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Spectrometric studies of cytotoxic protoberberine alkaloids binding to double-stranded DNA

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Abstract—The noncovalent complexes of five cytotoxic protoberberine alkaloids, that is, berberine, palmatine, jatrorrhizine, coptisine, and berberrubine with several double-stranded oligodeoxynucleotides were systematically investigated by using electrospray ionization mass (ESI-MS) and fluorescence spectrometric methods, with the aim of establishing the structure–activity relationships. ESI-MS spectrometric studies indicated that these five alkaloids showed both 1:1 and 1:2 binding stoichiometries with $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, and $d(AAGCATGCTT)_2$. Their relative binding affinities toward these three double-stranded DNA were semi-quantitatively evaluated by measuring the ratios of the complex signals ($[ds+alkaloid–5H]^{4-}$+$[ds+2alkaloid–6H]^{4-}$) to those of the duplexes ($[ds\text{-}4H]^{4-}$) and also by ESI-MS competitive binding experiments. These experiments established the relative binding affinities of five protoberberine alkaloids in the order of palmatine > jatrorrhizine > coptisine > berberine > berberrubine with $d(AAGAATTCTT)_2$, palmatine ⩾ coptisine > jatrorrhizine ⩾ berberine > berberrubine with $d(AAGGATCCTT)_2$ and palmatine > jatrorrhizine ⩾ coptisine > berberine > berberrubine with $d(AAGCATGCTT)_2$. Significantly, these alkaloids except berberrubine bound to $d(AAGGATCCTT)_2$ and $d(AAGCATGCTT)_2$ with the affinities comparable to Hoechst 33258, a typical DNA minor groove binder. The relative binding preferences of berberine, palmatine, and coptisine with these three double-stranded DNA were further quantitatively assessed by their association constants obtained from fluorescence titration experiments. The values revealed the order of relative binding affinities as berberine > coptisine > palmatine with $d(AAGAATTCTT)_2$ and coptisine > berberine > palmatine with $d(AAGGATCCTT)_2$ and $d(AAGCATGCTT)_2$. These results were not in full agreement with those obtained from ESI-MS experiments, maybe due to the different measuring solution conditions. The results from ESI-MS and fluorescence titration experiments indicated that the sequence selectivities of these five alkaloids were not significant and remarkable AT- or GC-rich DNA binding preferences were not obtained, in contrast to the report that berberine binds preferentially to AT-rich DNA. To provide further insight into the sequence selectivities, the association constants of berberine with $d(AAGATATCTT)_2$, 5′-AAGTAATCTT-3′/5′-AAGATTACTT-3′, $d(AAGGGCCCTT)_2$, $d(AAGGCGCCTT)_2$, and 5′-AAGGCCGCTT-3′/5′-AAGCGGCCTT-3′, that is double helical DNA from AT-rich to GC-rich sequences, were further measured by fluorescence titration methods. No significant differences in their association constants were observed, suggesting that berberine showed no remarkable sequence selectivities.

1. Introduction

The specific and noncovalent interactions of small organic molecules with DNA and RNA have attracted considerable interests in chemical biology,[1] not only because they provide molecular basis for antitumor, antiviral, and antibiotic drugs to elucidate their structure–activity relationships and to better understand their bioactivity mechanisms, but also because they can guide the rational design of sequence specific DNA-binding molecules. Among the various small organic molecules, natural products, which exert their activities through site-specific noncovalent binding to DNA with well-established binding affinities and modes, are attractive as versatile platforms for the development of DNA

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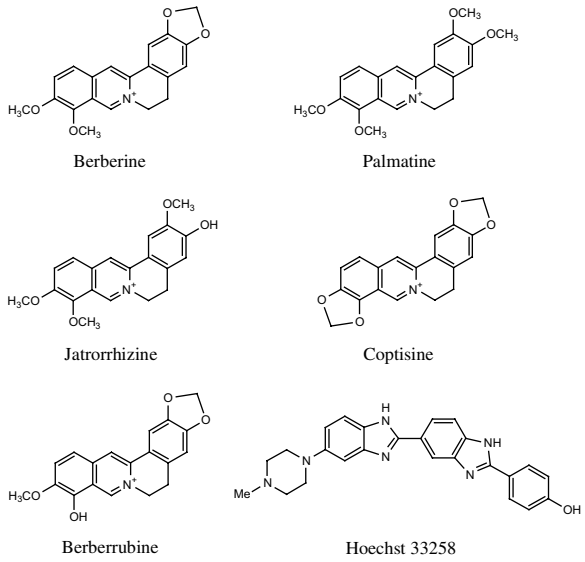
ligands because the structural modification of natural compounds may lead to more efficient DNA-binding agents.[2]

Recently, significant attentions have been paid to the noncovalent interactions of cytotoxic protoberberine alkaloids with DNA.[3] Protoberberine compounds represent an important class of isoquinoline alkaloids, which are widely present as the active constituents of some Chinese herbal medicines such as *Rhizoma Coptidis* ('Huang-Lian') and *Cortex Phellodendri* ('Huang-Bo'). These naturally occurring compounds exhibit multiple pharmacological activities,[4] such as antibacterial and antitumor activities, and therefore berberine-containing plants have been widely used in Chinese medicines and in folk medicines. The ability of cytotoxic protoberberine alkaloids to act as topoisomerase I or II poisonings was reported to be related to the antitumor activity, in which DNA binding process was of vital importance.[5] As a typical protoberberine alkaloid, berberine has been proved to strongly bind to DNA. Its binding affinities have been elucidated by several analytical techniques including absorption, fluorescence and NMR spectroscopies.[3] Recent results from NMR data suggested that berberine interacted with DNA as a minor groove binder with preferential AT-rich sequences.[3f] These studies are so far limited to berberine, and no work has been reported on the systematic study of the complexes of protoberberine-type alkaloids with DNA. Consequently, their structure–activity relationships remain to be established.

In our previous communications,[6] we reported for the first time the electrospray ionization mass (ESI-MS) spectrometric investigation of the noncovalent complexes of four protoberberine alkaloids, that is, berberine, palmatine, jatrorrhizine, and coptisine, with double-stranded DNA. Some preliminary structure–activity relationships, such as the relative binding affinities and sequence selectivities, were rapidly established by using ESI-MS techniques. Actually, ESI-MS spectrometry has been used as a sensitive and effective analytical technique for the characterization of specific drug-oligonucleotide duplex noncovalent complexes,[7] due to its specificity, sensitivity and quickness as well as its advantage to determine binding stoichiometries. Such applications have been validated by examining complexes, which have been well studied by other techniques.

In this full paper, we describe the systematic ESI-MS and fluorescence spectrometric study of the complexes of five protoberberine alkaloids, that is, berberine (B), palmatine (P), jatrorrhizine (J), coptisine (C), and berberrubine (Bu) (Scheme 1) with double-stranded oligodeoxynucleotides of different sequences, aiming at clarifying their structure–activity relationships and sequence selectivities. For comparison, the binding affinities of Heochst 33258 (H), a typical minor groove binder, was also investigated in a similar way. Three self-complementary double helix oligodeoxynucleotides, $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, and $d(AAGCATGCTT)_2$, were chosen as models in the ESI-MS studies. Recent NMR investigation of the complex of berberine with $d(AAGAATTCTT)_2$ suggested that berberine bound to the minor groove site of this double helix at the A_4–T_7 and A_5–T_6 base pairs,[3f] therefore the consecutive AATT was changed to GATC, CATG, ATAT, TAAT/ATTA, GGCC, GCGC, and GCCG/CGGC, and their binding constants with berberine were measured accordingly to examine whether berberine shows any sequence selectivities.



Scheme 1. Structures of cytotoxic protoberberine alkaloids and Hoechst 33258.

2. Results and discussion

2.1. ESI-MS spectrometric investigation

2.1.1. Observation of the noncovalent complexes of five individual alkaloids with $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, and $d(AAGCATGCTT)_2$. In our previous report,[6] we established the optimum conditions for the observation of the specific duplexes by conducting the electrospray of $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, and $d(AAGCATGCTT)_2$. As a result, these three double-stranded oligo-deoxynucleotides behaved similarly and afforded in the negative ESI-MS spectra the quadruply-charged double-stranded oligodeoxynucleotides ($[ds\text{-}4H]^{4-}$) with high relative abundances and low-intensity triply-charged double-stranded oligodeoxynucleotides ($[ds\text{-}3H]^{3-}$). The ESI-MS experiments using the berberine/$d(AAGAATTCTT)_2$ molar ratios varying from 0.5 to 2, revealed that berberine had 1:1 ($[ds+B–5H]^{4-}$) and 1:2 ($[ds+2B–6H]^{4-}$) binding stoichiometries. With the above-mentioned measuring conditions in hand, we studied the complexes of five alkaloids with $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, and $d(AAGCATGCTT)_2$, in 2:1 alkaloid/DNA molar ratios under the condition of which high relative abundance of the complexes could be obtained. The obtained negative ESI-MS spectra of these complexes are shown in Figure 1. It is found that all five alkaloids can form complexes with these three

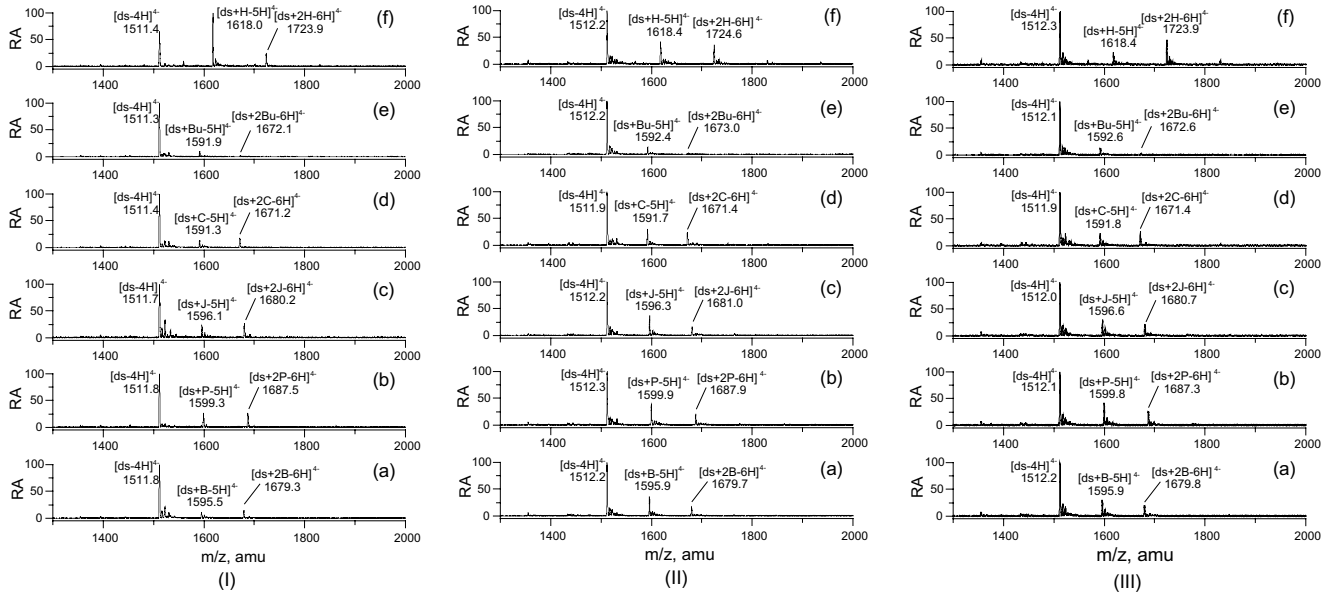


Figure 1. Negative ESI-MS spectra of the complexes of berberine (a), palmatine (b), jatrorrhizine (c), coptisine (d), berberrubine (e), and Hoechst 33258 (f) with d(AAGAATTCTT)$_2$ (I), d(AAGGATCCTT)$_2$ (II) and d(AAGCATGCTT)$_2$ (III) in 2:1 alkaloid/DNA molar ratios.

double-stranded oligodeoxynucleotides, and exhibit similar binding behaviors, that is, they all have both 1:1 ([ds+alkaloid–5H]$^{4-}$) and 1:2 ([ds+2alkaloid–6H]$^{4-}$) binding stoichiometries. On the other hand, protoberberine alkaloids commonly tend to self-associate in solution. The dimerization constant of berberine is reported to be $1.0 \times 10^5\,M^{-1}$ by UV spectroscopic dilution experiments.[3f] Higher than 1:1 complexes are also observed by ESI-MS and NMR in the binding of some minor groove binders, for example, distimycin to the same minor groove sites of duplex oligodeoxynucleotides, d(CGCGAATTGCG)$_2$[8] and d(CGCAAATTTGCG)$_2$,[9] in head-to-tail and side-by-side ways.[10] However, how the two protoberberine alkaloid molecules bound to the double-stranded DNA is currently unclear.

2.1.2. Relative binding affinities of five alkaloids with d(AAGAATTCTT)$_2$, d(AAGGATCCTT)$_2$, and d(AAGCATGCTT)$_2$. The amounts of formed alkaloid–DNA complexes can be determined from the relative intensities of the corresponding peaks present in the mass spectra, therefore under the same measuring conditions, the relative binding affinities of five alkaloids with d(AAGAATTCTT)$_2$, d(AAGGATCCTT)$_2$, and d(AAGCATGCTT)$_2$ can be evaluated by measuring the ratios of the complex signals ([ds+alkaloid–5H]$^{4-}$+[ds+2alkaloid–6H]$^{4-}$) to those of the free duplexes ([ds-4H]$^{4-}$).[11,12] The results are outlined in Figure 2 from which two observations can be extracted. Firstly, five alkaloids exhibited slightly different binding affinities. They bound in the order of palmatine > jatrorrhizine > coptisine > berberine > berberrubine to

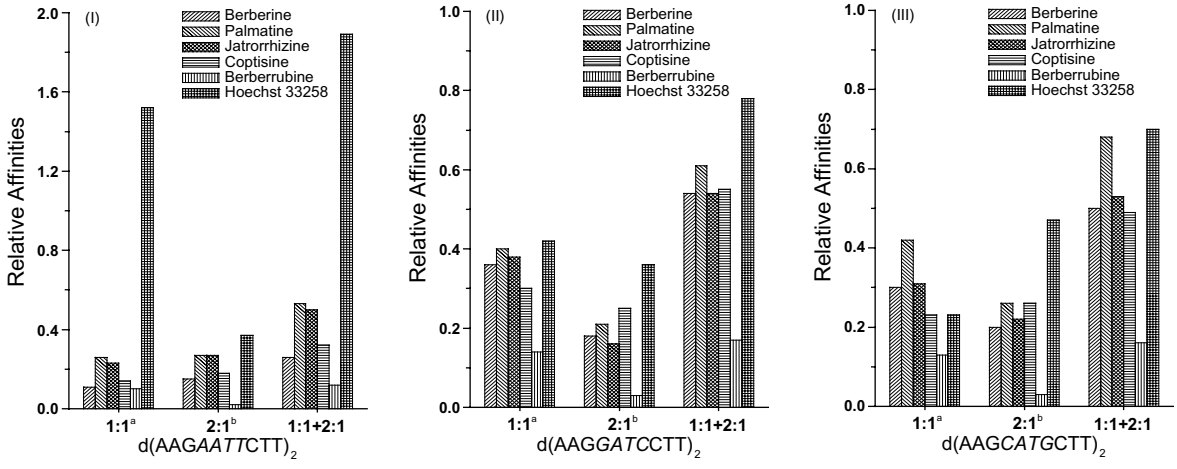


Figure 2. Relative binding affinities of five alkaloids and Hoechst 33258 toward d(AAGAATTCTT)$_2$ (I), d(AAGGATCCTT)$_2$ (II), and d(AAGCATGCTT)$_2$ (III). [a]The ratio of [ds+alkaloid–5H]$^{4-}$/[ds-4H]$^{4-}$; [b]The ratio of [ds+2alkaloid–6H]$^{4-}$/[ds-4H]$^{4-}$.

$d(AAGAATTCTT)_2$, palmatine ⩾ coptisine > jatrorrhizine ⩾ berberine > berberrubine to $d(AAGGATCCTT)_2$ and palmatine > jatrorrhizine ⩾ coptisine > berberine > berberrubine to $d(AAGCATGCTT)_2$. It should be noted, however, that berberine, palmatine, jatrorrhizine, and coptisine did not show significant differences in their binding affinities in the formation of both 1:1 and 1:2 complexes with three double helical oligodeoxynucleotides, which suggests that the slight differences in the structures of these four alkaloids have no great effect on their binding activities toward DNA. This conclusion was further supported from their association constants obtained from fluorescence titration experiments (vide infra). Berberrubine showed much lower affinities than the other four alkaloids, especially that its ability to form 1:2 complexes was rather low. Secondly, these alkaloids except berberrubine bound to $d(AAGGATCCTT)_2$ and $d(AAGCATGCTT)_2$ with the binding affinities comparable to Hoechst 33258, a typical DNA minor groove binder, however, all five alkaloids bound to $d(AAGAATTCTT)_2$ much less weakly than Hoechst 33258 which shows a binding preference for AT rich sequences.[13]

The relative binding affinities of five alkaloids toward $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, and $d(AAGCATGCTT)_2$ were further investigated by ESI-MS competitive binding experiments in which the oligodeoxynucleotide duplexes of fixed concentration were mixed with equimolar amounts of two alkaloids and the corresponding ESI-MS spectra (Fig. 3) were measured. ESI-MS competitive binding experiments have proved to be very promising in terms of sensitivity and specificity,[14] and allow the easy determination of which alkaloid of the two alkaloids in the mixture binds to $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, and $d(AAGCATGCTT)_2$ preferentially, because the preferential binding of the alkaloid usually leads to greater relative abundance in the ESI-MS spectra. Taking the competitive binding of five alkaloids to $d(AAGAATTCTT)_2$ as an example (Fig. 3I), on the basis of the sum of the relative abundances of the 1:1 and 1:2 noncovalent complexes, we could establish the following order of the pairs of the complexes: palmatine > berberine (Fig. 3IA), coptisine > berberine (Fig. 3IB), palmatine ⩾ jatrorrhizine (Fig. 3IC) and jatrorrhizine > coptisine (Fig. 3ID). Thus, the overall order of relative binding affinities with $d(AAGAATTCTT)_2$ was palmatine > jatrorrhizine > coptisine > berberine > berberrubine, completely in agreement with foregoing results obtained from ESI-MS independent experiments. Similarly, the binding preferences of five alkaloids in the order of palmatine ⩾ coptisine > jatrorrhizine ⩾ berberine > berberrubine for $d(AAGGATCCTT)_2$

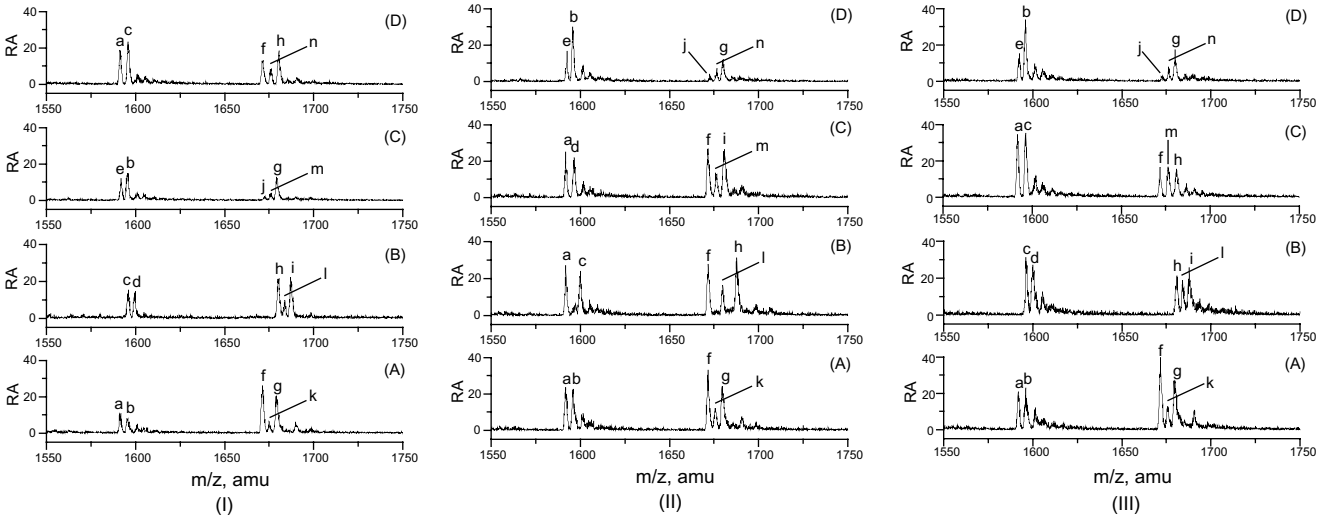


Figure 3. Negative ESI-MS spectra of the competitive binding complexes of five alkaloids with $d(AAGAATTCTT)_2$ (I), $d(AAGGATCCTT)_2$ (II), and $d(AAGCATGCTT)_2$ (III) in 2:2:1 alkaloid 1/alkaloid 2/DNA molar ratios. (I): berberine–coptisine (A), palmatine–jatrorrhizine (B), berberine–berberrubine (C) and jatrorrhizine–coptisine (D). Peak 'a' is assigned to $[ds+C-5H]^{4-}$ (*m/z* 1591.7), 'b' to $[ds+B-5H]^{4-}$ (*m/z* 1595.6), 'c' to $[ds+J-5H]^{4-}$ (*m/z* 1596.1), 'd' to $[ds+P-5H]^{4-}$ (*m/z* 1599.4), 'e' to $[ds+Bu-5H]^{4-}$ (*m/z* 1591.8), 'f' to $[ds+2C-6H]^{4-}$ (*m/z* 1671.2), 'g' to $[ds+2B-6H]^{4-}$ (*m/z* 1679.2), 'h' to $[ds+2J-6H]^{4-}$ (*m/z* 1680.4), 'i' to $[ds+2P-6H]^{4-}$ (*m/z* 1687.3), 'j' to $[ds+2Bu-6H]^{4-}$ (*m/z* 1672.4), 'k' to $[ds+B+C-6H]^{4-}$ (*m/z* 1675.0), 'l' to $[ds+P+J-6H]^{4-}$ (*m/z* 1683.8), 'm' to $[ds+B+Bu-6H]^{4-}$ (*m/z* 1676.1) and 'n' to $[ds+J+C-6H]^{4-}$ (*m/z* 1675.9), respectively. (II): berberine–coptisine (A), palmatine–coptisine (B), jatrorrhizine–coptisine (C), and berberine–berberrubine (D). Peak 'a' is assigned to $[ds+C-5H]^{4-}$ (*m/z* 1591.9), 'b' to $[ds+B-5H]^{4-}$ (*m/z* 1596.1), 'c' to $[ds+P-5H]^{4-}$ (*m/z* 1599.7), 'd' to $[ds+J-5H]^{4-}$ (*m/z* 1596.6), 'e' to $[ds+Bu-5H]^{4-}$ (*m/z* 1592.6), 'f' to $[ds+2C-6H]^{4-}$ (*m/z* 1671.7), 'g' to $[ds+2B-6H]^{4-}$ (*m/z* 1679.4), 'h' to $[ds+2P-6H]^{4-}$ (*m/z* 1687.9), 'i' to $[ds+2J-6H]^{4-}$ (*m/z* 1680.9), 'j' to $[ds+2Bu-6H]^{4-}$ (*m/z* 1672.5), 'k' to $[ds+B+C-6H]^{4-}$ (*m/z* 1675.5), 'l' to $[ds+C+P-6H]^{4-}$ (*m/z* 1680.0), 'm' to $[ds+C+J-6H]^{4-}$ (*m/z* 1676.3) and 'n' to $[ds+B+Bu-6H]^{4-}$ (*m/z* 1676.5), respectively. (III): berberine–coptisine (A), palmatine–jatrorrhizine (B), jatrorrhizine–coptisine (C), and berberine–berberrubine (D). Peak 'a' is assigned to $[ds+C-5H]^{4-}$ (*m/z* 1591.7), 'b' to $[ds+B-5H]^{4-}$ (*m/z* 1596.0), 'c' to $[ds+J-5H]^{4-}$ (*m/z* 1596.1), 'd' to $[ds+P-5H]^{4-}$ (*m/z* 1600.1), 'e' to $[ds+Bu-5H]^{4-}$ (*m/z* 1592.4), 'f' to $[ds+2C-6H]^{4-}$ (*m/z* 1671.7), 'g' to $[ds+2B-6H]^{4-}$ (*m/z* 1679.3), 'h' to $[ds+2J-6H]^{4-}$ (*m/z* 1680.8), 'i' to $[ds+2P-6H]^{4-}$ (*m/z* 1687.8), 'j' to $[ds+2Bu-6H]^{4-}$ (*m/z* 1672.6), 'k' to $[ds+B+C-6H]^{4-}$ (*m/z* 1675.5), 'l' to $[ds+P+J-6H]^{4-}$ (*m/z* 1684.1), 'm' to $[ds+C+J-6H]^{4-}$ (*m/z* 1676.2) and 'n' to $[ds+B+Bu-6H]^{4-}$ (*m/z* 1676.3), respectively.

(Fig. 3II) and palmatine > jatrorrhizine ges coptisine > berberine > berberrubine for $d(AAGCATGCTT)_2$ (Fig. 3III) were obtained.

Additionally, during the competitive binding experiments we always observed the mixed complexes (ion peaks k–n in Figure 3I–III) in which two different alkaloids were simultaneously bound to three individual DNA sequences, that is, $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, $d(AAGCATGCTT)_2$. For example, when berberine competitively bound with coptisine to $d(AAGAATTCTT)_2$ (Fig. 3IA), one new ion peak k was present at *m*/*z* 1675.0, which could be unambiguously assigned to a quadruply charged mixed complex ($[ds+B+C-6H]^{4-}$) in which one molecule of berberine and one molecule of coptisine were bound to $d(AAGAATTCTT)_2$. Their lower abundances than the two homogeneous 2:1 complexes indicated the mixed complexes could not form randomly.[12]

2.1.3. Sequence selectivities of five protoberberine alkaloids. It is reported that berberine was noncovalently bound in the minor groove of $d(AAGAATTCTT)_2$, with a preference for AATT sites over ATCG sites.[3f] If berberine and other four alkaloids have such selectivities, it is reasonable to deduce that the replacement of AATT by other bases may lead to dramatic decrease in their binding affinities. To test this hypothesis, we changed the consecutive AATT in $d(AAGAATTCTT)_2$ to GATC and CATG, corresponding to two oligodeoxynucleotide duplexes, $d(AAGGATCCTT)_2$ and $d(AAGCATGCTT)_2$, respectively, and measured the ESI-MS spectra of their complexes with five alkaloids. The ratios of the complex signals ($[ds+alkaloid-5H]^{4-}$ + $[ds+2alkaloid-6H]^{4-}$) to those of the unbound duplexes ($[ds-4H]^{4-}$) were used to probe the sequence selectivities. The results are outlined in Figure 4. It can be seen that berberine and coptisine bound to three oligodeoxynucleotide duplexes in the order of $d(AAGGATCCTT)_2 \geqslant d(AAGCATGCTT)_2 > d(AAGAATTCTT)_2$, that is, exhibited some binding preferences for $d(AAGCATGCTT)_2$ and $d(AAGCATGCTT)_2$, but it should be noted that their sequence selectivities were not marked. Palmatine, jatrorrhizine, and berberrubine bound to three oligodeoxynucleotide duplexes without any prominent preferences.

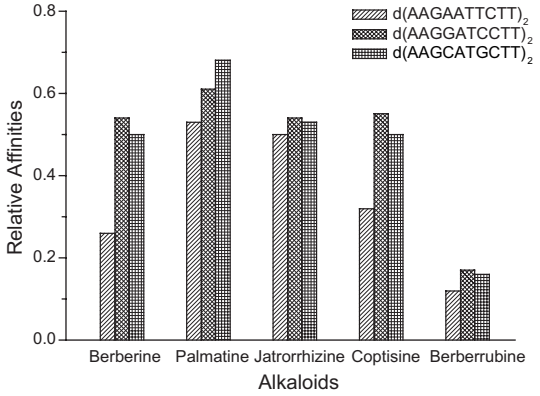


Figure 4. Relative binding affinities of five alkaloids with $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, and $d(AAGCATGCTT)_2$.

2.2. Fluorescence spectrometric investigation

The foregoing ESI-MS results were in disagreement with the reported NMR results.[3f] This may be ascribed to their different experimental conditions, that is, the present ESI-MS experiments were carried out in the aqueous methanol solutions whereas the NMR measurement was conducted in acidic aqueous buffer. To avoid the uncertainties arising from the different measuring conditions, the association constants of berberine, palmatine, and coptisine with these three and other five double-stranded oligodeoxynucleotides were measured in Tris–HCl buffer (pH 6.35) by means of fluorescence spectrometry. Fluorescence spectrometry is a sensitive analytical technique and widely used in the investigation of noncovalent complexes of small organic molecules with biomolecules such as DNA.[1a,b] The complexation is reflected in the change (enhancement or quenching) of fluorescence intensities. In Tris–HCl buffer (pH 6.35) used in this study, five alkaloids have strong absorbance peaks at around 350 nm and relatively weak and broad absorbance peaks at longer than 400 nm. They weakly fluoresce at around 520 nm when excited at 350 or 450 nm.[3c,3d,3e] Except phenolic hydroxyl group-containing jatrorrhizine and berberrubine, their weak fluorescences are greatly enhanced upon the complexation with DNA. These fluorescence spectroscopic changes allow us to study the binding activities of berberine, palmatine, and coptisine toward DNA. Analyses of the relationships between the fluorescence intensities and the DNA concentrations by nonlinear curve fitting methods afford the association constants (K_a's) of berberine, palmatine, and coptisine with double-stranded DNA (Table 1).

Some interesting observations can be extracted from Table 1. Firstly, berberine, palmatine, and coptisine exhibited comparable binding affinities with $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, and $d(AAGCATGCTT)_2$. It can be seen from the association constants that these three alkaloids bound in the order of palmatine > coptisine $\geqslant$ berberine to $d(AAGAATTCTT)_2$ and coptisine > berberine > palmatine with $d(AAGGATCCTT)_2$ and $d(AAGCATGCTT)_2$. Though maybe due to the different measuring solution conditions, these relative binding affinities were not in full agreement with those obtained from ESI-MS experiments in which palmatine always showed the highest binding affinities, the association constants of berberine, palmatine, and coptisine with three oligodeoxynucleotide duplexes were not significantly disparate. The greatest difference was observed in the binding of palmatine and coptisine to $d(AAGCATGCTT)_2$, but even in this case, coptisine bound only 4.3-fold more strongly than palmatine.

Secondly, no predominant AT or GC sequence selectivities were observed in berberine, palmatine, and coptisine. It can be seen that berberine and coptisine bound to three DNA in the order of

Table 1. Association constants (K_a's, M^{-1}) of berberine, palmatine and coptisine with double-stranded oligodeoxynucleotides[a]

DNA	Berberine	Palmatine	Coptisine
$d(AAGAATTCTT)_2$[b]	$(1.24 \pm 0.07) \times 10^4$[c]	$(1.78 \pm 0.06) \times 10^4$	$(1.26 \pm 0.18) \times 10^4$
$d(AAGGATCCTT)_2$	$(2.42 \pm 0.09) \times 10^4$	$(1.05 \pm 0.06) \times 10^4$	$(3.92 \pm 0.13) \times 10^4$
$d(AAGCATGCTT)_2$	$(1.85 \pm 0.09) \times 10^4$	$(0.72 \pm 0.09) \times 10^4$	$(3.12 \pm 0.15) \times 10^4$
$d(AAGATATCTT)_2$	$(1.38 \pm 0.08) \times 10^4$		
5′-AAGTAATCTT-3′/ 5′-AAGATTACTT-3′	$(1.51 \pm 0.07) \times 10^4$		
$d(AAGGGCCCTT)_2$	$(1.76 \pm 0.12) \times 10^4$		
$d(AAGGCGCCTT)_2$	$(1.34 \pm 0.17) \times 10^4$		
5′-AAGGCCGCTT-3′/ 5′-AAGCGGCCTT-3′	$(0.90 \pm 0.08) \times 10^4$		

[a] Measured by fluorospectrometric methods in 50 mM Tris–HCl (pH 6.35) at room temperature unless specified. These values were derived from the experimental data by nonlinear curve fitting methods using Microcal Origin software (version 6.0).
[b] The association constant of Hoechst 33258 with $d(AAGAATTCTT)_2$ was $(1.21 \pm 0.49) \times 10^7 M^{-1}$ in the present 50 mM Tris–HCl (pH 6.35).
[c] The value obtained by UV–vis was $(2.0 \pm 0.2) \times 10^4 M^{-1}$ at pH 5.8 (0.02 M NaCl) (see Ref. 3f).

$d(AAGGATCCTT)_2 \geqslant d(AAGCATGCTT)_2 > d(AAGAATTCTT)_2$ and palmatine in the order of $d(AAGAATTCTT)_2 > d(AAGGATCCTT)_2 > d(AAGCATGCTT)_2$, but there were no significant differences in their association constants, suggesting no marked sequence selectivities. To provide further insight into the sequence selectivities, the association constants of berberine with other five double-stranded oligodeoxynucleotides from AT-rich sequences to GC-rich sequences, $d(AAGATATCTT)_2$, 5′-AAGTAATCTT-3′/5′-AAGATTACTT-3′, $d(AAGGGCCCTT)_2$, $d(AAGGCGCCTT)_2$ and 5′-AAGGCCGCTT-3′/5′-AAGCGGCCTT-3′, were further measured. The obtained values varied in the range from $9.0 \times 10^3 M^{-1}$ to $2.4 \times 10^4 M^{-1}$ and their greatest differences did not exceed more than three folds. This result indicated that berberine showed no remarkable binding preferences for AT-rich or GC-rich DNA. This finding was in accord with the report that berberine and palmatine could interact with AT and GC sequences without any marked selectivities.[3h]

Additionally, the association constants of berberine, palmatine, and coptisine with the double-stranded oligodeoxynucleotides explored in this study were in the range of $0.9–4.0 \times 10^4 M^{-1}$ and thus 2–3 order of magnitude smaller than those of natural antibiotics. These findings necessitate appropriate structural modification of berberine and its related analogues to develop novel protoberberine alkaloid-based DNA-binding agents with enhanced binding affinities and sequence selectivities. This is actively in progress in our labs and will be reported elsewhere in due course.[15]

3. Conclusion remarks

The noncovalent complexes of five protoberberine alkaloids, that is, berberine, palmatine, jatrorrhizine, coptisine, and berberrubine, from Chinese herbal medicines, with double-stranded oligodeoxynucleotides $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$, $d(AAGCATGCTT)_2$, $d(AAGATATCTT)_2$, 5′-AAGTAATCTT-3′/5′-AAGATTACTT-3′, $d(AAGGGCCCTT)_2$, $d(AAGGCGCCTT)_2$, and 5′-AAGGCCGCTT-3′/5′-AAGCGGCCTT-3′, have been systematically investigated by use of ESI-MS and fluorescence spectrometries.

ESI-MS spectrometric studies indicated that five alkaloids showed both 1:1 and 1:2 binding stoichiometries with $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$ and $d(AAGCATGCTT)_2$, independent on the alkaloid/DNA molar ratios. An ESI-MS semi-quantitative evaluation of the relative binding affinities of five alkaloids was made by measuring the ratios of the complex signals ($[ds+alkaloid–5H]^{4-}+[ds+2alkaloid–6H]^{4-}$) to those of the duplex ($[ds\text{-}4H]^{4-}$) and also by competitive binding experiments. These experiments established the binding affinities of these five protoberberine alkaloids in the order of palmatine > jatrorrhizine > coptisine > berberine > berberrubine with $d(AAGAATTCTT)_2$; palmatine $\geqslant$ coptisine > jatrorrhizine $\geqslant$ berberine > berberrubine with $d(AAGGATCCTT)_2$ and palmatine > jatrorrhizine $\geqslant$ coptisine > berberine > berberrubine with $d(AAGCATGCTT)_2$. Significantly, these alkaloids bound to $d(AAGGATCCTT)_2$ and $d(AAGCATGCTT)_2$ with the affinities comparable to Hoechst 33258, however much more weakly to $d(AAGAATTCTT)_2$ than Hoechst 33258. The relative binding affinities of berberine, palmatine, and coptisine with $d(AAGAATTCTT)_2$, $d(AAGGATCCTT)_2$ and $d(AAGCATGCTT)_2$ were further evaluated with their association constants, which were obtained from fluorescence titration experiments. The results from both ESI-MS and fluorescence experiments indicated that the slight structural differences of these five alkaloids had no significant impact on their activities toward DNA.

Although berberine is reported to bind preferentially to AT-rich DNA, our results from ESI-MS and fluorescence titration experiments indicated that the sequence selectivities of five alkaloids were not significant and remarkable AT- or GC-rich DNA binding preferences were not obtained. No significant differences in the association constants of berberine with double helical oligodeoxynucleotides from AT-rich sequences to GC-rich sequences, further suggested that berberine showed no remarkable binding preferences for AT- or GC-rich DNA.

These findings may provide some important guidance for the development of novel alkaloid-based DNA-binding agents with enhanced binding affinities and sequence selectivities.

4. Experimentals

UV–vis absorption and fluorescence spectra were measured on Jasco UV-530 UV/vis Spectrophotometer and Perkin–Elmer Luminescence Spectrometer LS55, respectively. ESI-MS analyses were conducted on PE-SCIEX API 365 LC-MS spectrometer (Perkin–Elmer). Sartorius electronic pH meter was used to adjust pH value. The concentrations of single-stranded oligodeoxynucleotides were determined from UV–vis absorbance at 260 nm.

All chemicals were of highest available purity otherwise noted. All aqueous solutions were prepared with distilled and deionized water. Berberine (monoisotopic mass = 336.1 Da), palmatine (monoisotopic mass = 352.2 Da) and jatrorrhizine (monoisotopic mass = 338.1 Da) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products, China. Coptisine (monoisotopic mass = 320.1 Da) was a product from ChromaDex, Inc. Berberrubine (monoisotopic mass = 322.1 Da) was prepared in 60% yield by heating berberine at 190 °C for 15 min, according to the reported protocols.[16] Hoechst 33258 (monoisotopic mass = 424.2 Da) was purchased from Amersham Biosciences.

Oligodeoxynucleotides were purchased from Invitrogen Life Technologies and purified by open reversed-phase ODS column chromatography before use. The elution solution ranged from 5% acetonitrile aqueous solution to 10% acetonitrile aqueous solution containing 10 mM ammonium acetate. The solutions for ESI-MS measurement were prepared as follows: oligodeoxynucleotide stock solutions (1.0 mM) were annealed in 1 M ammonium acetate by heating at 90 °C for 5 min and then chilling slowly to room temperature to form double-stranded oligodeoxynucleotides (0.5 mM). Then the annealed solution (25 μL) of duplex oligodeoxynucleotides was mixed with the 10% methanol aqueous solution of berberine, palmatine, jatrorrhizine, coptisine, berberrubine, and Hoechst 33258 (each 2 mM), respectively, to make the individual complex. The resulting mixtures were diluted with spray solvent (50/50 v/v MeOH/100 mM aqueous ammonium acetate) to 100 μL and subject to negative ion ESI-MS spectrometric analysis, according to the reported protocols.[11] The monoisotopic masses of 5′-AAGAATTCTT-3′, 5′-AAGGATCCTT-3′, and 5′-AAGCATGCTT-3′ are 3024.6, 3025.6, and 3025.6 Da, respectively.

The fluorescence titration experiments were conducted in the following way: the solutions of single-stranded oligodeoxynucleotides in 50 mM Tris–HCl (pH 6.35) were heated at 95 °C in water bath for 4 min and then chilled to room temperature slowly to form double-stranded oligodeoxynucleotides. The annealed solution of duplex oligodeoxynucleotides was titrated into the solution of alkaloids of fixed concentrations in 50 mM Tris–HCl (pH 6.35) and the corresponding fluorescence spectra were measured at room temperature (excitation at 355 nm). The concentrations of alkaloids and DNA were ca.2.0×10^{-6} M and $0 \sim 2.0 \times 10^{-4}$ M in 50 mM Tris–HCl buffer (pH 6.35), respectively. The association constants (K_a's) were derived from the analysis of the relationship between the fluorescence intensity changes and the DNA concentrations by nonlinear curve fitting to the equation $I = I_0 + ((I_\infty - I_0)/(2[\text{alkaloid}]_0)) \times \{([\text{DNA}]_0 + [\text{alkaloid}]_0 + 1/K_a) - (([\text{DNA}]_0 + [\text{alkaloid}]_0 + 1/K_a)^2 - 4[\text{DNA}]_0[\text{alkaloid}]_0)^{1/2}\}$,[17] wherein I, I_0 and I_∞ represent the fluorescence intensities of the sample, alkaloid alone and the intensity when the alkaloid is totally bound, respectively; $[\text{DNA}]_0$ and $[\text{alkaloid}]_0$ are the initial concentrations of DNA and alkaloids, respectively.

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