

NMR Studies on Antitumor Drug Candidates, Berberine and Berberrubine

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Berberine and berberrubine, which display antitumor activity, have also demonstrated distinct enzyme-poisoning activities by stabilizing topoisomerase II-DNA cleavable complexes. The protoberberine berberrubine differs in chemical structure with berberine at only one position, however, it shows a prominent activity difference from berberine. Solution structures of berberine and berberrubine determined by NMR spectroscopy are similar, however, the minor structural rearrangement has been observed near 19 methoxy or hydroxyl group. We suggest that the DNA cleavage activities of topoisomerase II poisons could be correlated with both chemical environments and minor structural change together with hydrophobicity of interacting side chains of drugs with DNA molecule.

Keywords : Berberine, Berberrubine, Anti-tumor drug, ROESY, NMR.

Introduction

One of the most important molecular targets for anti-tumor drugs is DNA topoisomerase II, which forms a covalent linkage to both strands of the DNA helix by breaking and resealing the sugar-phosphate backbone bonds of DNA.^{1,2} Moreover, it has been reported that anti-tumor agents^{3,4,5} stabilize the covalent topoisomerase II-associated DNA complexes^{6,7} in eukaryotic systems^{8,9} which cleave and religate the DNA. Berberine (Figure 1A), a plant alkaloid, has been used in Ayurvedic and Chinese medicine for many years. The berberine alkaloid found in the roots, rhizomes, and stem bark of *Berberis vulgaris* L. plants has demonstrated significant antimicrobial activity against a variety of organism including bacteria, viruses, fungi, protozoans and chlamydia.^{10,11,12} Recently, it has been reported that berberine possesses anti-tumor properties^{10,11} and the 19-position in berberine analogues is an important determinant of DNA topoisomerase II inhibition,¹³ demonstrating that the protoberberine, berberrubine (Figure 1B) induces topoisomerase II-mediated DNA cleavage.¹⁴ Recent studies already demonstrated that berberrubine exhibits anti-tumor activity in animal models and they suggest that the hydroxyl group at the 19-position of berberrubine is essential for anti-tumor activity.^{15,16,17}

In this report, we present detailed NMR studies for both berberine and berberrubine related with their biological activities by two dimensional nuclear magnetic resonance (NMR) spectroscopy.

Experimental Section

Berberine was purchased from Sigma and berberrubine is derived from berberine as described.¹⁸ The (GCCGTCGTTTACA)₂ of DNA which contains cleavage sites of berber-

rubine was purchased from J. L. Science (Taejon, Korea). Etoposide and topoisomerase II were also purchased from TopoGEN (Columbus, Ohio). For drug titration experiments, 5 mg of DNA was dissolved in 50 mM phosphate, 90% H₂O/10% D₂O solution.

Biological assays for topoisomerase II-mediated DNA cleavage were accomplished using methods of Kim *et al.*¹⁴ The reaction buffer solution contained 0.3 mg of pBS (Stratagene) and cleavage buffer mix (30 mM Tris-HCl, pH 7.6, 60 mM KCl, 8 mM MgCl₂, 15 mM 2-mercaptoethanol, 3 mM ATP, 30 μg/mL bovine serum albumin). The reaction was initiated by adding drug and human topoisomerase II. After incubation for 30 min at 37 °C, the cleavage complexes were trapped by addition of 2 μL of 10% SDS followed by topoisomerase digestion with proteinase K for 30 min at 45 °C. The reaction products were purified with phenol/chloroform extraction and electrophoresed on a 1.2%

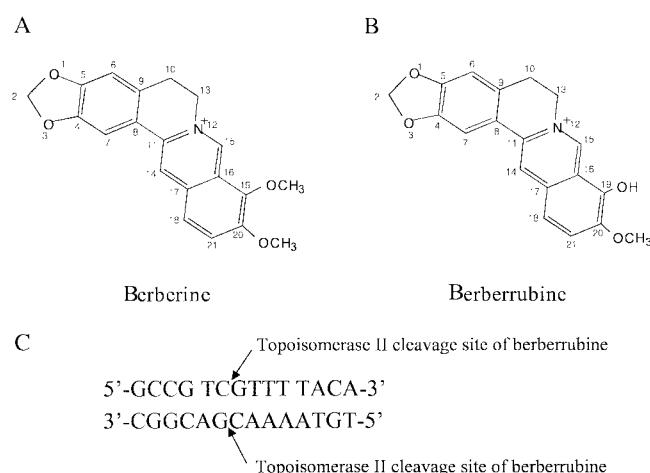


Figure 1. Chemical structures of berberine (A) and berberrubine (B). Berberine has two methoxy groups at positions 19 and 20, whereas berberrubine has hydroxyl group at position 19. (C) A consensus DNA 14-mer containing a putative topoisomerase II-mediated cleavage site.

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agarose gel containing 0.5 $\mu\text{g/mL}$ ethidium bromide for 2h at 0.25 V/cm. The amount of DNA products was quantified by densitometric analysis by Eagle Eye II (Stratagene).¹⁴ The level of DNA cleavage was arbitrarily set to 1 in the absence of drug. Data from DNA cleavage assays represent the average of three independent experiments.

For NMR samples, 2-4 mg of the compounds (berberine and berberrubine) were dissolved in both 500 μL DMSO and 90% $\text{H}_2\text{O}/10\%$ D_2O solution. NMR spectra were acquired at 298 K with a Bruker DRX 500 spectrometer equipped with triple axis gradients. Data were collected with a 7002Hz spectral width, 2048 complex points in t2 and 128 increments in t1 domain. Two-dimensional double quantum-filtered (DQF) COSY,^{19,20} rotating frame nuclear Overhauser enhancement spectroscopy (ROESY)²¹ and ^{13}C - ^1H -hetero-nuclear multiple-bond-correlated (HMBC) experiments²² were performed. The two dimensional ROESY with mixing times of 150 ms and DQF-COSY experiments were served to obtain inter-proton distance and dihedral angle constraints for structural information. All data were transferred to a SGI Indigo² workstation and processed using an XWIN-NMR package (Bruker Instruments, Rheinstetten, Germany).

NMR structures of both berberine and berberrubine were calculated using a simulated annealing method starting from an initial structure constructed by Builder routine in the Insight II program (Biosym/Molecular Simulations Inc., San Diego, CA). The structural calculations were performed with modified parameter and topology files generated by the Hetero-compound Information Center of Uppsala University (HICUP) using XPLOR 3.5.^{24,25} Angle information from initial structure optimized by Insight II program was used to identify the conformational relation of two vicinal protons. The distance restraints from ROESY spectra were assigned as strong, medium, and weak. All categories had a lower limit of 1.8 \AA , with upper limits of 2.7, 3.3, and 5.0 \AA for the strong, medium and weak intensities, respectively. Initial structures were generated using distance geometry and then used during the simulated annealing protocol from XPLOR version 98.0 (Molecular Simulations Inc.). The 20 <SA>_k solution structures, which were finally selected by lowest energy values, were displayed and analyzed using Insight II program (version 98.0).

Results and Discussion

Topoisomerase II mediated DNA cleavage activity of berberine and berberrubine. In spite of the structural similarity between berberine and berberrubine, DNA cleavage activity of berberrubine is much stronger than that of berberine (Table 1). Interestingly, the religation ability of berberrubine treated DNA was determined to be much higher than that of etoposide treated DNA (data not shown), suggesting that berberrubine would stabilize nicked DNA strands. The results of DNA cleavage assays and religation reactions indicate that berberrubine is a more effective topoisomerase II poison than berberine.

Resonance assignments. The structures of berberine and

Table 1. Effect of drug concentration on topoisomerase II-mediated DNA cleavage^a

	Drug concentration (μM)					
	0	10	50	100	150	200
Berberrubine	1.0	2.28	4.03	4.16	4.20	4.22
Berberine	1.0	1.25	1.49	1.61	1.62	1.63
Etoposide	1.0	3.01	5.43	6.65	6.70	6.75

Relative DNA cleavage

^aThe reaction products were analyzed on a 1.2% agarose gel containing 0.5 $\mu\text{g/mL}$ ethidium bromide. The relative level of DNA cleavage was arbitrarily set to 1 in the absence of drug. Data from DNA cleavage assays represent the average of three independent experiments.

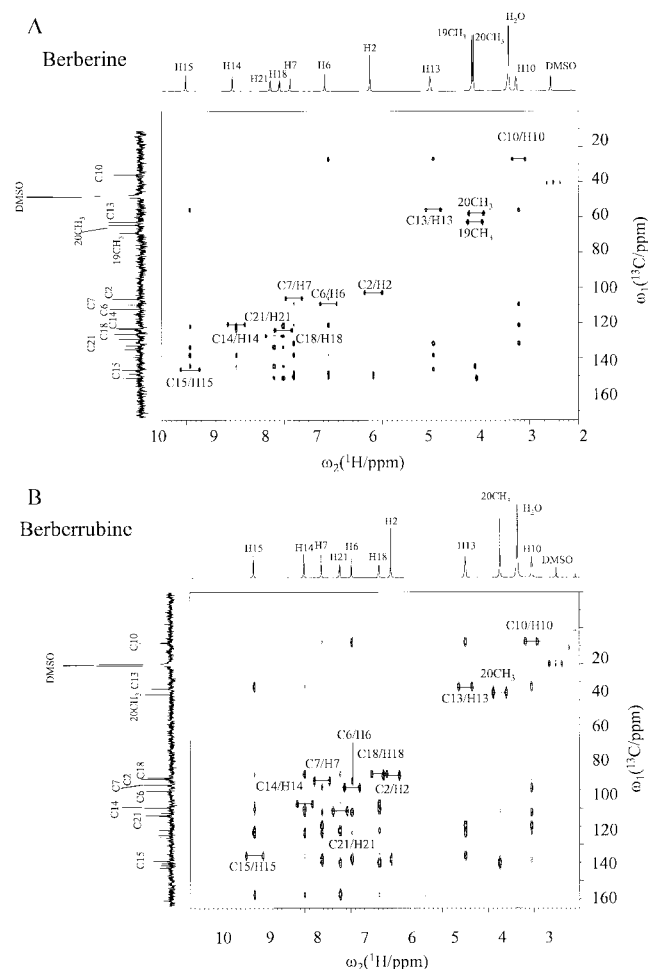


Figure 2. ^{13}C - ^1H HMBC spectra of berberine (A) and berberrubine (B) are displayed. The protons of both berberine and berberrubine are easily assigned based on 2D DQF-COSY spectra. Two protons bonded to the same carbon nuclei are connected with solid lines.

berberrubine were verified by one-dimensional proton and carbon NMR spectra combined with 2D HMBC and DQF-COSY spectra. Since no additional peaks were observed, we assume that no stereoisomers were present in these samples.

All carbon and proton resonances of berberine and berberrubine were easily assigned from the combined use of HMBC (Figure 2) and DQF-COSY. Berberine consists of five rings and two methoxy groups at positions 19 and 20,

whereas berberrubine has a hydroxyl group at position 19. Interestingly, the proton resonances near the hydroxyl group in berberrubine are mostly shifted to upfield regions compared to those in berberine.

In results, even though berberrubine differs only at position 19 from berberine, the NMR resonances are different, suggesting that the chemical environments of the two drugs are quite different. This data may explain different topoisomerase II mediated DNA cleavage activity between berberine and berberrubine.

Solution structures of berberine and berberrubine. Based on 2D-ROESY data, a total of ten distance restraints were derived for both berberine and berberrubine (Figure 3). The differences in ROEs between berberine and berberrubine are mainly caused by the methoxy group in berberine. After performing a simulated annealing calculation and refinement procedure, the twenty lowest energy structures for both berberine and berberrubine were selected for further analysis. All structures demonstrated a relatively rigid planar

arrangement and were well superimposed. Figure 5 shows REM average structures calculated from 20 <SA>_k structures of both berberine and berberrubine. The solution structure of berberine shows that the two methoxy groups are oriented opposite each other, possibly due to steric hindrance. Since both berberine and berberrubine consist of six-membered planar rings in the core structure, the two structures are very similar (Figure 5). Based on biochemical and NMR data, we propose that the minor structural differences between berberine and berberrubine could induce a different binding mode to consensus DNA (Figure 1C), resulting in a DNA cleavage efficiency of berberrubine that is quite different from that of berberine.

Berberine alkaloids have been also reported as multi-functional compounds, such as anti-fungal drug candidates and tumor suppressing agents. Recently, we have reported that the protoberberine alkaloid, berberrubine effectively induces DNA cleavage in a site-specific and concentration-dependent way, implying that berberrubine could be a new antitumor drug in cancer treatment.¹⁴ However, even though berberine and berberrubine are similar to each other in chemical structure except one functional group replacement, the ability to mediate DNA cleavage by topoisomerase II

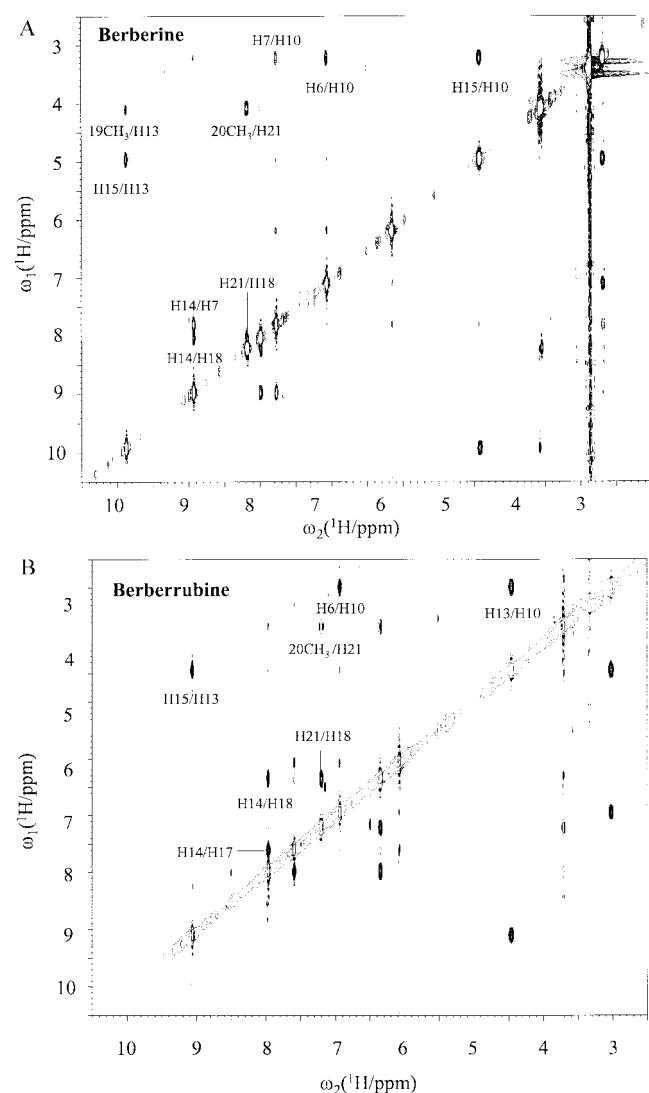


Figure 3. ROESY spectra of berberine (A) and berberrubine (B). All ROE peaks used for structural calculations are labeled.

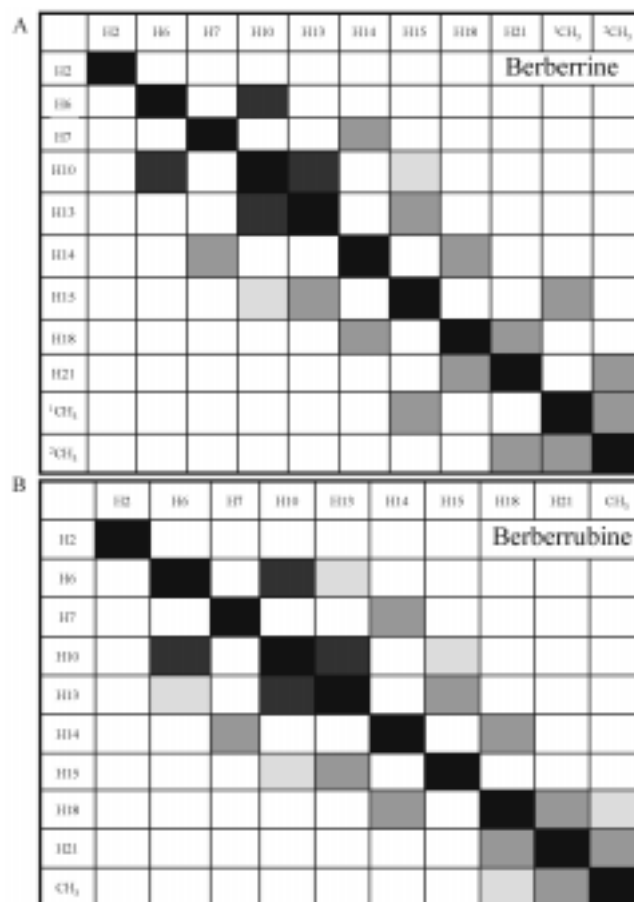


Figure 4. Observed ROEs are summarized for berberine (A) and berberrubine (B). The atomic numbering is arranged by order of each heterocompound. ROE intensities were classified by thickness of gray scale.

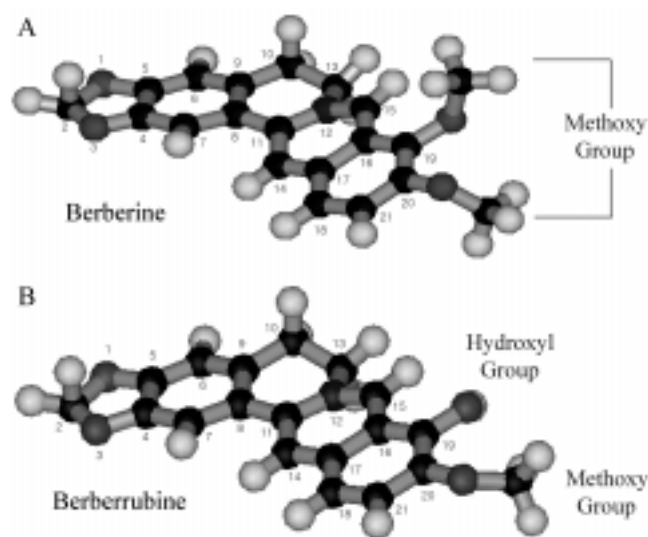


Figure 5. REM average structures of berberine (A) and berberrubine (B) calculated from NMR data. Both structures retain a topology of planarity, however, different orientation of functional groups at 19 position and minor structural difference are observed.

differ dramatically (Table 1). Our NMR results reported here provide an explanation for this, proving that the chemical environments and solution structures of the two drugs differ as well (Figure 4). In addition, the DNA binding pattern of berberrubine is dissimilar to that of berberine. A detailed mechanism of DNA binding of each of these drugs would provide a clearer picture about the specificity of protoberberine functional groups. Structural studies with these DNA-drug complexes are currently in progress.

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