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DNA binding properties of 9-substituted harmine derivatives

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Abstract

The β-carboline alkaloids have been characterized as a group of potential antitumor agents. The underlying mechanisms of harmine and its derivatives were investigated by DNA binding assay and Topoisomerase (Topo) inhibition assay. Meanwhile, the DNA photocleavage potential of these compounds and their cytotoxicity were also examined by DNA photocleavage assay and cytotoxicity assay in vitro. Harmine and its derivatives exhibited remarkable DNA intercalation capacity and significant Topo I inhibition activity but no effect with Topo II. Introducing an appropriate substituent into position-9 of β-carboline nucleus enhanced the affinity of the drug to DNA resulting in remarkable Topo I inhibition effects. These results suggested that the ability of these compounds to act as intercalating agents and Topo I inhibitors was related to the antitumor activity. Moreover, these data showing a correlation between cytotoxicity and Topo I inhibition or DNA binding capacity are very important as they strongly suggested that the Topo I-mediated DNA cleavage assay and DNA binding assay could be used as a guide to design and develop superior analogues for antitumor activities.

Keywords: Harmine; β-Carboline; Intercalating; Topoisomerase; Photocleavage; Cytotoxic; T_m

Many plant-derived products are used to combat malignant tumors. The chemotherapeutic drugs which are widely used in clinical oncology include drugs extracted from plants such as taxol and etoposide, or derived from plant alkaloids by simple chemical modification such as the camptothecin (CPT) derivatives topotecan and irinotecan.

The β-carboline alkaloids, present in medicinal plants such as *Peganum harmala* and *Eurycoma longifolia*, have recently drawn an increasing interest due to their antitumor properties. Such plants have been used traditionally for hundreds of years to treat many diseases including cancers and malaria in Oriental medicine. The well-known members of the class are harman and norharman. The structural simplicity of β-carboline alkaloids hides a multitude of in vitro and in vivo effects which make these molecules of interest from both a biophysical and a medicinal perspective. However, to date, the scientific basis for the use of these compounds as antitumor agents is poorly understood.

Harmine (**1**), the most representative naturally occurring β-carboline alkaloid, was originally isolated from *P. harmala* L [1]. Recently, it was also extracted from *Peganum multisectum Maxim*, a plant indigenous to western China by Duan et al. [2]. Subsequent investigations [3,4] demonstrated that harmine was highly cytotoxic against human tumor cell lines. However, by far, the underlying mechanism of action for the antitumor effects of harmine has not been completely defined. Some β-carboline alkaloids such as harman and norharman have been reported to intercalate into DNA base pairs leading to alter DNA replication fidelity or to influence on enzymatic activities in DNA-repair processes and inhibit Topo I [5–11].

Recently, we described the synthesis and the structure-activity relationships (SAR) of a series of 9-substituted harmine derivatives, which exhibit a significant antitumor activity against a panel of human tumor cell lines at

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micromolar concentrations [4]. By analogy with related structures (e.g., harman and norharman), harmine and its derivatives are thought to intercalate into DNA and might interfere with topoisomerase activities. In the present work, we attempt to elucidate the underlying molecular mechanism of these compounds as novel potent antitumor agents. The ability of DNA binding, Topo I inhibition as well as DNA photocleavage was examined by DNA binding assay, topoisomerase inhibition assay, and photocleavage assay. Meanwhile, the cytotoxic activity of these compounds against human tumor cell lines was investigated.

Materials and methods

Chemicals. Harmine **1** (purity 99.85%) was extracted from *Peganum multisectum Maxim*, a plant indigenous to western China, according to the method by Duan et al. [2]. Compounds **2**–**8** (Fig. 1) were synthesized, purified, and characterized as previously described [4].

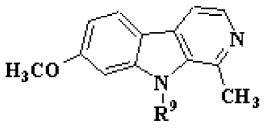
Calf thymus DNA (denoted CT-DNA), amsacrine, doxorubicin hydrochloride, camptothecin, and Human Topoisomerase II-alpha were purchased from Sigma. No further purification was performed. Dithiothreitol (DTT), bovine serum albumin (BSA), sperimidine and other chemicals were from Sino-American Biotechnology Company. Human recombinant Topoisomerase I (hTopo I) [13] was kindly provided by Prof. Zhou Shi-ning and Dr. Yang Guo-wu of Sun Yat-sen University.

Plasmids pBR322. Plasmid pBR322 was prepared using *Escherichia coli* culture and purified using an E.Z.N.A. Plasmid Minipreps Kit I (Omegabio-Tek). The plasmid was suspended in Tris–EDTA buffer contained 90–95% Form I (native supercoiled form) and 5–10% Form II (open circular form resulting from single strand breaks) DNA.

Determination of ΔT_m. T_m measurements were performed using a Shimadzu UV 2501PC Spectrometer and following the methods described [12] with slight modification. Experiments were carried out in PE buffer (1 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.4) in a thermostatically controlled cell hold and the quartz cuvette (1 cm path length) was heated by circulating water at a heating rate of 0.5 °C/min from 25 to 95 °C. Amsacrine and doxorubicin hydrochloride were used as standards. In all cases, the ratio of compound to CT-DNA is 0.5. The 'melting' temperature T_m was taken as the mid-point of the hyperchromic transition.

DNA binding studies. The interaction of the selected 9-substituted harmine derivatives with CT-DNA was studied by UV spectrometry following the methods described [12] with some modifications. Measurements were taken in the same buffer as those for ΔT_m determination directly in a 1-cm pathlength quartz cuvette at room temperature using a Shimadzu UV 2501PC Spectrometer. The cuvette initially held 0.75 ml of a 40μM solution of compounds **2** and **6**, respectively, and then was progressively titrated by increasing amounts of CT-DNA to obtain the spectrum of fully bound drugs in the presence of a large excess of DNA by means of a dispenser equipped with a 25-μl syringe and adequate Teflon tubing.

DNA photocleavage examination. Experiments were performed according to the methods described by Toshima et al. [15]. Briefly, the experiments were carried out in a volume of 10 μl containing 0.25 μg pBR322 plasmid DNA in Tris–HCl buffer (50 mM Tris–HCl, pH 7.5) and 100 μM various harmine derivatives, respectively. Reaction volumes were held in polyethylene microcentrifuge tubes and then irradiated under a mercury-vapor ultraviolet light (8 W, 365 nm, 5 cm distance). Samples were irradiated for 2, 1 h, 30, and 15 min, respectively, at room temperature. Identical treatments were placed in dark at room temperature. After irradiation, a 2-μl of a mixture containing 50% sucrose and 0.25% bromophenol blue was added to the irradiated solution. Samples were analyzed by electrophoresis on a 1% agarose horizontal slab gel containing 0.5 $\mu g/mL^{-1}$ ethidium bromide in Tris–EDTA buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.0). Untreated pBR322 plasmid DNA was used as control. Electrophoretic analyses were carried out at 10 V cm^{-1} for 2 h. Gels were photographed under UV light with Bio-Rad digital camera and analyzed with Gel-Pro software.



1 R^9=H
2 R^9=CH_3
3 R^9=CH_2CH_3
4 R^9=n-C_4H_9
5 R^9=CH_2CH_2OH
6 R^9=$CH_2C_6H_5$
7 R^9=$CH_2C_6F_5$
8 R^9=$(CH_2)_3C_6H_5$

Fig. 1. Chemical structures of harmine and its derivatives.

DNA relaxation reactions with Topo I. Experiments were performed following the procedures described by Yang et al. [13]. The reaction buffer (20 μl) consisted of 35 mM Tris–HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 2 mM spermidine, 0.1 mM EDTA, and 50 mg L^{-1} BSA, 0.25 μg supercoiled pBR322 DNA, and 1 U Human recombinant Topoisomerase I (hTopo I), and 100 or 50 μM compounds **1**–**8** were incubated at 37 °C for 30 min. Camptothecin (100 μM) was used as the positive control. The reactions were terminated by addition of 5× stop solution (5% SDS, 0.25% BPB, and 50% sucrose), and then the samples were electrophoresed through 1% agarose in Tris–acetate–EDTA at 30 V for 8 h. The gels were stained with ethidium bromide and photographed under UV light. After photography under UV light, the amount of DNA was quantified by scanning with an Image Master (Pharmacia Biotech).

DNA unwinding assays with Topoisomerase II. DNA unwinding activities of Topoisomerase II were examined according to the method described by Deveau et al. [14] with some modifications. Experiments were performed in the reaction buffer (50 mM Tris–HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT, and 30 mM BSA), 0.25 μg supercoiled pBR322 DNA, and 1 U Human Topoisomerase II-α, and 600 μM compounds **1**–**8** were incubated at 37 °C for 15 min 100 μM AMSA was used as positive control. Other procedures were the same as for the Topoisomerase I assays described above.

In vitro cytotoxic activity assays. Cytotoxicity assays in vitro were carried out using 96 microtiter plate cultures and MTT staining according to the procedures described by Al-Allaf [16] with a slight modification. Briefly, cells were grown in RPMI-1640 medium containing 10% (v/v) fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cultures were propagated at 37 °C in a humified atmosphere containing 5% CO_2. Cell lines were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Drug stock solutions were prepared in DMSO. The final concentration of DMSO in the growth medium was 2% (v/v) or lower, concentration without effect on cell replication. In all of these experiments, three replicate wells were used to determine each point.

Results

DNA thermal denaturation studies

As polycyclic planar *N*-heteroaromatics may exhibit intercalating properties, in the present investigation, melting temperature (T_m) measurements were deployed to evaluate harmine and its 9-substituted derivatives, relative affinity for DNA. The T_m of CT-DNA in the presence and absence of compounds **1**–**8** were obtained from melting curves and the results of T_m analysis performed with CT-DNA are shown in Fig. 2. Five typical melting curves are presented in the top panel, and the ΔT_m values ($\Delta T_m = T_m^{\text{drug-DNA complex}} - T_m^{\text{DNA alone}}$) are compared