Spectroscopic Studies on Interaction of Protoberberines with the Deoxyoligonucleotide d(GCCGTCGTTTTACA)$_2$

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The topoisomerase II poisoning effect of certain protoberberine alkaloids is associated with anti-cancer activity. Structure-activity relationships of protoberberine analogues substituted on the ring protons reveal that substitution at the C19 position is an important determinant of biological activity. In this study, the effects of substituent modification at the C19 position on the interaction of protoberberines with DNA are determined using UV and NMR spectroscopy. The line broadening effect on aliphatic resonances, chemical shift changes of the imino protons of HP14 upon berberine and berberrubine binding to HP14, and the rate of the exchange process between protoberberine analogs bound indicate that berberrubine binds HP14 more specifically than berberine. In addition, the free HP14 is altered by the substituent at the 19-position. UV spectra of berberrubine have shown a hypochromic effect together with a slight red shift, which are usually regarded as characteristics of DNA intercalation. These results are consistent with our previous report that the berberrubine is partially intercalated with HP14 with molar ratio 1 : 1, whereas a non-specific interaction is predominant between the berberine and HP14.

Key Words : Berberine, Berberrubine, Topoisomerase II poison, DNA binding, NMR

Introduction

Eukaryotic DNA topoisomerase II catalyzes the ATP-dependent relaxation of negative and positive supercoils, knotting, unknotting, catenation, and decatenation of DNA by passing the double-stranded DNA helix through a transient double-stranded break and then resealing the strand break. The mechanism of topoisomerase II activity involves DNA cleavage, strand passage, and religation, succeeded by enzyme turnover with the aid of ATP.[1,2] During this cycle, the enzyme covalently binds to DNA forming an intermediate called topoisomerase II-DNA covalent cleavable complex.[3-6] Topoisomerase presents a potentially selective target for the design of new antitumor drugs. Eukaryotic DNA topoisomerase II is the cellular target for a variety of active agents currently used for the treatment of human cancers.[3,7-9] Although many topoisomerase II-targeted agents have been identified for treatment of certain types of human cancer, complications such as resistance in solid tumors and subsequent genetic changes severely limit the efficacy of DNA topoisomerase II poisons.[10]

Berberrubine (Figure 1) is an isoquinoline alkaloid isolated from *Berberis Vulgaris L.* and is readily derived from berberine.[11] In previous studies structure-activity relationships of protoberberine analogues substituted on the ring protons revealed that substitution at the C19 position was an important determinant of biological activity.[12] The binding affinity associated with the interaction of berberrubine with DNA is significantly enhanced upon substitution at the C19 position. To gain understanding of the interactions of protoberberine analogs with DNA, we have characterized the DNA (HP14; Figure 1(c)) binding properties of berberine and berberrubine in previous works.[13] In this study, the effects of substituent modification at the C19 position on the interaction of protoberberines with DNA are determined using UV and NMR spectroscopy.

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Experimental Section

Materials & Methods. Berberine was obtained from Sigma and kept as a 10 or 40 mM stock solution in D_2O or 90% H_2O/10% D_2O. Berberrubine is synthesized from berberine as described.[11] The deoxyoligonucleotide d(GCC-GTCGTTTTACA)$_2$ (HP14) which contains cleavage sites of topoisomerase II was obtained from J. L. Science (Daejeon, Korea). The lyophilized HP14 (Figure 1(B)) sample was dissolved in 0.5 mL D_2O buffer or in 0.45 mL of the degassed H_2O buffer (50 mM potassium phosphate, 100 mM NaCl, 0.01 mM EDTA, pH 7.0). Then 0.05 mL of 99.96% D_2O was added to the H_2O buffer sample.

NMR Spectroscopy. All NMR spectra were acquired on Bruker DRX-600 and DRX-500 spectrometers in quadrature detection mode, equipped with a triple-resonance probe with an actively shielded pulsed field gradient (PFG) coil. All two-dimensional experiments were performed at 298 K. Solvent suppression was achieved by utilizing pulsed-field gradient techniques for all H_2O experiments.[14] The 1D spectra were acquired with spectral width 6000Hz for spectra acquired in D_2O and 12000 Hz for spectra acquired in H_2O. The repetition delays were set to 2 sec, and 40-64 scans were averaged for each FID. All experiments were

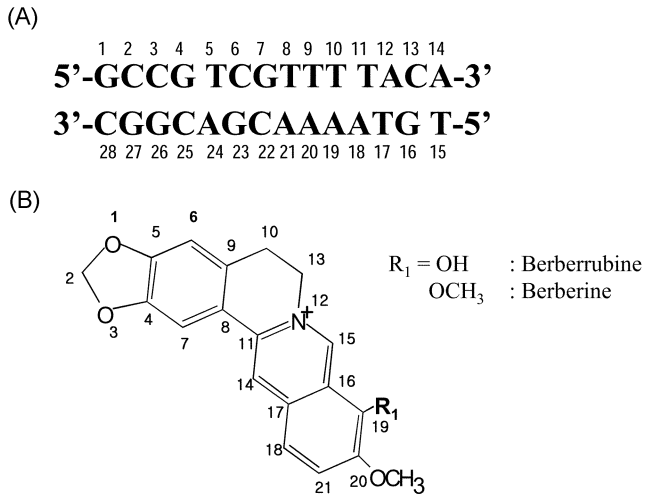

Figure 1. (A) A consensus DNA 14-mer HP14. (B) Chemical structures of berberine and berberrubine. The atomic numbering and ring lettering are indicated in their structure. Berberine has two methoxy groups at positions 19 and 20, whereas berberrubine has a hydroxyl group at position 19.

performed in the phase sensitive mode using the time proportional phase incrementation (TPPI) method. The NMR data were processed using the XWIN-NMR (Bruker Instruments) software. The NMR spectra were analyzed using Sparky 3.60 software.

UV Spectroscopy. All UV-Vis absorbance experiments were conducted on a Varian Cary400 (Sydney, Australia) spectrophotometer equipped with water circulated cellblock. A quartz cell with a 1 cm path length was used for all the absorbance studies. Absorbance versus temperature profiles were measured at 260 nm in aqueous buffer (50 mM potassium phosphate, 100 mM NaCl, 0.01 mM EDTA, pH 7.0). Melting transitions were monitored with the cellblock temperature and measured at 260 nm with a 1.5 sec averaging time. The temperature was raised in 1 °C/min increments, and samples were allowed to equilibrate for 30 sec at each temperature setting.

Results

Line Broadening Effect of the Aliphatic Resonances and Chemical Shift Changes of Imino Protons of HP14. Differences in binding to HP14 by protoberberine analogs are obvious in exchange dynamics and in resonance positions. Titrations of HP14 with berberine and berberrubine were carried out with the molar ratio HP14 : protoberberine analogs varied over the range of 1 : 1 to 6 : 1 (Figure 2). As we reported,[13] the binding process of protoberberines to HP14 occurs at an intermediate rate with respect to the NMR time scale, so no separate signals of HP14 were observed for the free and the bound species. We observed different line broadening effects of aliphatic resonances for berberrubine/HP14 and berberine/HP14 complex (Figure 2), which are indicative of differences in kinetic behavior between the two protoberberine analogs. As shown in Figure 2A, a steady

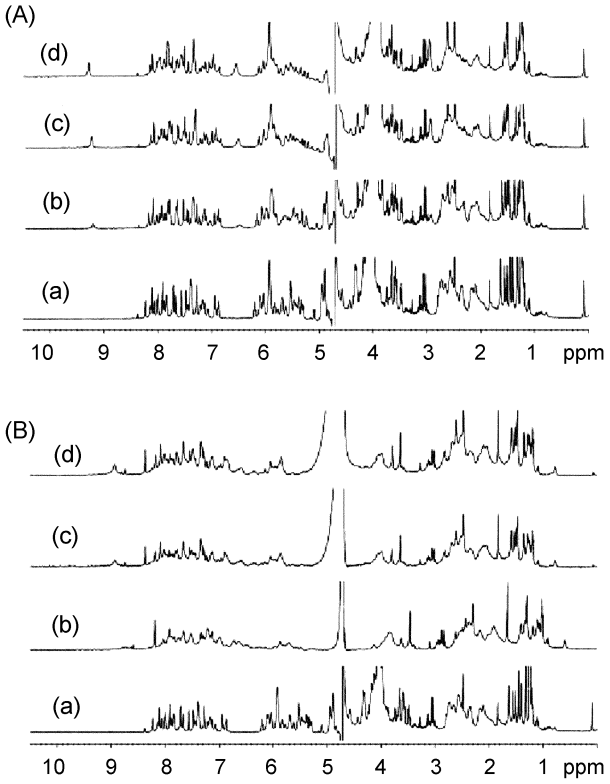

Figure 2. The aliphatic proton region of 1D-^{1}H NMR spectra of berberine/HP14 complex (A) and berberrubine/HP14 complex (B) acquired at 25 °C in 100 mM KCl, 50 mM phosphate buffer and pH 7.0 in D_2O. The molar ratio between HP14 and berberine is (a) 1 : 0. (b) 1 : 1. (c) 1 :3. (d) 1 : 6.

line broadening effect was observed in the signals corresponding to the aliphatic resonances of berberine as the molar ratio of berberine to HP14 was increased. In berberrubine/HP14 complex as compared to berberine/HP14, the line broadening effect was the most evident at molar ratio 1 : 1. This broadened resonance linewidth diminished as the ratio of the bound to free berberrubine decreased during titration with the drug. The rate of the exchange process between protoberberine analogs free and bound to HP14 is therefore altered by the substituent at the 19-position.

The addition of the protoberberine analogs to a solution of the HP14 also induced chemical shift perturbation on the resonances of imino proton region of HP14 even at low drug/DNA ratios. The chemical shift changes of the imino protons for HP14, observed by addition of the protoberberine analogs to HP14, are plotted in Figure 3. These 1D NMR studies in 90% H_2O/10% D_2O showed up-field shifts of the imino protons of HP14 upon binding of the protoberberines. Corresponding to the line broadening effect, a gradual chemical shift change was observed in drug titration as the molar ratio of berberine to HP14 was increased. However, the up-field shift of imino protons in berberrubine/HP14 complex was most evident at molar ratio 1 : 1 but became less pronounced as the ratio of the bound to free berberrubine decreased during titration with the drug.

UV-Vis Spectral Characterization. To evaluate the

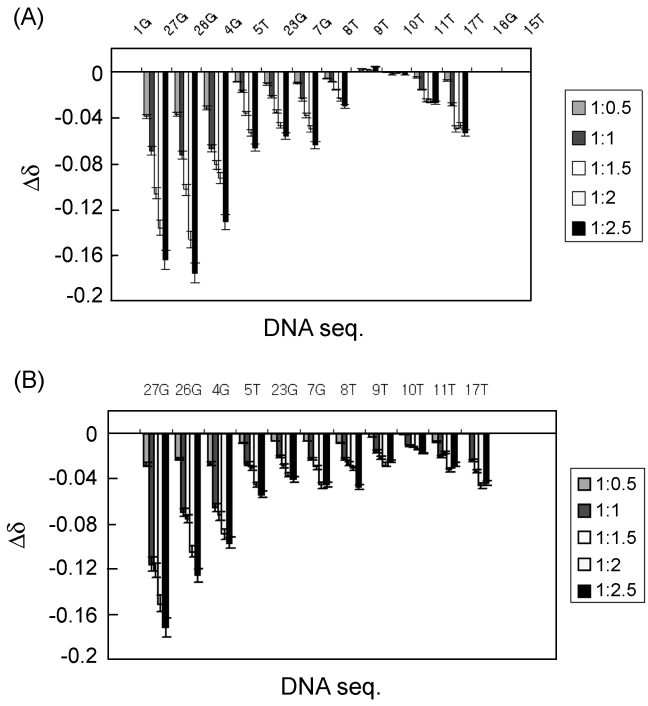

Figure 3. Plot of protoberberine-induced changes in ^{1}H chemical shifts ($\Delta\delta = \delta_{bound} - \delta_{free}$) of imino protons for berberine/HP14 complex (A) and berberrubine/HP14 complex (B). The mole ratio between HP14 and protoberberine is indicated in the box.

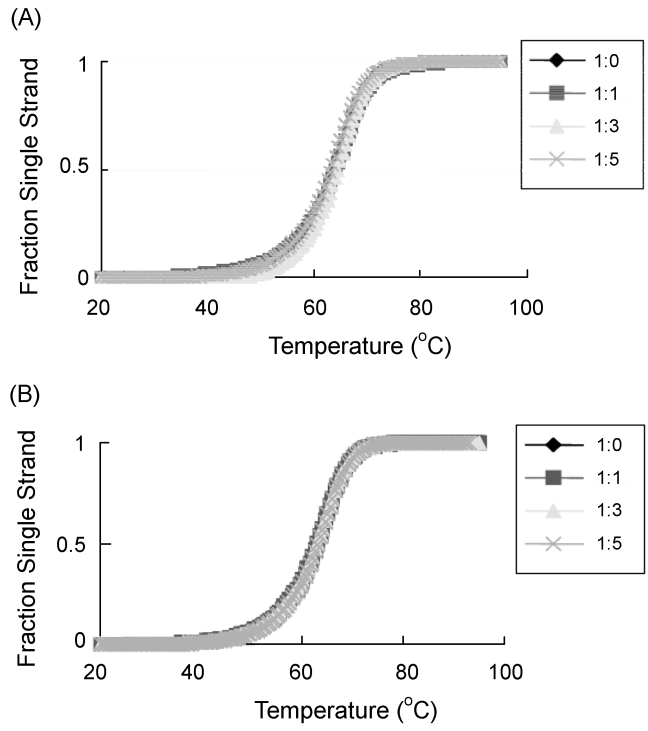

Figure 4. UV melting profiles for the HP14 duplex and their complexes with berberine (A) and berberrubine (B). The molar ratio between HP14 and protoberberine is indicated in the box. Solution conditions were 100 mM KCl, 50 mM phosphate buffer and pH 7.0. For clarity of presentation, the melting curves were normalized by subtraction of the upper and lower base lines to yield plots of fraction of single strand versus temperature.[26]

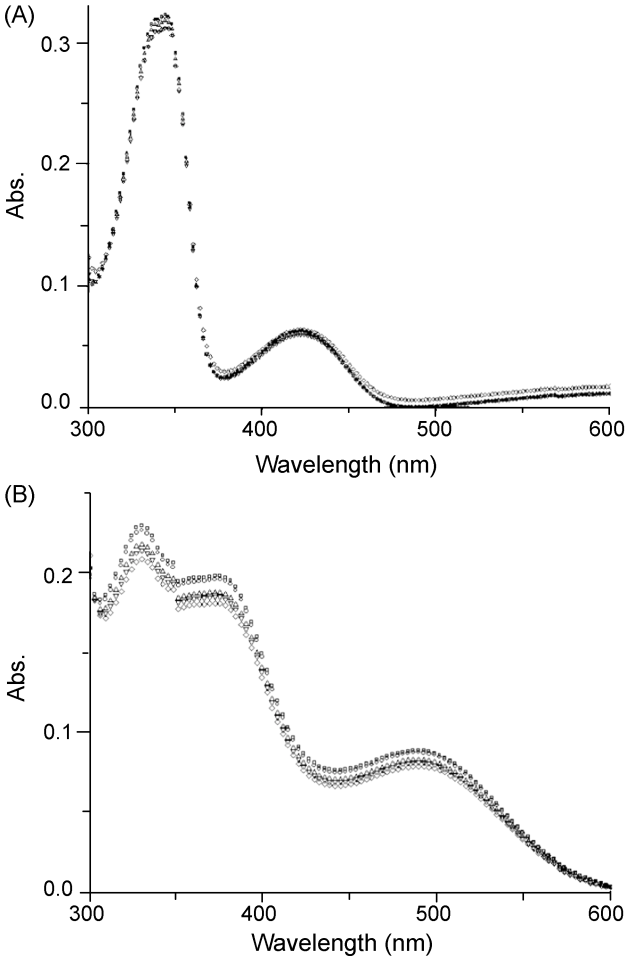

Figure 5. Influence of DNA binding on the visible absorption spectra of berberine (A) and berberrubine (B). Fixed concentrations of drug were titrated with increasing concentrations of HP14. Drug concentrations were maintained below 5 μM throughout the experiment.

effects of protoberberine analogs on the DNA double helix, the thermal stability of the HP14 double helix, as monitored by the change of the absorption maximum at 260 nm, was measured and reported in Figure 4. For clarity of presentation, the melting curves were normalized by subtraction of the upper and lower base lines to yield plots of fraction of single strand versus temperature. HP14 showed clear sigmoidal curves monitored at 260 nm, signifying that the double-stranded structure remains stable.

Upon complex formation with DNA, characteristic changes to the visible absorption spectra of both protoberberines are evident. A hypochromic effect in the region of the absorption of the berberine and the berberrubine was detected as shown in Figure 5. This effect was more evident in the case of berberrubine. Differences in the maximum wavelength were < 2 nm in both protoberberines.

Discussion

The topoisomerase II poisoning effect of certain protoberberine alkaloids is associated with anti-cancer activity.[1,7-9]

The substitution of methoxy group on 19-position had a significant effect on the relative activity of protoberberine analogs. Berberrubine was shown to be more a potent inducer of topoisomerase II-DNA cleavable complexes than the structurally similar berberine.[12]

To characterize the interaction between the protoberberine analogs and HP14, we monitored the NMR and UV spectral changes during titration of HP14 with berberine and berberrubine. In this study we have confirmed our previous results[13] and we have extended our understanding of the interactions of protoberberine analogs with the DNA oligonucleotide. In the course of titrations of HP14 with berberine and berberrubine, a line broadening effect of aliphatic resonances for berberrubine/HP14 and berberine/HP14 complex was observed (Figure 2). Upon complex formation the bound ligand would experience the longer rotational correlation time of the macromolecular assembly and a decrease in segmental mobility. Interaction is therefore apparent from changes in the linewidth of the proton resonances since complex formation results in an increased relaxation rate due to the longer correlation time of the oligonucleotides and the motional constraints imposed by binding of the protoberberine analogs to the oligonucletides. The progressive variation in linewidth and intensity for proton resonances occurring in the absence of any chemical shift changes is characteristic of relatively rapid exchange on the relaxation time scale.[15-18] Spectral linewidth increases of imino protons of HP14 on addition of protoberberine analogs to HP14 have been reported previously.[13]

Addition of berberrubine to HP14 at molar ratio 1 : 1 produced a marked line broadening in aliphatic resonances, indicating complex formation (Figure 2). This broad resonance linewidth decreased as the ratio of the bound to free berberrubine decreased during titration with the drug. This result clearly indicates that berberrubine interacts specifically with HP14 at molar ratio 1 : 1 and is consistent with the results of our previous NMR investigations.[13] On the other hand, the aliphatic resonances of berberine show a gradual line broadening effect as the drug ratio to HP14 is increased. This trend clearly indicates a non-specific interaction between the berberine and HP14.

In addition to linebroadening effects, chemical shift perturbation of the resonances of imino protons of HP14 was observed after addition of the protoberberine analogs. Proton chemical shifts were perturbed as a result of the change in the chemical environments of the protons in the interface of intermolecular interaction between biomolecule and ligand. Differences in the DNA binding mode of protoberberine analogs, berberine and berberrubine, are clearly manifested through chemical shift deviation of drug resonances after addition of drugs to HP14 (Figure 3). The imino proton resonances of HP14 were shifted up field upon binding of the protoberberines. The up-field shift of imino protons in berberrubine/HP14 complex was most obvious at molar ratio 1 : 1. The tendency of the up field shift decreased as the molar ratio of berberine to HP14 was increased. These data indicated that berberrubine interacts with HP14 at molar ratio 1 : 1 specifically. Compared to the berberrubine, the chemical shift of the protons of the berberine does not indicate specific, stoichiometric binding. The chemical shift changed successively by drug titration as the molar ratio of berberine to HP14 was increased. These chemical shift changes for the protons of the berberine upon binding to HP14 clearly also indicate a non-specific interaction between the berberine and HP14. The combination of gradual linebroadening effect and the lack of a specific-binding trend on chemical shift change of the berberine certainly suggest non sequence selectivity. From these NMR spectral changes, we expect that the berberine simply binds DNA less specifically than berberrubine and the rate of the exchange process between protoberberine analogs bound and free HP14 is altered by the substituent at the 19-position.

The melting profiles of the free and drug complexed HP14, as monitored by the change of the absorption maximum at 260 nm, is plotted in Figure 4. The intercalation of small molecules into the double helix is known to increase the DNA melting temperature,[19] at which the double helix denatures into single stranded DNA, owing to the increased stability of the helix in the presence of an intercalator. In contrast to a strong intercalator, the complex of berberine and berberrubine with the HP14 produce an almost inconsiderable enhancement in the stabilization of double helix HP14 (Figure 4). Intercalation to a regular DNA requires the spreading of local-base stacking before insertion of the molecule into the DNA. This energy, of course, would be compensated by the newly emerged stacking between the intercalated ring of molecule and both sides of the base pairs.[20] In the interaction of berberrubine with HP14, this stabilizing energy by new stacking is considerably small since the intercalating of berberrubine to HP14 is only partially achieved. The intercalation of berberrubine, therefore, would show no stability increase compared with that of free DNA.

The hypochromic effect is a powerful indicator of the oriented chromophores commonly seen in DNA,[21,22] where the effect is observed as the reduction in optical absorption intensity. In general, this effect refers to the stacking interaction between the dye and nucleobase.[23] The intercalative binding of ligand into the base pairs of DNA has such characteristics together with a slight red shift of the absorption band of ligand.[24]

The UV spectra showed small hypochromicity with an increase of berberrubine concentration, together with a slight red-shift of the maximum peak (Figure 5). Since the hypochromicity of chromophore generally results from *p*-electron transfer by stacking interaction with nucleic bases, the spectral change appears to reflect the contribution of intercalation into the nucleic base pair. On the other hand, the addition of berberine to HP14 induced only a much smaller hypochroism and no shifts in the absorption spectra. These UV spectral characteristics have been reported by Li, *et al*.[25]

In this study, we have observed that the structural difference of protoberberine analogs have a dramatic effect on

their DNA binding behavior. Our results suggest that a substitution of a hydroxyl group to a methoxy group on the 19-position in a berberine is associated with a non-specific DNA binding affinity and a reduced topoisomerase II poisoning of berberine. The methoxy substituent on 19-position of berberine may lead to more sterically hindered geometries for intercalation and to hinder DNA binding by berberine. These results are consistent with our previous result[13] that berberrubine interacts specifically with HP14 at molar ratio 1 : 1, whereas a non-specific interaction is predominant between berberine and HP14.

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