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Characteristics of the interaction of lycobetaine with DNA

LIU Jie, YANG Sheng-Li, XU Bin

(*Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China*)

ABSTRACT The characteristics of the interaction of lycobetaine (LBT) with DNA were examined by fluorescence spectrometer, disc electrophoresis and restriction enzyme analysis. The apparent binding constant of LBT with calf thymus DNA has been determined as 1.67×10^6 L/mol by ethidium bromide displacement method. Based on electrophoresis titration, the mode of DNA binding was found to be through intercalation. Fluorescence quenching assay showed that the intrinsic association constant and the binding site size of LBT to calf thymus DNA were 0.26×10^6 L/mol and 2.6 base pairs, respectively. Selective inhibition of LBT on action of some restriction enzymes showed that LBT intercalate preferentially into GC base pairs. Neither DNA strand break nor interstrand cross-link was produced by LBT. LBT did not bind to DNA covalently and did not cause DNA alkylation.

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KEY WORDS lycobetaine; DNA; ethidium; disc electrophoresis; fluorescence spectrometry

Lycobetaine (LBT) is a new anticancer agent derived from lycorine, a main alkaloid from *Lycoris radiata* Herb. It can inhibit the growth of many experimental tumors including P 388, leukemia L1210, Lewis lung carcinoma, ascites hepatoma and Ehrlich ascites carcinoma[1]. It has been reported that LBT altered the circular dichroism of calf thymus DNA in solution[2], inhibited DNA and RNA syntheses[3], and blocked the expression of actively transcriptional genes[4]. Extensive evidence showed that nucleic acids were probably the principal cell target site. It prompted the present study of interaction of LBT with DNA.

MATERIALS AND METHODS

LBT, homoharringtonine (HHRT) and hydroxycamptothecin (HCPT) were kindly provided by the Department of Phytochemistry, Shanghai Institute of Materia Medica. Doxorubicin (DOX) was purchased from Adria Laboratories Inc. Ethidium bromide was from Sigma Chemical Company; restriction enzymes from Boeringer Biochemical Company.

DNA samples Calf thymus DNA was obtained from Sigma Chemical Company. Plasmid pMYC and pPA4 DNAs were freshly prepared as described in reference[5].

Estimation of drug-binding apparent constants. Drug-DNA binding constants were estimated as described previously[6]. To 2 ml of Tris-EDTA buffer, pH 8.1, containing ethidium bromide 1.3 μmol/L, calf thymus DNA was added to reach a final concentration of 1.35 μmol/L. The fluorescence was measured after equilibrium for several minutes, using a Hitachi 650-10S spectrofluorometer equipped with a 150-W Xenon lamp, at an excitation wavelength of 525 nm and an emission wavelength of 600 nm. Aliquots of concentrated drug solutions were added and the fluorescence was measured.

Electrophoresis titration Electrophoresis titration of conformation changes of plasmid pMYC DNA which occurred upon interaction with LBT was performed by dye titration method, which was originally used to measure DNA superhelical density[7]. The 1.0% agarose gels (16 by 0.5 cm) were formed in 18-cm glass tubes, using TAE buffer containing Tris-acetic acid 50 mmol/L, pH 8.0, Na_2EDTA 2 mmol/L and sodium acetate 20 mmol/L. The gel in each tube contained a concentration of LBT 0, 0.4, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 or 4.0 μmol/L. The tubes containing the gels were immediately placed in conventional disc tube electrophoresis apparatus which was filled with the buffer described above. DNA solution 10 μl (0.5-1 μg) containing the same concentration of LBT were layered on top of the gel. Electrophoresis was then performed at 20 V and 26℃ for 6-8 h until bromphenol blue indicator migrated to the end of the gel. The gels were then extruded and stained for 20 min in TAE buffer containing ethidium bromide 0.5 μg/ml. Photographs of the gels were taken under long-wavelength uv light with a red filter.

Fluorescence quenching assay Fluorescence of LBT was measured at an excitation wavelength of 365 nm using the above spectrofluorometer in the presence of varying concentrations of calf thymus DNA. LBT was dissolved in HEPS buffer 25 mmol/L and EDTA 1.0 mmol/L at 0.1 or 1.0 μmol/L. The intrinsic association constant and the binding site size of LBT to calf thymus DNA were calculated[8].

Digestion of modified DNA with restriction enzymes Normal or modified plasmid pMYC (or pPA4) DNA 1 μl (50 ng) and 1 unit of restriction enzyme were added to a suitable reaction buffer as described in Boeringer Catalog 1987/1988. Final volume of the reaction mixtures was 20 μl. The mixtures were incubated at 37℃ until most DNA in control group was digested. The products of digestion were separated by 1% agarose gel electrophoresis, and the action of restriction enzyme on DNA treated with LBT was then observed under uv lamp after stained with ethidium bromide (1 μg/ml) solution.

Fluorescence assay for detecting DNA strand breakage, interstrand cross-linking and alkylation Reactions were performed at 22-26℃ in a total volume of 200 μl buffered with sodium phosphate 50 nmol/L at pH 7.0 for 4-6 h in dark or under irradiation of uv light. For strand breakage, the reaction solutions contained plasmid pMYC-CCC DNA 0.1 mg/ml (80% CCC) and

LBT 5–50 μmol/L or HCPT 5 μmol/L. For crosslinking and alkylation, the reaction solutions contained the same concentration of plasmid pMYC–linear DNA (100% linear) and the other above components. Aliquots of 50 μl were taken and analyzed for the extent of strand breakage or interstrand crosslinking by addition to the standard pH 11.8 assay solution 3 ml, which contained sodium phosphate 20 mmol/L, pH 11.8, EDTA 0.2 mmol/L and ethidium bromide 0.5 μg/μl. The same volume of reaction solution was taken and analyzed for the extent of alkylation by addition to standard pH 8.1 assay solution 3 ml. The measurements of ethidium bromide fluorescences were performed as described above or after heat denaturation at 96℃ followed by rapid cooling to 23℃.

RESULTS

Drug–DNA apparent binding constants The apparent binding constants of the drugs to DNA were estimated and compared by measuring the reduction in fluorescence of an ethidium bromide–DNA complex as an result of competitive displacement. The drug concentration that produced 50% inhibition of fluorescence was assumed to be inversely proportional to the apparent binding constant[9]. From Fig 1 the drug concentrations producing 50% inhibition were found to be 6.0, 0.6, 2100 and > 6600 μmol/L for LBT, DOX, HCPT and HHRT, respectively. Controls were performed to show that the drugs did not interfere with the fluorescence measurements at the levels employed. Taking the apparent binding constant of ethidium bromide to be 10^7 L/mol, then the apparent binding constants were calculated as 1.7×10^6, 1.7×10^7, 4.7×10^3 and 0 L/mol for LBT, DOX, HCPT and HHRT, respectively. It indicates that LBT has a binding ability to DNA which is markedly greater than that of HCPT and HHRT, but less than that of DOX.

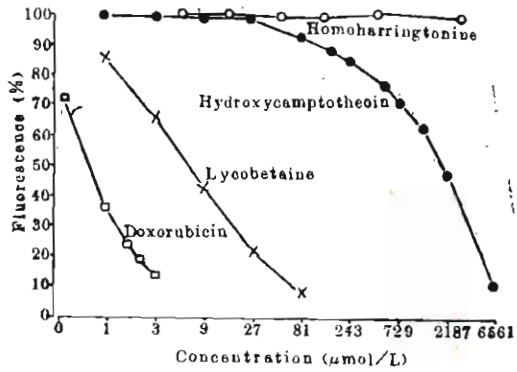

Fig 1. Inhibition of ethidium bromide binding to calf thymus DNA by addition of anticancer drugs.

Electrophoresis titration Native plasmid pMYC DNA is negatively supercoiled. The interaction of LBT with pMYC DNA has been shown to induce marked conformational changes. The negative supercoils were removed progressively and the positive supercoils were then introduced into the molecule with increasing LBT. LBT at 0.8 μmol/L removed all superhelical turns in closed circular DNA. In this case the mobility of CCC DNA are practically equal to that of the same circular DNA containing one or more nicks (Fig 2). Based on this experiment, the mode of LBT binding to

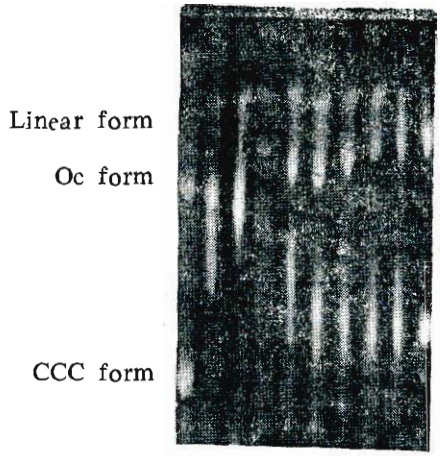

Fig 2. Electrophoresis titration of pMYC DNA conformational changes which occur upon interaction with lycobetaine. Electrophoresis was performed in gels containingincreasing concentrations of lycobetaine, from left to right: 0, 0.4, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 μmol/L. Oc: open circle; CCC: condensed covalently circle.

DNA was determined to be through intercalation.

Fluorescence quenching assay LBT has fluorescent property, and the fluorescence is quenched when it binds to DNA. Fig 3 showed the fluorescence spectrum of LBT, and the fluorescence quenching by DNA. No peak shift was seen during the interaction of LBT with DNA. Fig 4 showed the Scatchard analysis for the binding of LBT to calf thymus DNA, the experimental points generated from fluorescence quenching assay, the line representing the best fit to the data determined by least squares analysis. The intrinsic association constant and binding site size were calculated by the equation of McGhee and Hippel[8] to be 2.6×10^5 L/mol and 2.6 base pairs, respectively.

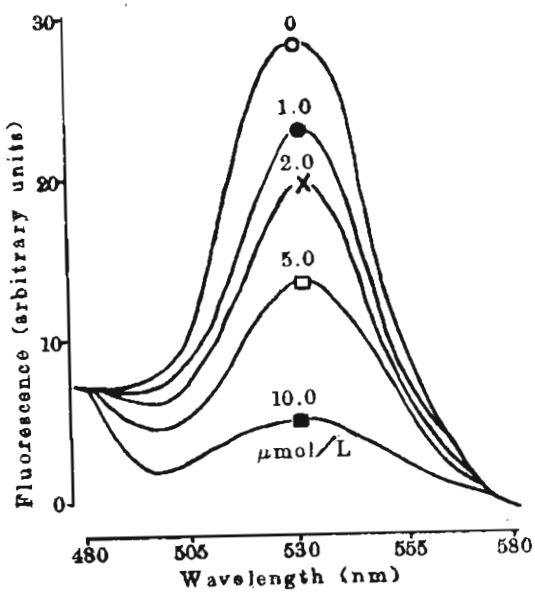

Fig 3. Fluorescence spectrum of lycobetaine (0.1 μmol/L) and its changes which occur upon interaction with calf thymus DNA (1-10 μmol/L).

Restriction enzymic studies Plasmid pMYC and pPA4 can be cut by several restriction enzymes, and are easily separated by agarose gel electrophoresis. When treated with LBT at 0.5, 1.0 or 2.0 μmol/L,

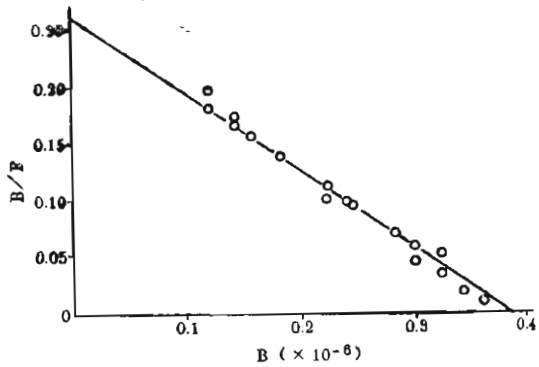

Fig 4. Scatchard plots of the binding data for the interaction of lycobetaine with calf thymus DNA. B = binding ratio (drug bound/base pair); F = free lycobetaine in molar concentration.

they showed different degrees of resistance to some enzymes (Tab 1). Through the analysis of enzyme recognition sites, it can be seen that LBT inhibited selectively the action of the enzymes of which recognition sequences more than 2 GC base pairs existed. It is suggested that LBT may intercalate mainly into GC base pairs.

Tab 1. Action of restriction enzyme on plasmid (pMYC or pPA4) DNA treated with different concentrations of lycobetaine. + + +: completely digested; + +, +: partly digested; −: completely undigested.

Enzyme	Cut site	Action of enzyme on DNA treated with lycobetaine at 0.5	1.0	2.0 μmol/L
Bgl II	AGATCT	+ + +	+ + +	+ + +
Cla I	ATGCTA	+ + +	+ + +	+ +
EcoR I	GAATTC	+ + +	+ + +	+ + +
EcoRV	GATATC	+ + +	+ + +	+ + +
Hae II	PuGCGCPy	+ +	+	−
Hind III	AAGCTT	+ + +	+ + +	+ +
Hpa I	GTTAAC	+ + +	+ + +	+ + +
Hpa II	CCGG	+ +	+	−
Pvu II	CAGCTG	+ + +	+ +	+
Sma I	CCCGGG	+ +	+	−

DNA damages In pH 11.8 assay system only CCC or interstrand cross-linked DNA will spontaneously renature after heat denaturation[10]. LBT at 5-50 μmol/L did not cause decrease in the fluo-

rescence of CCC-DNA or increase in that of linear DNA. HCPT, as a positive control, caused a marked decrease in the fluorescence of CCC DNA. These results indicated that neither strand breakage nor interstrand cross-linking was produced by LBT. In pH 8.1 assay system the loss of ethidium bromide fluorescence is directly proportional to the extent of alkylation[11]. LBT at 5-50 μmol/L did not decrease the fluorescence, which implied that LBT did not bind to DNA covalently or form DNA alkylation.

DISCUSSION

Considerable evidence accumulated from biochemical and pharmacological studies demonstrated that the biological activities of many anticancer agents such as anthracyclines are related to their ability to bind to DNA[12]. LBT has been shown to bind to DNA with a high apparent binding constant of 1.7×10^6 L/mol. The binding is thought to be due to intercalation of the planar ring structure into GC base pairs, and the ionic interaction and H-bond might be important for stabilization of the binding. The planar aromatic chromophore is recognized as an essential requirment for intercalation. It is probably related to the cytotoxic effect of LBT, and should be as the basis of LBT structure reformation. The essential features of the intercalation model for LBT into GC base pairs will need NMR and X-ray diffration analysis. The specificity of LBT to GC base pairs should be determined using synthetic poly (dAdT)-poly (dAdT) and poly (dGdC)-poly (dGdC).

In general, intercalators appear to fall into three distinct categories based on either equal or preferential inhibition of DNA and/or RNA synthesis[13]. The present compound is similar in this respect to actinomycin D, which inhibits preferentially RNA synthesis and does not damage DNA structure. Other intercalators, examplified by doxorubicin and anthrapyrazole, inhibit DNA synthesis equally to or much more potently than RNA synthesis. They are often photosensitizers and can induce the formation of single-or double-strand breaks or cross-links *in vitro* after illumination at the wavelength of absorption maximum[10]. The discrimination of LBT from other intercalators most likely derives from the difference in binding affinity which related to drug-DNA complex and the orientation of the bound molecule.

The interaction with DNA has been considered to be an important characteristic of effective anticancer drugs. It is understandable that the ethidium bromide displacement method will be very useful for rapid screening of the effective antitumor substances. It is rapid, convenient and only very small quantities of samples are needed.

Sedimentation-velocity titration and viscometric titration are the commonly used methods to determine whether a compound is an intercalator[14,15]. Electrophoresis of CCC DNA in agarose gels can be used for following the DNA conformational changes, and thus offers a new convenient and potential method for determining the property of intercalator. The electrophoresis titration, as described, is rapid, requires small amount of DNA (as little as 0.2 μg) and can use unpurified extracts.

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石蒜内铵与 DNA 交互作用的特点

刘 杰、杨胜利、胥 彬 （中国科学院上海药物研究所，上海 200031，中国）

提要 用荧光测定、圆盘电泳和限切酶分析等方法研究了石蒜内铵(LBT)与 DNA 的交互作用。发现，LBT 是一种 DNA 嵌入剂，主要插入 GC 碱基对之间。它与 DNA 的表观结合常数和内部结合常数分别为 1.67×10^6 和 0.26×10^6 L/mol，其结合位点大小为 2.6 bp 左右。LBT 不与 DNA 共价结合，也不引起 DNA 烷基化、交联或链断裂。

关键词 石蒜内铵；DNA；乙锭；圆盘电泳；荧光光谱测定法