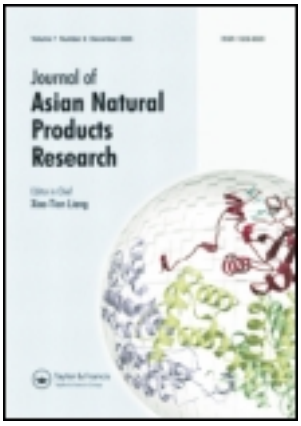

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Anticancer effect and neurotoxicity of *S*-(+)-deoxytylophorinidine, a new phenanthroindolizidine alkaloid that interacts with nucleic acids

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Phenanthroindolizidine alkaloids are a family of plant-derived compounds with significant antineoplastic activity as well as other effects like antiamebicidal, antiviral, and anti-inflammatory activities. The specific biomolecular targets of these compounds have not yet been clearly identified. *S*-(+)-Deoxytylophorinidine (CAT) is a new phenanthroindolizidine alkaloid, originally extracted from the roots of *Tylophora atrofolliculata* and *Tylophora ovata*. Potent anticancer activity was observed *in vitro* and *in vivo*. Neurotoxicity of CAT was also studied and it was far less serious than that of vinblastine. Interactions between this compound and DNA had been studied in detail in our laboratory previously, and we further studied its interactions with RNA.

Keywords: *S*-(+)-deoxytylophorinidine; phenanthroindolizidine alkaloid; anticancer; circular dichroism; nucleic acid; neurotoxicity

1. Introduction

Phenanthroindolizidine alkaloids typified by tylophorine are a family of plant-derived alkaloids isolated from Asclepiadaceae and Moraceae plant family [1–3]. Many of these alkaloids possess significant anticancer activity (both *in vitro* and *in vivo*) and also anti-inflammatory, antiamebicidal, and antiviral activities [4–6]. However, the specific cellular target of the phenanthroindolizidines is still unclear, though a significant amount of cellular activities of these compounds are known [7]. Protein and nucleic acid synthetic suppression has been reported previously, and 40S ribosomal subunit has been postulated to host the binding site of these drugs [8]. Other targets, such as thymidylate synthase [9], dihydrofolate reductase [10], and NF-κB [11], were also reported. Nucleic acids [12,13], both DNA and RNA, were suspected to be the primary target of this kind of compound although other unclear mechanisms may also work. Interactions of such compounds with DNA may directly interfere with the process of duplication and transcription. And, in a similar way, such interactions with rRNAs in the ribosomes may directly interfere with the process of protein synthesis. So, the direct interactions between this compound and nucleic acid were determined in our laboratory.

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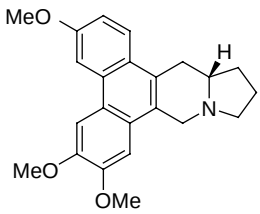

Figure 1. Structure of *S*-(+)-deoxytylophorinidine.

S-(+)-Deoxytylophorinidine (short for CAT) (Figure 1) is a new phenanthroindolizidine alkaloid, originally extracted from the roots of Chinese medicinal plant *Tylophora atrofolliculat*a and *Tylophora ovata* in our institute. The purity was 99.84% determined by the HPLC method. Potent anticancer activity was observed *in vitro*, with the inhibitory concentration (IC_{50}) about 10^{-7} mol/l in several tumor cell lines. *In vivo* pharmacodynamic evaluations showed potent anticancer activities. Circular dichroism (CD) is a powerful tool for the study of the secondary structures and conformations adopted by nucleic acids. The CD spectrum of DNA is sensitive to changes in conformation resulting from ligand binding. CD is particularly powerful for monitoring structural changes resulting from changes in environmental conditions [14]. We already found that this compound could concentration-dependently and AT-repeated sequence-specifically interact with DNA by CD and fluorescence emission spectra. And the same methods were used to further determine the interactions between this compound and RNA. Neurotoxicity of phenanthroindolizidine alkaloids was reported elsewhere [4]. Neurotoxicity of CAT was also studied and it was far less serious than that of vinblastine.

2. Results and discussion

2.1 In vitro *anticancer activities of CAT on various human cancer cell lines*

Several kinds of cancer cell lines were chosen for *in vitro* evaluations. We tried to contain all the common kinds of human tumors for wide use future**.** Synthesized CAT was used for the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. The effects of synthesized CAT on the cell growth in these cell lines were examined. All the cell lines were exposed to gradient concentrations of the compound and dose-dependent growth inhibition was observed. As shown in Table 1, IC_{50} of CAT was determined to be in the similar order of magnitude, 10^{-7} mol/l, in the used cell lines.

2.2 In vivo *anticancer activity of CAT on H22 murine hepatoma xenografts*

To further assess the therapeutic effects of CAT *in vivo,* animal studies were carried out using Kunming (KM) mice with H22 mouse murine hepatoma xenografts. Citrate of CAT was used instead of CAT, due to its poor solubility. The activity of its salt was compared with CAT itself *in vitro*, and they have similar efficacies**.** The tumor weight and tumor inhibition rates are shown in Table 2. The inhibition rate for CAT 15 mg/kg was 75.90% and that of CAT 12.5 mg/kg was 48.37% compared to that for control group. However, as shown in Figure 2, the average body weight of CAT 15 mg/kg group decreased rapidly to about 20 g in the last day. The decreased body weight was an obvious indication of severe toxicity.

2.3 In vitro *assay for neurotoxicity*

PC12 neurite outgrowth assay is a suitable and reliable model for predicting the neurotoxicity of various compounds. PC12 is a rat pheochromocytoma cell line. Microtubules in neurites, axons, and dendrites of nerve cells have cell-specific posttranslational modifications that are distinct from nonneuronal microtubules. After treatment with vincristine (VCR), the axons lost their uniform shape and exhibited an irregular, shortened structure. In contrast, the neurites of CAT-treated

Table 1. *In vitro* growth inhibition of CAT on various kinds of cancer cell lines.

	IC_{50} (10^{-7} mol/l)									
	A2780	A375	A549	BGC823	HCT8	HepG2	MCF-7	SH-3Y-5Y	SW1990	U251
CAT	3.42 ± 0.58	3.7 ± 0.45	2.98 ± 0.41	6.18 ± 0.80	1.16 ± 0.29	1.45 ± 0.44	9.11 ± 2.46	1.84 ± 0.20	5.16 ± 0.93	3.34 ± 1.04

Note: Data represent the mean of three independent experiments ± SD.

Table 2. Effects of CAT on H22 murine hepatoma xenografts.

Group	Dose (mg/kg × d)	Animal no. (initial/final)	Body weight (g) (initial/final)	Tumor weight (g, mean ± SD)	Inhibition (%)
Control		9/9	26.56 ± 0.88/35.22 ± 4.87	1.03 ± 0.46	
CTX (i.p.)	100 × 1	8/8	26.13 ± 0.64/29.00 ± 1.60	0.07 ± 0.04	93.47**
CAT low	10.0 × 4	9/9	26.11 ± 0.78/25.00 ± 5.39	0.83 ± 0.52	19.22
CAT medium	12.5 × 4	9/9	26.00 ± 0.71/20.33 ± 2.24	0.53 ± 0.20	48.37*
CAT high	15.0 × 4	9/6	25.89 ± 0.93/19.50 ± 2.74	0.25 ± 0.14	75.90**

Note: $*p < 0.05$, $**p < 0.01$ compared with control group.

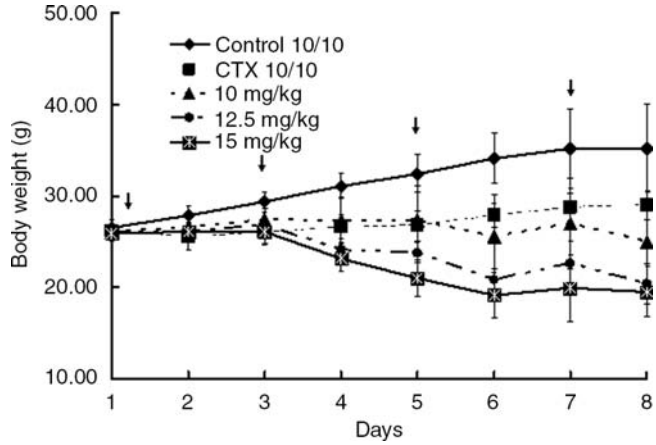

Figure 2. Effects of *S*-(+)-deoxytylophorinidine on body weight of mice. Dosages of all the salts were equimolar to the dosage used for *S*-(+)-deoxytylophorinidine. H22 hepatoma was implanted s.c. into KM mice in the axillary fossa. Treatment was started the day after the implantation. CAT was administered by p.o. once every other day, for four times in all, and cyclophosphamide was administered by i.p. injections once. Body weights were recorded every day.

PC12 cells retained their structural integrity, even in relatively high concentration, and appeared similar to cells treated with vehicle control (Figure 3).

2.4 Concentration-dependent interactions between CAT and RNA determined by CD and fluorescence emission spectra

We further studied the interactions between CAT and RNA. Intrinsic circular dichroic spectra of a series of concentrations of RNA were shown in Figure 4(A). The concentration range was from 1×10^{-5} to 100×10^{-5} M(nt) which is calculated by the amount of nucleotides of RNA. The concentration of CAT was fixed to be 1.2×10^{-4} M, and circular dichroic spectra were obtained in the absence and the presence of the same series of concentrations of RNA from 1×10^{-5} to 100×10^{-5} M(nt). Induced circular dichroic (ICD) spectra, which are the net CD spectra after subtraction of the CD spectra of CAT and the CD spectra of correspondent concentration of RNA, were shown in Figure 4(B). And the minimum ICD spectra at the wavelength of 250–265 nm were plotted with the concentration of RNA in Figure 4(C). These results suggest that CAT can concentration-dependently interact with RNA. Fluorescence emission spectra were also used to check the interaction between Baker's yeast RNA and CAT. The concentration of CAT was fixed to be 6×10^{-5} or 3×10^{-5} M, and fluorescence emission spectra were obtained in the absence and the presence of series of concentrations of RNA from 1×10^{-6} to 1×10^{-2} M(nt). Fluorescence emission spectra of CAT (6×10^{-5} M or 3×10^{-5} M) in the absence and presence of the series of concentrations of RNA ($1 \times 10^{-6} - 1 \times 10^{-2}$ M) were obtained by 260 nm excitation and 350–450 nm emission (Figure 5(A),(B)). The 380 nm fluorescence intensities of the spectra are shown in Figure 5(C),(D).

2.5 Discussion

The specific cellular target of the phenanthroindolizidines is unknown, but it was determined that some of the phenanthroindolizidines inhibited protein synthesis and to a lesser extent RNA and DNA synthesis [3]. CAT is a newly extracted and synthesized phenanthroindolizidine that exerts its potent anticancer effects both *in vitro* and *in vivo*. We already found

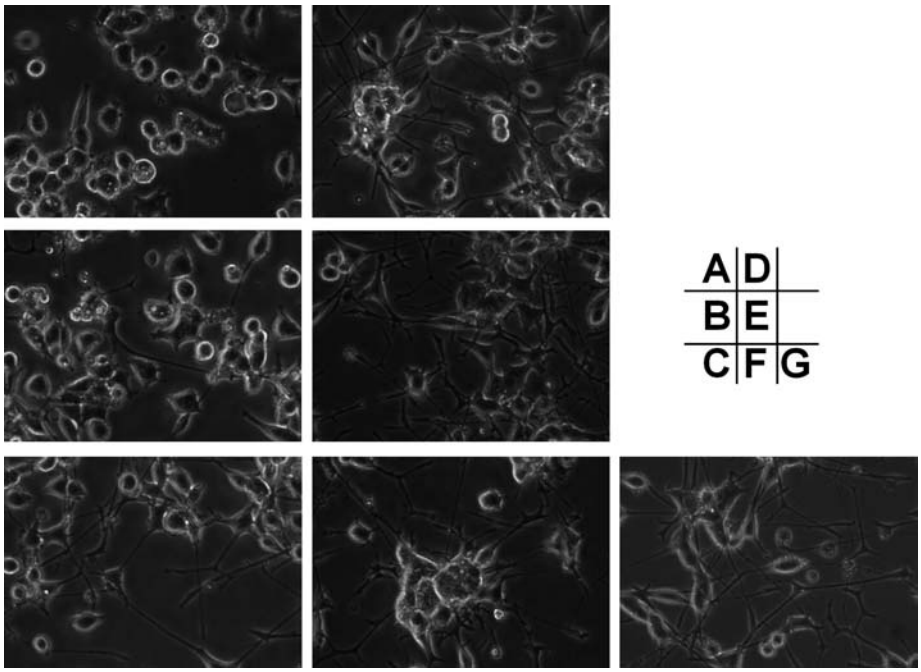

Figure 3. *In vitro* neurotoxicity evaluation of *S*-(+)-deoxytylophorinidine. Rat pheochromocytoma PC12 was used to evaluate the *in vitro* neurotoxicity of *S*-(+)-deoxytylophorinidine and VCR. Low concentration of VCR (1×10^{-9} M) can inhibit the NGF-induced neurite outgrowth of rat pheochromocytoma cells (PC12) and up to 1×10^{-7} M *S*-(+)-deoxytylophorinidine only slightly inhibit the neurite outgrowth. (A–C) NGF-pretreated rat pheochromocytoma cells (PC12) treated by VCR, 1×10^{-7}, 1×10^{-8}, and 1×10^{-9} M, respectively. (D–F) NGF-pretreated rat pheochromocytoma cells (PC12) treated by *S*-(+)-deoxytylophorinidine, 1×10^{-7}, 1×10^{-8}, and 1×10^{-9} M, respectively. (G) NGF-induced neurite outgrowth of rat pheochromocytoma cells (PC12).

that this compound could concentration-dependently and AT-repeated sequence-specifically interact with DNA previously. The AT-rich sequences, located at a fixed distance upstream of the transcription start site, have been identified in essentially all animals, plants, and fungi that have been examined [15,16]. The preference of CAT to intercalate into AT-repeated sequences strongly suggests that CAT interferes with the normal function of transcription, maybe by blocking the recognition of promoter site by protein factors. CAT as a representative of phenanthroindolizidine alkaloid was also proved to interact with RNA by CD and fluorescence emission spectra. Such interactions are in accordance with previous reports that certain phenanthroindolizidine and phenanthroquinolizidine alkaloids (tylocrebrine, tylophorine, and cryptopleurine) adopt 40S ribosome subunit as their site of action. And inhibition of protein synthesis was regarded as the main mechanism for the action [8,17]. These conclusions were obtained by cross-resistance patterns of independently isolated mutants to the compounds. And a specific mutation in yeast *cry* gene was found to be the key for the mutation to these compounds [8]. The gene *cry* was checked to be the RPS14A (NCBI accession: AAA34530) in NCBI Map viewer, a ribosomal protein that is a component of 40S small subunit of ribosome. The mutation of this protein leads to the resistance to the phenanthroindolizidine. We postulate that the normal interactions between ribosomal RNA and such proteins are altered to some extent by

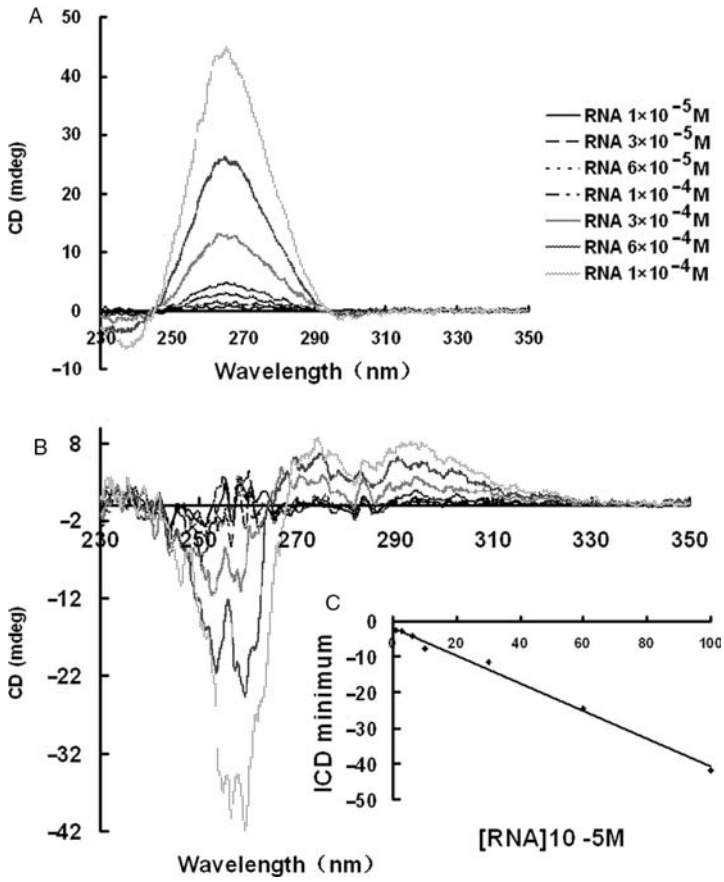

Figure 4. (A) Circular dichroic spectra of a series of concentrations of RNA ranging from 1×10^{-5} to 100×10^{-5} M(nt) calculated by the amount of nucleotides of RNA. (B) ICD spectra of *S*-(+)-deoxytylophorinidine in the presence of the same concentration series of RNA. ICD is the net CD spectra after subtraction of the CD spectra of *S*-(+)-deoxytylophorinidine and the CD spectra of correspondent concentration of RNA. (C) Plot of the minimum ICD of the spectra at the wavelength of 250–265 nm with the concentration of RNA.

phenanthroindolizidine, which in turn interferes the normal functions of ribosome. The ribosome RNA is the direct target of phenanthroindolizidine, which can be exemplified by the CAT–RNA interactions. And the normal structural and functional aspects of ribosome were altered concomitantly. And the synthesis of protein should be inhibited. This may be another important mechanism of the anticancer effects of phenanthroindolizidines in addition to their interactions with DNA sequence-specifically, which inhibit the processes of DNA replication and transcription. Such interactions between RNA and CAT are to be further studied in our laboratory in future. Neurotoxicity of CAT was proved to be far less serious than that of vinblastine. This partly proves that CAT does not act on cellular microtubule system. Body weight of animals decreased in CAT-treated group and this is an indicative of its severe toxicity. However, CAT and other phenanthroindolizidines may still deserve further developing for their favorable oral absorption. Preliminary pharmacokinetics (data not shown) of CAT and its salts showed this compound can penetrate the blood–brain barrier and distribute in brain tissues. So, it may be an appropriate property to treat

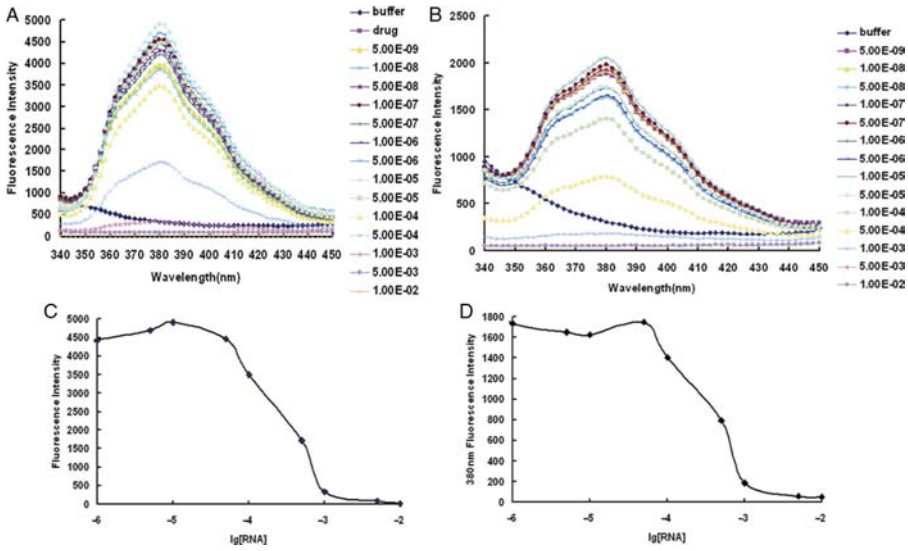

Figure 5. Fluorescence emission spectra of *S*-(+)-deoxytylophorinidine (A, 6×10^{-5} M and B, 6×10^{-5} M) in the absence and the presence of the Baker's yeast RNA ($1 \times 10^{-6} - 1 \times 10^{-2}$ M(nt)) were obtained by 260 nm excitation and 350–450 nm emission. Constructed curve of the maximum, at around 280 nm vs. logarithm of the [RNA] (M/M(nt)) is shown in (C) and (D), respectively.

neural tumor such as glioma. Few antitumor drugs except temozolomide and lomustine can be used by oral administration in clinical practice to treat brain tumor effectively.

3. Materials and methods

3.1 Cell culture and MTT assay

Human gastric cancer BGC823, human liver cancer Bel7402, human colon cancer HCT-8, human ovarian cancer A2780, human lung cancer A549, human melanoma A375, human neuroblastoma SH-SY5Y, human glioma BT-325, human glioma U251 cell lines, and rat pheochromocytoma PC12 cell line were mainly purchased from American Type Culture Collection (ATCC) or Cell Culture Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. They were all maintained in Roosevelt Park Memorial Institute medium 1640 (RPMI 1640) supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37°C. The MTT assay was performed in triplicate. Cells (1500/well) were added to each well of the 96-well plates. After 24 h, cells were treated with serial concentrations of samples for 72 h. After 72 h, incubated at 37°C in 5% CO_2, the CAT-containing medium was removed and replaced by fresh medium. The cells in each well were then incubated in culture medium with 100 μl of 0.5 mg/ml MTT solution for 4 h. After the medium was removed, 150 μl of DMSO was added to each well. The plates were gently agitated and measured for the absorbance of the wells at 570 nm with a reference wavelength of 450 nm by a microplate reader (Wellscan MK3, Labsystems Dragon Co., Helsinki, Finland). The 50% IC_{50} is defined as the concentration that reduced the absorbance of the untreated wells by 50% of the vehicle in the MTT assay [18].

3.2 Animal study

Male KM mice were obtained from the Laboratory Animal Center, Academy of Military Medical Science, and acclimated to laboratory conditions 1 week before

tumor implantation. The mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. H22 murine ascites hepatoma (purchased from Cell Culture Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences) xenografts were established by injecting s.c. 1×10^6 H22 cells into axillary fossa. Treatment was initiated the day after the implantation. The citrate of CAT was used due to the poor solubility of CAT. Citrate of CAT was dissolved in pure water along with solvent served as control. Cyclophosphamide was dissolved in normal saline. CAT was administered by p.o. once every other day, for four times in all, and cyclophosphamide was administered by i.p. injections once. There were nine mice in each CAT treating group. The body weights of the mice were monitored daily for toxicity. At the end of treatment, the mice were sacrificed and the tumors were peeled off and weighted. To evaluate the anticancer activity of CAT, percentage of inhibition was calculated as following:

$$\text{Inhibition}(\%) = \left(1 - \frac{\text{mean of tumor weight of treated group}}{\text{mean of tumor weight of control group}}\right) \times 100\%.$$

3.3 *PC12 pheochromocytoma cell line as an* in vitro *assay for neurotoxicity*

After incubation with β-nerve growth factor (Recombinant Rat β-NGF, R&D catalog number: 556-NG) in 6-well plate for 6 days in order to stimulate neurite outgrowth, rat pheochromocytoma PC12 cells were treated for 24 h with VCR or CAT, at concentrations of 10^{-7}, 10^{-8}, and 10^{-9} mol/l. Then, they were observed by phase contrast microscopy [19,20].

3.4 *Circular dichroic spectroscopy*

CD spectra were measured on a Jasco J-815 CD spectrometer (displaying CD in millidegrees ellipticity) using 0.5 cm cell. All the CD experiments were carried out at 25°C in BPES buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, 185 mM NaCl, pH 7.0) [14]. Baker's yeast RNA (Sigma-Aldrich Corp., St Louis, MO, USA, cat. No. R6750) were dissolved in BPES buffer as stock solution and diluted when used. All the concentrations of RNA used were calculated by the amount of nucleotides (M(nt)). DNA and CAT were diluted with BPES buffer with the designated concentrations and ratios to make 1 ml volume system and stirred constantly before being scanned from 350 to 220 nm. The samples were scanned every 0.5 nm for four times and average values were used for the spectra automatically. Baseline of solvent CD signals was deducted from each spectrum. All the utensils, buffer solutions, and pipette tips used for RNA were all RNAse free to protect RNA from degrading. ICD spectra were obtained by subtracting the spectra of single drug and the spectra of single RNA from the spectra of their mixture of corresponding concentrations [14,21–23].

3.5 *Fluorescence spectroscopy*

Fluorescence emission spectra were recorded for CAT in the presence and absence of baker's yeast RNA (Sigma-Aldrich, cat. No. R6750) in the range 340–450 nm upon excitation at 260 nm at 23°C, using a slit width of 2 nm. Fluorescence emission spectra were measured on a fluorescence spectrophotometer (Spectra Max Gemini XS, New York, USA). Samples of RNA and CAT are treated in the same way as in the CD spectroscopy. Designated concentrations and RNA/CAT ratios were added and mixed before being scanned. Baker's yeast (*Saccharomyces cerevisiae*) RNA was used in this experiment and all the utensils, buffer solutions,

and pipette tips used were all RNAse free to protect RNA from degrading [23,24].

Acknowledgements

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