Asian Journal of Chemistry; Vol. 25, No. 5 (2013), 2597-2600

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.13502

Studies on the Binding Properties of Temozolomide with DNA

JIAN-HUA WEI, RUI-HE ZHOU, YAN PENG and YAN-CHENG LIU*

State Key Laboratory Cultivation Base for the Chemistry and Molecular Engineering of Medicinal Resources, School of Chemistry and Chemical Engineering, Guangxi Normal University, Guilin 541004, P.R. China

*Corresponding author: E-mail: ycliugxnu@yahoo.cn

(*Received*: 23 January 2012; *Accepted*: 15 November 2012) AJC-12410

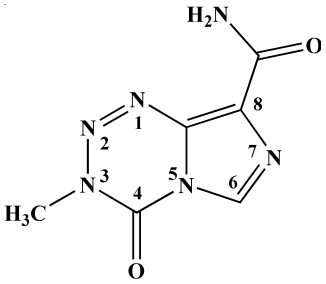
The interaction mechanism of temozolomide with DNA was studied by UV-VIS absorption spectroscopy, fluorescence emission spectroscopy, circular dichromic absorption spectroscopy and agarose gel electrophoresis method. The results of spectral analyses suggest moderate intercalative binding ability of temozolomide with ct-DNA, but without considerable exterior electrostatic interaction between temozolomide and ct-DNA. While the result of gel electrophoretic mobility shift assay on pUC19 DNA suggests covalent binding mode of temozolomide to DNA. Based on the above research results, a multi-mode binding mechanism of temozolomide to DNA macromolecule was concluded.

Key Words: Temozolomide, DNA binding, Spectroscopy, Gel electrophoresis.

INTRODUCTION

Deoxyribonucleic acid is the primary intracellular target of many anticancer drugs for their pharmaceutical activity. Studies on the interaction between DNA and small molecular drugs will help to provide molecule-level evidences for their anticancer mechanism and structure-activity relationship (SAR). Based on molecular and electronic spectroscopic analyses, the interactional mechanism between small molecules and biomacromolecules such as DNA can be deduced and discussed[1,2].

Temozolomide (TMZ) is a kind of new alkylating agent for anticancer chemotherapy (**Scheme-I**). It was firstly synthesized in UK and then distributed by Schering-Plough (USA). Temozolomide was finally authorized for clinical application in Europe at 1998 and followed in USA since 1999 under certification by FDA, with trade name as Temodal. Temozolomide mainly exhibits significant curative effect on glioblastoma multiform and it is also therapeutic on melanoma, leukemia and other solid tumors[3,4]. And the crystal structure of temozolomide has also been characterized by P.R. Lowe, *et al.*[5]. However, molecular level studies on the interaction between temozolomide and DNA by spectroscopic methods has not been emphasized[6]. In our present work, the binding properties of temozolomide with DNA was studied by UV-VIS absorption spectroscopy, fluorescence emission spectroscopy, circular dichroism as well as agorose gel electrophoresis, which provided important proofs for detailed understanding the anticancer property of temozolomide.



Scheme-I: Chemical structure of temozolomide

EXPERIMENTAL

Temozolomide was commercially available with purity of 98 % and used as received without further purification. Calf thymus DNA (ct-DNA) and sodium dodecyl sulfonate (SDS) were purchased from Sigma-Aldrich. Ct-DNA gave a UV absorbance ratio at 260 nm to 280 nm of *ca.* 1.85:1, indicating that the DNA was effectively free of protein[7]. The DNA concentration per nucleotide was determined spectrophotometrically by employing a molar absorption coefficient (6600 M^{-1} cm^{-1}) at 260 nm[8]. The stock solution of pUC19 plasmid DNA (250 μg/mL) was purchased from Takara Biotech. Tris-NaCl buffer solution, TBS, (5 mM Tris, 50 mM NaCl, pH adjusted to 7.35 by titration with hydrochloric acid using a Sartorius PB-10 pH meter) was prepared using double distilled water. The TBE buffer (1 ×) and DNA loading buffer (6 ×) were commercially available. ESI-MS for the stability studies on temozolomide was recorded on a Bruker HCT

electrospray ionization mass spectrometer. UV-VIS absorption titration was performed on a PerkinElmer Lambda45 UV-visible spectrophotometer. Fluorescence emission titration was performed on a Shimadzu RF-5301/PC spectrofluorophotometer. The circular dichroism spectra of DNA were obtained by using a JASCO J-810 automatic recording spectropolarimeter. All spectroscopic analyses were operated at 25 ºC.

ct-DNA stock solution of 2×10^{-3} M was prepared by TBS and stored at 4 ºC for no more than 5 days before use. Temozolomide aqueous solution of 2×10^{-3} M was freshly prepared in double-distilled water before use and stored as stock solution within 24 h. The stability of temozolomide in aqueous solution was determined by ESI-MS, which showed temozolomide molecule is the predominant species in solution in 24 h (*m/z*: 216.95, $[TMZ+Na]^+$).

In UV-Vis spectral analysis, the working solution of temozolomide was set as 1×10^{-4} M. Ct-DNA or sodium dodecyl sulfonate stock solution was separately added with increasing concentrations. After each addition, the solution was allowed to incubate for 10 min before the absorption spectra were recorded. The intrinsic binding constant K_b was determined by the following equation:[9]

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/K_b(\varepsilon_b - \varepsilon_f) \quad (1)$$

where, [DNA] is the concentration of the DNA based on nucleotide, ε_a is the apparent absorption coefficient ($\varepsilon_a = A_{obsd}$/[compound]), ε_f and ε_b are the extinction coefficients for the free compound and the compound fully bound to DNA. The intrinsic binding comstant, K_b, of temozolomide are derived from the ratio of the slope to the Y intercept by linear fitting of [DNA]/(ε_a-ε_f) with [DNA] from the above equation. A solution containing 10^{-4} M DNA and 10^{-5} M ethidium bromide (EB) ([DNA]/[EB] = 10 : 1) was set for EB-DNA competitive binding studies. The quenching constant of temozolomide was determined by the classic Stern-Volmer equation[10]:

$$I_0 / I = 1 + K_q \times [Q] \quad (2)$$

where, I_0 and I are the peak emission intensity of the EB-DNA system in the absence and presence of the quencher, [Q] is the concentration of each quencher compound and K_q is the Stern-Volmer quenching constant, which is obtained by the linear fit of plotting I_0/I *versus* [Q] and determines the efficiency of the quencher. In the circular dichroism absorption spectral analysis, DNA working solutions of 2×10^{-4} M in the absence and presence of temozolomide with [DNA]/[TMZ] ratios 1:0.2 and 1:1 were scanned, respectively. The circular dichroism spectrum was recorded in the region of 200-400 nm under 100 nm/min scan rate and the circular dichroism signals of TBS were subtracted as the background. All the spectroscopic experiments were kept at 25 ºC. In agarose gel electrophoresis experiment, every 0.5 μg pUC19 DNA were reacted with temozolomide of a series of concentrations and fixed up to a total 25 μL by 1 × TBE buffer, so that the same experiment can be repeated twice. All samples were incubated at 37 ºC in dark for 2 h. Then 12 μL of each sample mixed with 2 μL DNA loading buffer was electrophoresed through 0.8 % agarose gel for 60 min under 5 V/cm electric field. The gel was stained with EB (1.2 mM) in dark for 0.5 h and then visualized on a BIO-RAD imaging system with a UV-VIS transilluminator.

RESULTS AND DISCUSSION

DNA contains the genetic information responsible for transformation and expression, plays an important role in biological systems. Conformational changes of DNA will directly affect the genetic expression, which is closely related to carcinogenesis and anticarcinogenesis[11]. Interactions between small molecular drugs and DNA, including covalently binding mode and non-covalently binding mode (such as intercalation, groove binding, electrostatic attraction, *etc*.), always rank among the primary action mechanisms of antitumour activity[12]. To investigate the binding properties of temozolomide with DNA, spectroscopic studies including UV-VIS, fluorescence and circular dichroism spectral analyses as well as gel electrophoretic mobility shift assay were carried out.

UV-Vis absorption spectral analysis: It is well documented that hypochromicity along with bathochromicity on UV-Vis spectrum of aromatic planar molecules is characteristic of intercalative binding mode, due to π-π stacking from the aromatic planar structure and the neighboring base pairs of DNA[13]. Considering the planar character of heterocyclic temozolomide, intercalative binding mode of temozolomide with DNA should be proposed. So the UV-Vis absorption spectrum of temozolomide was primarily adopted to discuss the binding mode of temozolomide to ct-DNA.

The UV-Vis absorption spectra of temozolomide in the absence and presence of ct-DNA are shown in Fig. 1. In the absence of DNA, temozolomide showed characteristic absorption peaks at *ca*. 329 nm, which is ascribed to the $\pi \rightarrow \pi^*$ electronic transition of the heterocyclic conjugated system of temozolomide. The addition of ct-DNA with increasing [DNA]/[temozolomide] ratios range from 0.2 : 1 to 1.45 : 1, the maximum absorbance at 329 nm decreased from 0.904 to 0.777 with 13.3 % hypochromicity, but without bathochromicity. It suggests the characteristic intercalative binding mode of temozolomide to DNA, but the binding intensity is moderate[14]. For further quantitative comparison, the intrinsic binding

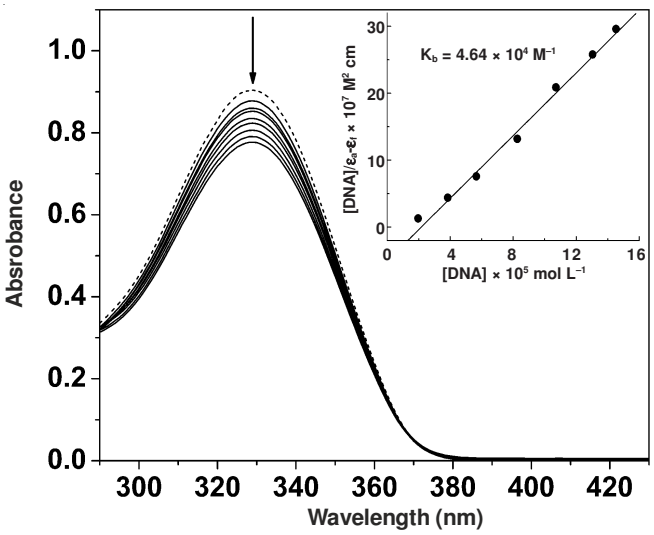


Fig. 1. UV-VIS spectra of temozolomide in the absence (dashed line) and in the presence (solid lines) of ct-DNA with increasing concentrations ([temozolomide] = 1×10^{-4} M, [DNA]/[temozolomide] ranges from 0.2 to 1.45)

constant K_b for temozolomide was also calculated according to eqn. 1. The K_b values for temozolomide is 4.64×10^4 M^{-1} (inset Fig. 1), which also suggests moderate intercalative binding of temozolomide between the neighboring base pairs of DNA double helix.

Although the intercalative binding mode of temozolomide with ct-DNA is suggested, other binding modes of temozolomide should not be excluded. In the reference experiment, sodium dodecyl sulfate (SDS) was selected as probe for electrostatic interaction. Sodium dodecyl sulfonate tends to form polyanionic groups similar to DNA polyanionic phosphate backbone, which is able to instead DNA for confirming electrostatic interaction between temozolomide and DNA. Since sodium dodecyl sulfonate has no UV-Vis absorbance in the absorption band of temozolomide, it can avoid the perturbation from the characteristic UV-Vis absorption of DNA[15]. We found addition of sodium dodecyl sulfonate to temozolomide solution (8×10^{-5} M) did not induce obvious spectral perturbation. With increasing [SDS]/[TMZ] ratios by sodium dodecyl sulfonate titration till up to 21:1, the maximum absorbance of temozolomide at 329 nm slightly decreased from 0.702 to 0.648 with only 7.7 % hypochromicity. The weak hypochromicity should be mainly contributed to the dilution effect of temozolomide solution by sodium dodecyl sulfonate addition, which suggests that no considerable electrostatic interaction between temozolomide and DNA.

Competitive binding studies by fluorescence spectroscopy: The binding abilities of temozolomide with ct-DNA were further investigated by competitive binding with ethidium bromide (EB) as an intercalative probe[16]. In the competitive binding experiments, the EB-DNA system with [EB]/[DNA] = 1:10 ([EB] = 1×10^{-5} M, [DNA] = 1×10^{-4} M) showed the characteristic strong emission at around 588 nm when excited under 347 nm with maximum emission intensity I_0 = 865.32 (slit width = 5 nm/10 nm), indicating that the intercalated ethidium bromide molecules had been sufficiently protected by the neighboring base pairs in the DNA from being quenched by polar solvent molecules. When increasing the concentration of temozolomide with [temozolomide]/[EB] ratios range from 1:1 to 12.1:1, the characteristic emission of ethidium bromide was significantly decreased down to I = 626.67 with 27.6 % quenching ratio, as shown in Fig. 2, which indicates moderate quenching ability of temozolomide on fluorescence emission of EB the fluorescence emission of EB. Furthermore, the quenching constants K_q was also calculated by restricted linear fitting of $[I_0/I]$ to [Q] from the Stern-Volmer equation (eqn. 2). The calculated K_q for temozolomide is 3.0×10^3, which is obviously lower than those of stronger intercalative agents range in 10^4-10^5.[17] However, it further suggests that temozolomide may compete with EB to bind DNA through intercalative binding mode at the similar binding site of EB[18].

Circular dichroism spectral analysis: Circular dichroism is a useful technique to assess whether nucleic acids undergo conformational changes as a result of complex formation or changes in environment[19]. The circular dichroism spectrum of DNA is very sensitive to its conformational changes. It is generally accepted that covalent binding and intercalative binding can influence the tertiary structure of DNA and induce

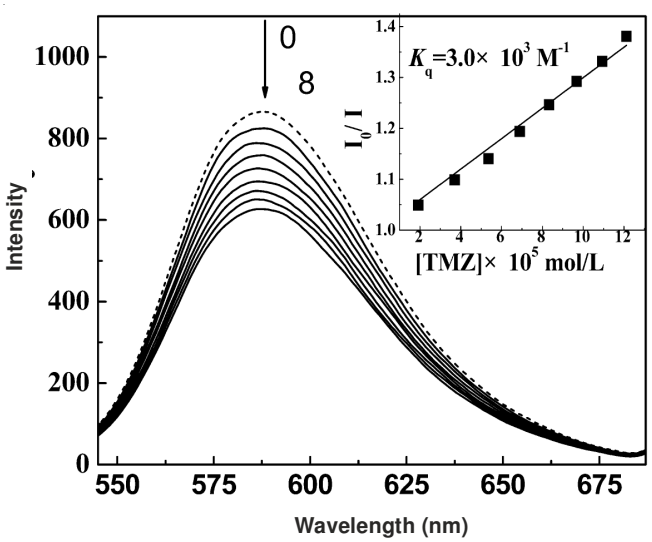


Fig. 2. Fluorescence spectra of EB-ctDNA system competitively bound by temozolomide ([DNA] = 2.30×10^{-5} M, [EB] = 1.00×10^{-5} M, [TMZ] = 0.00, 1.92, 3.70, 5.36, 6.90, 8.33, 9.68, 10.90, 12.10×10^{-5} M, respectively corresponding to curves 0→8 with λ_{Ex} = 347 nm and slit = 5 nm/10 nm)

changes in the circular dichroism spectra of DNA, whereas other non-covalent binding modes such as electrostatic interaction or groove binding does not cause significant perturbation[20].

The circular dichroism spectra of ct-DNA in the absence and presence of temozolomide was shown in Fig. 2. DNA alone shows a positive absorption band at around 273 nm and a negative absorption band at around 246 nm, corresponding to the π-π stacking of the DNA base pairs and right-hand helicity, respectively. As indicated from Fig. 3, with the addition of temozolomide at [DNA]/[TMZ] = ratio of 1 : 0.2, no significant change was observed. When the concentration of temozolomide added increased up to the [DNA]/[TMZ] = ratio of 1:1, obvious and moderate decreases on both positive and negative absorbance of circular dichroism spectrum of ct-DNA were found, with 6.9 % positive absorbance decrease (273 nm) and 12.0 % negative absorbance decrease (246 nm), respectively. When small molecules intercalated between the neighboring base pairs of DNA, decrease on circular dichroism positive absorption due to more intensive π-π stacking and decrease on its

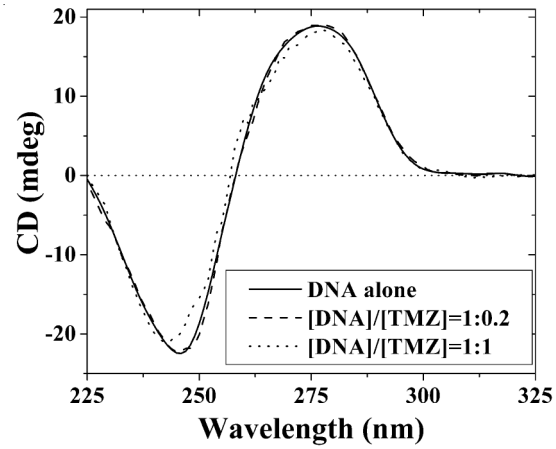


Fig. 3. Circular dichroism spectra of ct-DNA in the absence (solid curve) and presence of temozolomide with [DNA]/[TMZ] = 1 : 0.2 (dashed curve) and 1 : 1 (dot curve), respectively, in which [DNA] = 2×10^{-4} M

negative absorption due to loosening the DNA superhelix which weakens the chiral character of DNA right-hand helicity are usually observed[21]. So the intercalative binding mode of temozolomide with ct-DNA can be further confirmed viewed from the circular dichroism spectral analysis.

Agarose gel electrophoresis assay: The binding modes of temozolomide to pUC19 plasmid DNA were further examined by agarose gel electrophoresis assay. Natural-derived plasmid DNA mainly presents a closed-circle supercoiled form (form I), as well as linear form (form III) and nicked form (form II) as small fractions. It is generally accepted that when interacted with small molecules, the conformation of plasmid DNA will be changed corresponding to the binding modes. An intercalation mode to plasmid DNA can loosen the supercoiled form DNA to decrease its electrophoretic migration rate, while covalently binding mode to DNA tend to make it to adopt a linear form and simple electrostatic interaction by small molecules do not change the supercoiled form of plasmid DNA[22].

As shown in Fig. 4, upon addition of temozolomide with increasing concentrations range from 2 to 50 μM in the absence of any external redox reagent or light, the conformational changes of plasmid DNA can be reflected by gel electrophoresis especially at higher temozolomide concentrations. At temozolomide concentrations lower than 10 μM, no obvious changes were observed. At 20 μM temozolomide, nicked form (form II) DNA decreased significantly, while at 50 μM, nicked form disappear and linear form (form III) DNA formed. Since the formation of linear form DNA can be ascribed to the covalently binding of small molecules with DNA, temozolomide covalently binding with plasmid DNA should also be suggested[23].

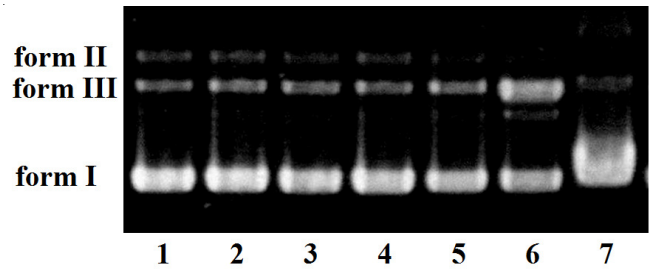


Fig. 4. Agarose gel electrophoresis assay on pUC19 plasmid DNA bound by temozolomide in various concentrations (lane 1: DNA alone; lanes 2-6: DNA + TMZ of 2, 5, 10, 20, 50 μM, respectively; lane 7: DNA + EB of 10 μM, EB was set for reference as intercalative binding agent)

Conclusion

As a new developed anticancer drug, temozolomide has been suggested as pro-drug to give a metabolite by non-enzymic hydrolysis, which covalently binds DNA to exert its anticancer efficacy.

ACKNOWLEDGEMENTS

The authors thank Natural Science Foundation of Guangxi Province (Nos. 0991012Z, 0991003, 2010GXNSFF013001), Science Foundation of Education Department of Guangxi (No. 200807MS014) and Key project of Guangxi Normal University (No. 2010ZD007) for financial support.

REFERENCES

1. L. Strekowski and B. Wilson, *Mutat. Res.*, **623**, 3 (2007).
2. S. Wang, B.S. Gaylord and G.C. Bazan, *J. Am. Chem. Soc.*, **126**, 5446 (2004).
3. C. Sansom, *The Lancet*, **353**, 472 (1999).
4. F.M. Iwamoto, A.M. Omuro, J.J. Raizer, C.P. Nolan, A. Hormigo, A.B. Lassman, I.T. Gavrilovic and L.E. Abrey, *J. Neurooncol.*, **87**, 85 (2008).
5. P.R. Lowe, C.E. Sansom, C.H. Schwalbe, M.F.G. Stevens and A.S. Clark, *J. Med. Chem.*, **35**, 3377 (1992).
6. B.J. Denny, R.T. Wheelhouse, M.F.G. Stevens, L.L.H. Tsang and J.A. Slack, *Biochemistry*, **33**, 9045 (1994).
7. C.V. Kumar, J.K. Barton and N.J. Turro, *J. Am. Chem. Soc.*, **107**, 5518 (1985).
8. M.F. Reichmann, S.A. Rice, C.A. Thomas and P. Doty, *J. Am. Chem. Soc.*, **76**, 3047 (1954).
9. C.V. Kumar and E.H. Asuncion, *J. Am. Chem. Soc.*, **115**, 8547 (1993).
10. (a) O. Stern and M.Z. Volmer, *Z. Phys.*, **20**, 183 (1919); (b) J.R. Lakowicz and G. Weber, *Biochemistry*, **12**, 4161 (1973).
11. C.X. Zhang and S.J. Lippard, *Curr. Opin. Chem. Biol.*, **7**, 481 (2003).
12. I. Kock, D. Heber, M. Weide, U. Wolschendorf and B. Clement, *J. Med. Chem.*, **48**, 2772 (2005).
13. G.I. Pascu, A.C.G. Hotze, C. Sanchez-Cano, B.M. Kariuki and M.J. Hannon, *Angew. Chem. Int. Ed.*, **46**, 4374 (2007).
14. M.A. Galindo, D. Olea, M.A. Romero, J. Gomez, P. del Castillo, M.J. Hannon, A. Rodger, F. Zamora and J.A.R. Navarro, *Chem. Eur. J.*, **13**, 5075 (2007).
15. D.L. Ma and C.M. Che, *Chem. Eur. J.*, **9**, 6133 (2003).
16. J. Olmsted and D.R. Kearns, *Biochemistry*, **16**, 3647 (1977).
17. Z.F. Chen, Y.C. Liu, L.M. Liu, H.S. Wang, S.H. Qin, B.L. Wang, H.D. Bian, B. Yang, H.K. Fun, H.G. Liu, H. Liang and C. Orvig, *Dalton Trans.*, 262 (2009).
18. (a) D.L. Boger, B.E. Fink, S.R. Brunette, W.C. Tse and M.P. Hedrick, *J. Am. Chem. Soc.*, **123**, 5878 (2001); (b) G.H. Clever, Y. Söltl, H. Burks, W. Spahl and T. Carell, *Chem. Eur. J.*, **12**, 8708 (2006).
19. V. Rajendiran, M. Murali, E. Suresh, M. Palaniandavar, V.S. Periasamy and M.A. Akbarsha, *Dalton Trans.*, 2157 (2008).
20. L. Messori, P. Orioli, C. Tempi and G. Marcon, *Biochem. Biophys. Res. Commun.*, **281**, 352 (2001).
21. Y.M. Zhao, J.H. Zhu, W.J. He, Z. Yang, Y.G. Zhu, Y.Z. Li, J.F. Zhang and Z.J. Guo, *Chem. Eur. J.*, **12**, 6621 (2006).
22. C.K.L. Li, R.W.Y. Sun, S.C.F. Kui, N.Y. Zhu and C.M. Che, *Chem. Eur. J.*, **12**, 5253 (2006).
23. Y.C. Liu, Z.F. Chen, L.M. Liu, Y. Peng, X. Hong, B. Yang, H.G. Liu, H. Liang and C. Orvig, *Dalton Trans.*, 10813 (2009).