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Short communication

Alkaloid chelirubine and DNA: Blue and red luminescence

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ABSTRACT

Extracts from Himalayan herb *Dicranostigma lactucoides* containing alkaloid chelirubine have been used for centuries in Chinese herbal medicine. We have found a new utilization for the alkaloid: it can be used as a DNA fluorescent probe showing blue (free form) and red (intercalated to DNA) luminescence emission after irradiation by near-UV light. Besides quantification of DNA (LOD=6 ng ml^{-1}) it can also be used as a supravital cell probe because chelirubine molecules can effectively enter into the living cell through the cell membrane.

1. Introduction

Alkaloids are natural substances with many interesting therapeutical [1–3], biological [4] and chemical [5,6] effects. Besides the well-known alkaloids (quinine, xanthine, caffeine, nicotine, cocaine, *etc.*), there are also rare alkaloids with little information about their properties in literature. One of the less known alkaloids is chelirubine (CHR) from group of quaternary benzo[c]-phenanthridine alkaloids (QBAs) [7]. CHR is present in small quantities (as a "minor" alkaloid) in several plants from family *Papaveraceae* and must be separated from the mixture of other alkaloids [8]. To our knowledge, the best source for isolation of CHR is a herb *Dicranostigma lactucoides* [9].

Sanguinarine (SR), chelerytrine and nitidine are readily available QBAs and therefore they have been studied in detail by several scientific groups [10–12]. According to these studies it could be stated that QBAs share many properties, such as equilibrium between iminium and alkanolamine form depending on solution pH (Scheme 1) [13], interaction of iminium form with nucleic acids (intercalation) [12,14–19], and rapid penetration into cells [20]. The main differences between individual QBAs are their different luminescence spectral properties [21] – an important feature from the analytical point of view.

CHR chloride used in our experiments was obtained from raw plant material (*Dicranostigma lactucoides*). Distribution and biosynthesis of the alkaloid was described elsewhere [9,22,23]. Dried ground roots were extracted in methanol. Then, the extract was concentrated and dissolved in sulfuric acid. After filtration, the extract was alkalized and CHR and other two QBAs were extracted into diethyl ether. Following a concentration step, diethyl ether residue contained a mixture of three alkaloids, which were separated by low pressure LC [9]. During first preparation, the structure of pure CHR chloride was checked by ^{1}H and ^{13}C NMR spectroscopy [24]. Further on, CHR was separated according to its retention time. Resulting product was more than 99% pure. For details see technical notes.

In contrast to other QBAs, CHR has unique spectral properties. When an aqueous solution of the alkaloid is excited by appropriate wavelength, there are two emission bands in the emission spectrum with maxima at 420 nm (violet–blue fluorescence) and 620 nm (red luminescence), respectively (Fig. 1, Figure A in Supporting Information). Under acidic or neutral aqueous conditions the 420 nm emission band is dominant. However, when double stranded DNA (*ds*DNA) is added to the system, 420 nm emission band is suppressed, and *vice versa* "red" band is enhanced. On the

Abbreviations: QBAs, quaternary benzo[c]phenanthridine alkaloids; CHR, chelirubine; SR, sanguinarine; EB, ethidium bromide

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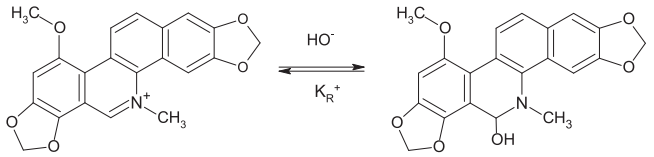

Scheme 1. Structure of CHR in a quaternary cation form (charged iminium) and a pseudo-base form (neutral alkanolamine).

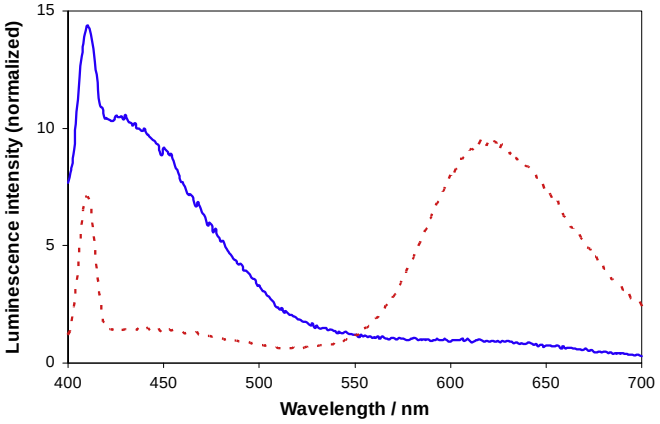

Fig. 1. Emission spectra in presence (- -) and absence (solid line) of CT DNA. The sharp peak at 410 nm is related to Raman scattering.

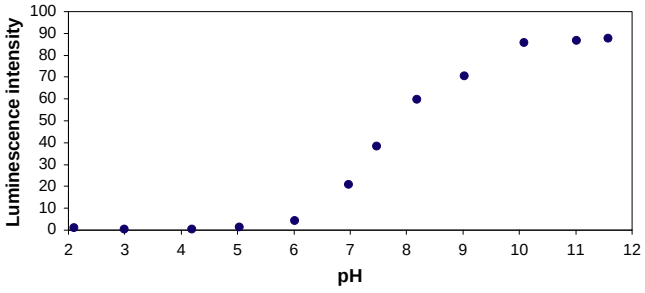

Fig. 2. Fluorescence emission at 420 nm (excitation 360 nm) of CHR ($c=3\times10^{-6}$ M at different pH, $I=0.1$ M).

contrary, both emission bands of SR are quenched by the presence of *ds*DNA [21].

We have also found that CHR-*ds*DNA complex can be excited not only by light of wavelength around 360 nm but also by wavelengths about 500 nm (Figure B in Supporting Information). Both absorption bands are near to maxima of widely used excitation lasers (third harmonic 355 nm Nd:YAG and 488 nm Ar^+ lasers). While excitation by wavelength of 355 nm enables observation of both 420 and 620 nm emission bands, excitation at 488 nm is not so destructive for living cells and still allows observing interaction with DNA at 620 nm.

Because of the superior purity of our CHR, constant pK_{R+} characterizing equilibrium between quaternary cation and pseudo-base (hydroxylated) form (Scheme 1) was estimated by luminescence spectroscopy and compared with result published earlier [25]. The value of 7.62 ± (0.08) (Fig. 2) was obtained that is very close to value 7.70 from previous study. Such a small difference could be caused by tiny amounts of other QBAs contaminating the preparation used in older study (other QBAs have higher pK_{R+} than CHR [25]). In our further studies, slightly acidic phosphate buffer at pH 6.20 was used throughout the *in vitro* experiments as a compromise between high iminium form content and optimal pH for *ds*DNA.

Table 1 summarizes association constants for oligonucleotides with different sequences. The 12-membered oligonucleotides 5′-$(GC)_6$ -3′ (Fig. 3), 5′–CGCGAATTCGCG-3′ (*Dickerson*), and 26-membered oligonucleotide 5′–CAATCGGATCGAATTCGATCCGAT-TG-3’ (*ds*26) were chosen to represent different combination of common bases. Association constants were found not to be much dependent on A–T or G–C content (Table 1) and in general, they are slightly higher than those published for sanguinarine and chelerythrine [12].

The linear increase of fluorescence intensity at 620 nm as a response to increasing amount of *ds*DNA allows using CHR for quantification of DNA. LOD (3σ method) obtained from calibration curve (Figure C in technical notes) was calculated to be 6 ng ml^{-1} of *ds*DNA. This is comparable to Hoechst 33258 and slightly worse than cyanine dyes (Table A in Supporting Information). The advantages of CHR over other dyes are the possibility to choose excitation wavelength and good cell membrane permeability.

The use of QBAs as supravital DNA probes has been presented in our previous study with focus on macarpine [20]. Beside macarpine, CHR was identified as another possible fluorescent probe. Therefore, more detailed study of CHR ability to stain cell nucleus *in vivo* was conducted. In living HeLa cells stained with CHR (100 μg ml^{-1}) we have observed various fluorescence of cellular structures under particular filter combinations. Under near-UV excitation (Fig. 4a) we have observed bright red nucleus and blue cytoplasm containing bright blue vesicles (probably lysosomes). Similar picture was observed under excitation by violet light (Fig. 4b) – orange nuclei and light blue cytoplasm and vesicles. However, under irradiation by green (Fig. 4c) or blue (Fig. 4d) light, only red nuclei with distinguishable nucleoli were visible. Last setup corresponds to wavelength available in Ar^+ laser (488 nm), so fluorescence could also be observed on microscopes equipped with this laser. These results demonstrated unique property of CHR, *i.e.* dual fluorescence emission under near-UV excitation, which was observed also during fluorometric measurements. Hence, we suppose that red fluorescence of nuclei corresponds to the iminium form of CHR intercalated to DNA and blue fluorescence of cytoplasm is caused by free form of CHR.

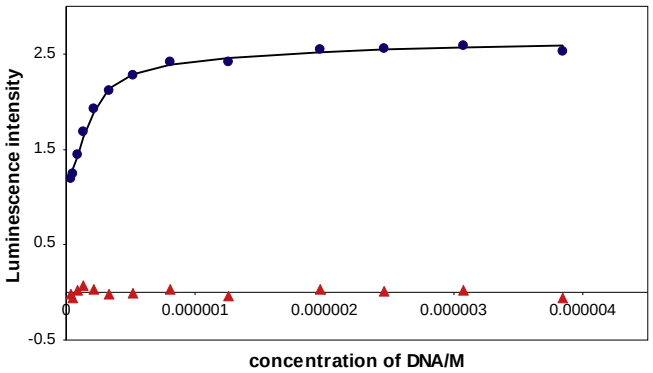

Fig. 3. Dependence of luminescence intensity (emission at 620 nm, excitation at 360 nm) on concentration of oligonucleotide $(GC)_6$. Measured values (○), calculated values (solid line) and residues (Δ) are plotted in the figure.

Table 1
Stepwise association constant of CHR complexes with different oligonucleotides (stoichiometry 6:1 (CHR:DNA), assuming identical binding sites).

Sequence	$(GC)_6$	Dickerson	*ds*26
log *K*	5.83 ± 0.08	5.44 ± 0.04	5.63 ± 0.02

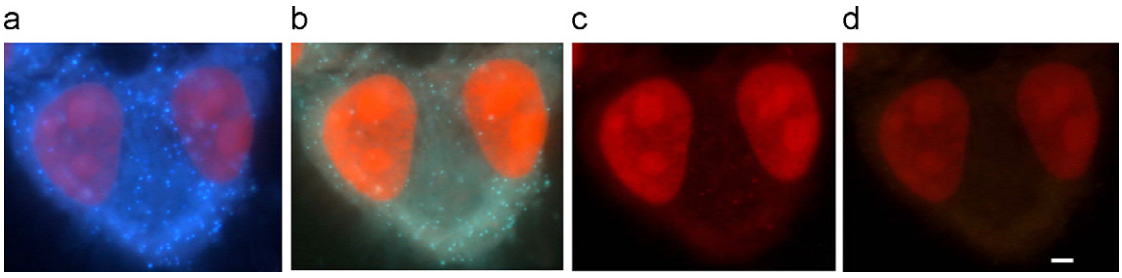

Fig. 4. Living HeLa cells stained with CHR at concentration 100 μg/ml observed with Leica DM 5000B fluorescence microscope using following excitation: (a) UV (filter A), (b) blue and UV (filter D), (c) green (filter N2.1), (d) blue (filter I3). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

In conclusion, thanks to its unique properties including blue and red fluorescence emission in presence/absence of *ds*DNA, CHR can be used for quantification of *ds*DNA content by means of luminescence spectroscopy. We have demonstrated that CHR can be used as an alternative DNA dye to DAPI, Hoechst 33258, PicoGreen, cyanine dyes, SYBR-type dyes, ethidium bromide, etc [26,27]. Moreover, CHR is cell membrane-permeable and can be used for DNA-specific staining of living cells. Although the extraction of QBAs from plants is time consuming, it might be possible in future to substitute it by synthetic [28–30] or semi-synthetic (from other alkaloids) preparation to obtain CHR in larger amounts.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.10.045.

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