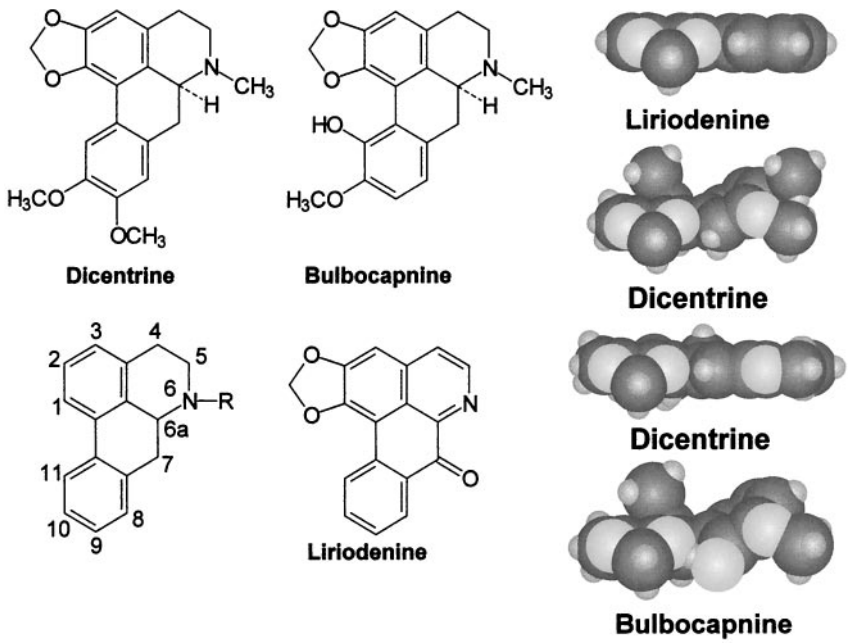

FIG. 1. Molecular structures of dicentrine, bulbocapnine, and liriodenine. The space-filling model of dicentrine is shown before and after rotations around single bonds to achieve a planar conformation.

inhibition is detected as a concentration-dependent increase in the catenated SV40 daughter chromosomes [7, 8] since the final (decatenation) step of SV40 DNA replication is carried out by topoisomerase II. The GF/C filter assay for topoisomerase-DNA cross-links stable to strong denaturants has been described [9]. This assay is used to measure topoisomerase poisoning *in vivo* and *in vitro* [1].

In Vitro *Topoisomerase II Assays*

The assay for catalytic inhibition of topoisomerase II measures decatenation of highly catenated kDNA networks by purified topoisomerase II [1, 7]. Topoisomerase II and kDNA were from TopoGen. The topoisomerase II poisoning assay measures cross-linking of purified topoisomerase II to purified [^{3}H]Tdr-labeled SV40 DNA [1].

Assay for DNA Unwinding

Supercoiled (form I) or relaxed (form II) pUC19SV40 DNA (the simian virus 40 genome cloned into the plasmid pUC19) (0.05 μg) was incubated with excess human topoisomerase I (TopoGen) for 30 min at 37°. The reaction mixture contained 10 mM Tris–Cl, pH 7.5, 150 mM NaCl, 0.1 mM spermidine, 1 mM EDTA, 5% glycerol, 0.1% BSA (Sigma), and various concentrations of drugs. Drugs were dissolved in DMSO, and all samples, including controls without drugs, contained 5% DMSO. The reactions were stopped by the addition of stop/loading dye (1% sarkosyl, 0.025% bromophenol blue, and 5% glycerol), and DNA was extracted with chloroform:isopropanol (24:1) to remove the drug from the reaction mixture. Samples were electrophoresed through a 1% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA). Gels were stained with ethidium bromide (0.5 μg/mL), destained, and photographed on a UV transilluminator. The unwinding assay was modified from that used previously [7, 10].

RESULTS AND DISCUSSION

Dicentrine inhibited the topoisomerase II-dependent decatenation step of SV40 DNA replication, as shown by the concentration-dependent increase in the intensity of the A-, B-, and C-family catenated SV40 daughter chromosomes (Fig. 2). Increasing inhibition of topoisomerase II increases the catenation linking number of the viral chromosomes, causing bands with progressively higher electrophoretic mobility [11, 13]. There was also a marked accumulation of pulse label in the late replication intermediates (LC, Fig. 2). The two phenomena are thought to be linked mechanistically [8]. No protein-DNA cross-links were detected by the GF/C filter assay (data not shown), indicating that dicentrine is a catalytic topoisomerase II inhibitor but not a topoisomerase II poison [14]. *In vitro* assays with purified topoisomerase II confirmed that dicentrine is a strong catalytic inhibitor of topoisomerase II with an IC_{50} of 27 μM, but is not a topoisomerase II poison (data not shown).

Bulbocapnine was found to be completely inactive in the *in vivo* (SV40) assay, where it caused neither accumulations of catenated SV40 dimers nor protein-DNA cross-links in either the HCl form (data not shown) or in the free base form (Fig. 2). Both the bulbocapnine HCl and free base forms were also inactive in *in vitro* assays for topoisomerase II inhibition and topoisomerase poisoning (data not shown).

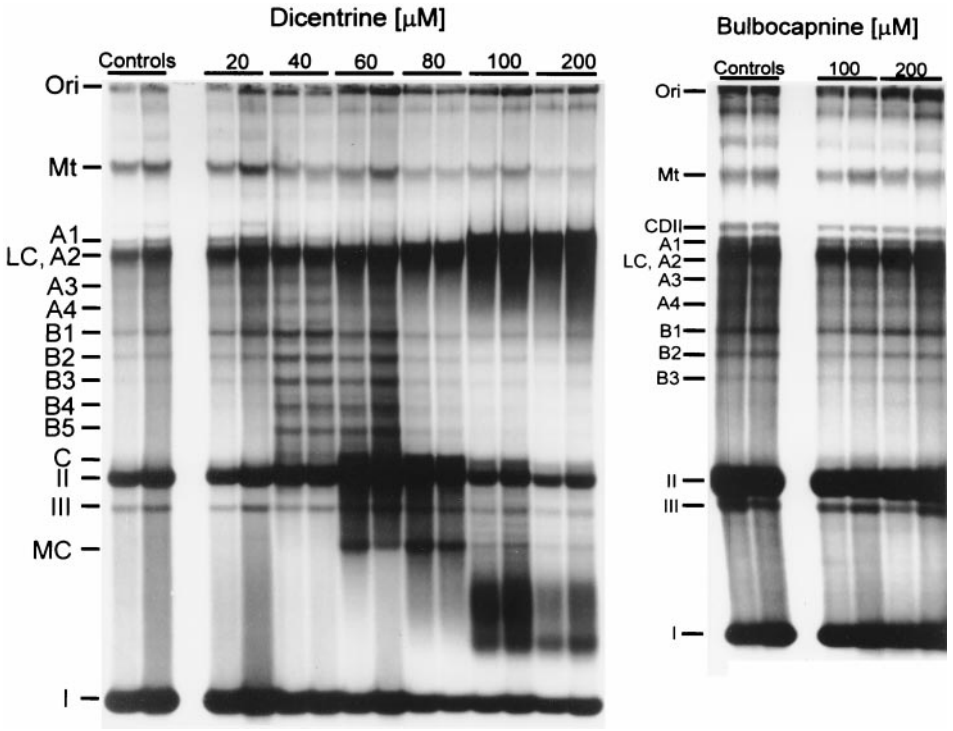

FIG. 2. Dicentrine inhibition of topoisomerase II in SV40-infected monkey kidney cells. Key: Controls, solvent (DMSO) at the highest concentration used in the study; Ori, origin of electrophoresis; Mt, mitochondrial DNA; LC, late Cairns structure (a late "figure eight" replication form in which the terminal 5% of the chromosome remains to be replicated); B_1–B_5, B-family catenated dimers (topologically linked daughter chromosomes in which one daughter chromosome is superhelical and one is relaxed due to a single-strand DNA break) with catenation linking numbers of 1 through 5, respectively; II, form II (nicked circular) SV40 genomes; III, form III (double-stranded linear viral genomes); I, form I (superhelical circular) SV40 genomes. MC is the point at which B-family catenated dimers are no longer resolved from one another. It appears as a pseudo-band on one-dimensional gel patterns. The material between MC and form I in some lanes is caused by DNA intercalating topoisomerase II inhibitors [11] but not by the non-intercalating inhibitor ICRF-193 [12] and so may represent additional DNA unwinding due to intercalation. Bulbocapnine does not inhibit topoisomerase II *in vivo*.

Since topoisomerase II inhibitors are often DNA intercalating drugs that unwind the Watson–Crick DNA helix, dicentrine was tested for DNA unwinding. In the presence of purified topoisomerase I, dicentrine did unwind DNA (Fig. 3, lanes 5 and 6). The introduction of supercoils into covalently closed, relaxed DNA circles by dicentrine in the presence of topoisomerase I (Fig. 3, lanes 13–16) shows that the effects of dicentrine in lanes 5 and 6 were not due to inhibition of topoisomerase I. These results show that maximal DNA unwinding was reached at the lowest ethidium and dicentrine concentrations tested (lanes 3 and 5). At these concentrations of ethidium and dicentrine, it is likely that all DNA binding sites are occupied. A lower concentration of ethidium caused partial unwinding in the bulbocapnine experiment. Bulbocapnine, even at high concentrations, did not unwind DNA (compare lane 2 with lanes 5–8). DNA unwinding is not unique to intercalators. Minor groove binding drugs such as netropsin have been shown to change the superhelicity of DNA. However, dicentrine does not have the extended, flexible structure and positive charges typical of minor groove binding drugs such as netropsin and 4′,6-diamidino-2-phenylindole (DAPI) [15]. Although these results implicate DNA intercalation in the inhibition of topoisomerase II by dicentrine, intercalation has not been proven, and inhibition by direct binding to topoisomerase II has not been disproven.

Liriodenine, like many other strong topoisomerase II inhibitors, is very planar (Fig. 1) and a likely DNA intercalator. The 10-hydroxyl and 10-methoxyl analogs of liriodenine also have been shown to inhibit topoisomerase II *in vitro* [16]. Although dicentrine unwinds DNA, it is not planar and does not seem to be a good candidate for DNA binding by the intercalative mode. Efficient, classic intercalators generally have two or three fused aromatic rings [17]. Dicentrine and bulbocapnine have only two aromatic rings, and they are twisted and bent with respect to one another because of the saturated rings that separate them. In addition, these two aromatic rings are surrounded by bulky groups such as the methylenedioxy group, methoxyl groups, and saturated rings, including a saturated heterocyclic ring with an *N*-methyl group. These bulky groups seem likely to interfere with any activity as a "minimal" or "partial" intercalator [18, 19]. However, dicentrine is flexible and can adopt a strained planar conformation by rotations around the single bonds at the 6a and 7 carbons of the saturated rings. Although intercalation is associated normally with very planar fused aromatic ring systems, intercalation by molecules without fused aromatic rings is

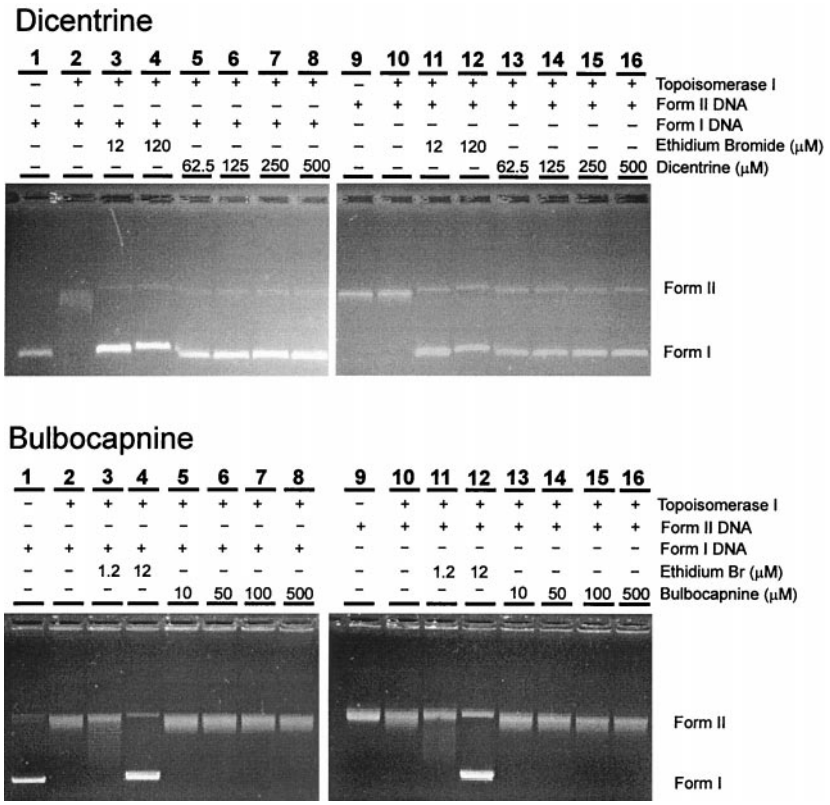

FIG. 3. DNA unwinding assays for dicentrine and bulbocapnine. Superhelical DNA (form I) was the substrate in reactions 1–8, and covalently closed relaxed circular DNA (form II) was the substrate for reactions 9–16 in both experiments.

possible. Pyrylium and thiopyrylium dyes, whose aromatic rings are linked by single bonds, can intercalate into DNA by assuming a flat geometry that brings their aromatic rings into the same plane [20]. Bulbocapnine, the inactive compound, cannot attain a planar conformation because of steric hindrance between the 11-hydroxyl group and the methylenedioxy ring (Fig. 1, bottom). Modeling also shows that a hydrogen bond between the 11-hydroxyl group and the oxygen in the methylenedioxy ring may be possible. A hydrogen bond might further stabilize the bent and twisted conformation.

A structure–activity relationship for inhibition of topoisomerase II by aporphine alkaloids is evident. Liriodenine, a very flat molecule, is a very strong topoisomerase II inhibitor (IC_{50} = 0.11 μM). Dicentrine, a molecule that can adopt a relatively flat conformation, is a strong topoisomerase II inhibitor, yet is significantly weaker than liriodenine (IC_{50} = 27 μM). Bulbocapnine, a molecule that cannot adopt a flat conformation, is inactive as a topoisomerase II inhibitor and does not unwind DNA. The requirement of a strained planar conformation for DNA intercalation by dicentrine would be expected to result in weaker DNA binding and topoisomerase II inhibition as compared with the normally planar liriodenine. Structural modifications making the planar conformation more strained should result in a weaker DNA intercalation and topoisomerase II inhibition. Our results suggest that it may be possible to modulate the biological activities of some drugs by modifications affecting their ability to achieve a planar conformation suitable for DNA intercalation. Control of this variable might also prevent topoisomerase II-directed cytotoxicity by drugs with other molecular targets.

This study was supported by grants from the Public Health Service: CA60914 to R.S. and P30CA16058 to the Ohio State University Comprehensive Cancer Center. We thank TopoGen for the gift of topoisomerase II and kDNA, and Edith F. Yamasaki for help with the manuscript.

References

1. Woo SH, Reynolds MC, Sun NJ, Cassady JM and Snapka RM, Inhibition of topoisomerase II by liriodenine. *Biochem Pharmacol* **54:** 467–473, 1997.
2. Lu ST, Wang SJ, Lai PH, Lin CM and Lin LC, Studies on the alkaloids of Formosan Lauraceous plants. XV. Alkaloids of *Lindera oldhamii* Hemsl. *Yakugaku Zasshi* **92**: 910–917, 1972.
3. Shafiee A, Ghanbarpour A, Lalezari I and Lajevardi S, Alkaloids of *Papaver* genus. XI. Alkaloids of *Glaucium vitellinum*, population Isfahan. *J Nat Prod* **42**: 174–178, 1979.
4. Mayo SL, Olafson BD and Goddard WAI, DREIDING: A generic force field for molecular simulations. *J Phys Chem* **94**: 8897–8909, 1990.
5. Snapka RM, Permana PA, Marquit G and Shin C-G, Two-dimensional agarose gel analysis of simian virus 40 DNA replication intermediates. *Methods* **3:** 73–82, 1991.
6. Snapka RM, Analysis of SV40 DNA replication intermediates. In: *The SV40 Replicon Model for Analysis of Anticancer*

Drugs (Ed. Snapka RM), pp. 15–37. Academic Press, San Diego, 1996.
7. Permana PA, Snapka RM, Shen LL, Chu DTW, Clement JJ and Plattner JJ, Quinobenoxazines: A class of novel antitumor quinolones and potent mammalian DNA topoisomerase II catalytic inhibitors. *Biochemistry* **33**: 11333–11339, 1994.
8. Snapka RM and Yamasaki EF, Topoisomerase II and termination of DNA replication. In: *The SV40 Replicon Model for Analysis of Anticancer Drugs* (Ed. Snapka RM), pp. 65–100. Academic Press, San Diego, 1996.
9. Shin C-G, Strayer JM, Wani MA and Snapka RM, Rapid evaluation of topoisomerase inhibitors: Caffeine inhibition of topoisomerases *in vivo*. *Teratog Carcinog Mutagen* **10**: 41–52, 1990.
10. Pommier Y, Covey JM, Kerrigan D, Markovits J and Pham R, DNA unwinding and inhibition of mouse leukemia L1210 DNA topoisomerase I by intercalation. *Nucleic Acids Res* **15**: 6713–6731, 1987.
11. Snapka RM, Powelson MA and Strayer JM, Swiveling and decatenation of replicating simian virus 40 genomes *in vivo*. *Mol Cell Biol* **8:** 515–521, 1988.
12. Snapka RM, Woo SH, Blokhin AV and Witiak DT, Inhibition of topoisomerase II by ICRF-193, the meso isomer of 2,3-bis(2,6-dioxopiperazin-4-yl)butane. Critical dependence on 2,3-butanediyl linker absolute configuration. *Biochem Pharmacol* **52**: 543–549, 1996.
13. Permana PA, Ferrer CA and Snapka RM, Inverse relationship between catenation and superhelicity in newly replicated simian virus 40 daughter chromosomes. *Biochem Biophys Res Commun* **201**: 1510–1517, 1994.
14. Chen AY and Liu LF, DNA topoisomerases: Essential enzymes and lethal targets. *Annu Rev Pharmacol Toxicol* **34**: 191–218, 1994.
15. Störl K, Burckhardt G, Lown JW and Zimmer C, Studies on the ability of minor groove binders to induce supercoiling in DNA. *FEBS Lett* **334**: 49–54, 1993.
16. Harrigan GG, Gunatilaka AA, Kingston DGI, Chan GW and Johnson RK, Isolation of bioactive and other oxoaporphine alkaloids from two annonaceous plants, *Xylopia aethiopica* and *Miliusa* cf. *banacea*. *J Nat Prod* **57**: 68–73, 1994.
17. Atwell GJ, Bos CD, Baguley BC and Denny WA, Potential antitumor agents. 56. "Minimal" DNA-intercalating ligands as antitumor drugs: Phenylquinoline-8-carboxamides. *J Med Chem* **31**: 1048–1052, 1988.
18. Gabbay EJ and DePaolis A, Topography of nucleic acid helices in solutions. Steric requirements for intercalation. *J Am Chem Soc* **93**: 562–564, 1971.
19. Gabbay EJ, Scofield RE and Baxter CS, Steric effects on the intercalation of aromatic cations to deoxyribonucleic acid. *J Am Chem Soc* **95**: 7850–7858, 1973.
20. Yamamoto N, Okamoto T and Kawaguchi M, Novel intercalators of pyrylium dye into double-stranded DNA. *Nucleic Acids Symp Ser* **29**: 83–84, 1993.