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New series of non-toxic DNA intercalators, mitochondria targeting fluorescent dyes

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ABSTRACT

Five novel benzoxazolium and benzothiazolium derived asymmetric dicationic monomethine cyanine dyes, bearing a thioester group have been synthesised via condensation of quaternary nitrogen containing heterocyclic salts in various anhydrides. Their chemical structures were evaluated by NMR, ESI/MS, IR spectroscopy, and melting point temperatures. Photophysical studies on the title compounds were performed in methanol solutions. The longest wavelength absorption maxima λ_{max} of the studied dyes are in the region 453–508 nm. The corresponding fluorescence maxima λ_{fl} were registered within the 489–553 nm. Further spectroscopy and calorimetric techniques including circular dichroism (CD), UV–Vis, fluorescence, thermal denaturation and isothermal titration calorimetry have been applied to the studied dyes in the presence of biopolymers, in order to evaluate their staining ability as fluorescent markers. In aqueous solution the compounds exhibit no intrinsic fluorescence, however upon binding to dsDNA and dsRNA they show considerable enhancement of fluorescence intensity. Binding constants *Ks* of the complexes [dye–polynucleotide] have been evaluated from fluorescence spectral titrations. Although the intercalation is the predominant binding mode, the compounds show little or no effect on the cell proliferation. Upon fast cell internalization the compounds accumulate in the mitochondrial space.

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1. Introduction

Within the last two decades design of small molecules that are able to recognize DNA/RNA by non-covalent interaction often combines two or more non-covalent binding modes (intercalation, groove binding, or electrostatic sugar-phosphate backbone binding) [1,2]. Such dyes selectively report on structural differences of DNA and RNA secondary structure. Furthermore their subcellular positioning and fate can be in that way manipulated. Lipophilic di-cations usually target mitochondria, namely the mitochondrial matrix space due to the permeability of the outer mitochondrial membrane and the inner mitochondrial membrane potential $\Delta\psi_{mito}$. After passing the plasma membrane, where this potential is not so prominent ($\Delta\psi_{cell}$ = 30–60 mV), accumulation in the mitochondrial matrix is enabled by the driving force of $\Delta\psi_{mito}$ = 150–180 mV [3]. Although the cationic charge increases affinity for the mitochondrial matrix, it should be dispersed over a large surface area to enable easier passage through the phospolipid bilayers. There are numerous mitochondrial dyes and mitochondrial membrane potential probes available on the market, however they all exhibit serious drawbacks related to their use *in vitro/in cellulo*. Respiration inhibition, inhibition of mitochondrial ATPase, general cytotoxicity, photobleaching and sensitivity issues are problems which are described [4,5,6]. Inspired by our previously reported specific mitochondrial fluorescent probes [7,8], we prepared novel cyanine dyes, lipophilic di-cations which should: a) Have affinity towards DNA/RNA; b) Have limited or no effect on cell proliferation; c) Aim the mitochondria.

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The probes are benzothiazole and benzooxazole based cyanine dyes carrying additional mitochondria targeting cationic charge. Previously, we used triphenylphosphonium cation which targets mitochondria *in vitro* and *in vivo* [9,10], this series of compounds bear pyridinium ion, also known to facilitate mitochondrial uptake [11]. To avoid problems related to general cytotoxicity and anti proliferative activity we attached the bulky substituents on the long arm of the cyanine dyes, which by our experience limits the damage caused by the interaction with DNA/RNA [12].

2. Materials and methods

2.1. Study of DNA/RNA interactions

2.1.1. UV/visible spectrophotometry, circular dichroism (CD) and fluorescence spectroscopy

The UV/Vis spectra were recorded on a Varian Cary 100 Bio spectrophotometer, fluorescence spectra on a Varian Cary Eclipse fluorescence spectrophotometer and CD spectra were collected with a Jasco J-810 spectropolarimeter at 25 °C using 1 cm path quartz cuvettes.

The polynucleotides: $p(dAdT)_2$, $p(dGdC)_2$, pApU, and *calf thymus* (*ct*)-DNA (Sigma-Aldrich, St.Louis, USA) were dissolved in sodium cacodylate buffer, $I = 0.05$ mol dm^{-3}, pH = 7, calf thymus (*ct*-) DNA was additionally sonicated and filtered through a 0.45 μm filter. Aqueous solutions of compounds were buffered to pH = 7 (sodium cacodylate buffer, $I = 0.05$ mol × dm^{-3}). The polynucleotide concentration was determined as the concentration of phosphates by UV absorption [13]. Spectrophotometric and titrations were performed at pH = 7.0 (sodium cacodylate buffer, $I = 0.05$ mol dm^{-3}) by adding portions of polynucleotide solution into the solution of the studied compound. Thermodynamic equilibrium is reached within a minute of polynucleotide addition to dye and 2–3 min incubation time was maintained through all the experiments.

In fluorescence spectroscopy experiments the excitation wavelength above 450 nm ($\lambda_{exc} > 450$ nm) was used to avoid the possible inner filter effect caused by increasing absorbance of the polynucleotide. The emission spectra were collected in the range λ_{em} = 460–650 nm. The CD experiments were performed by adding aliquots of the aqueous solutions of compounds into the buffered solution of polynucleotide.

2.1.2. Thermal denaturation experiments

Thermal melting curves for $p(dAdT)_2$, pApU and ct-DNA and their complexes with studied compounds were determined by following the change in the absorption at 260 nm as a function of temperature. The absorbance of the ligands was subtracted from each curve and the absorbance scale was normalized. *T*m values are the midpoints of the transition curves, determined from the maximum of the first derivative and checked graphically by the tangent method. The Δ*T*m values were calculated subtracting *T*m of the free nucleic acid from *T*m of the complex. The Δ*T*m values (with the instrumental error ± 0.5 °C) reported are the average of at least duplicate measurements.

2.1.3. Isothermal titration calorimetry (ITC)

Titrations were performed on MicroCal VP-ITC (Malvern, UK), the polynucleotide solutions (ctDNA), $p(dAdT)_2$, $p(dGdC)_2$ prepared as in Section 2.1.1. Origin 7.5 software, supplied by the manufacturer was used for data analysis. Due to multifaceted binding interactions of all the compounds with pApU, the fitting procedure did not yield satisfactory results and those were omitted. The reference cell was filled with ultrapure water. In the experiments, one aliquot of 2 μL five aliquots of 5 μL and 24 aliquots of 10 μL of the compound **A1**, **A2**, **A3**, **A4** or **A7** were injected from a rotating syringe (220 rpm) into the isothermal cell, equilibrated at 25.0 °C, containing 1.4406 ml of the polynucleotide (C = 20 30 × 10^{-6} mol dm^{-3}).

The spacing between each injection was in the range 240–300 s. The initial delay before the first injection was 2000 s in all experiments. All solutions used in ITC experiments were degassed prior to use under vacuum (0.64 bar, 10 min) to eliminate air bubbles.

Microcalorimetric experiment, after fitting procedure, using built in Origin 7.5 software and one set of sites mode, directly gave three parameters: reaction enthalpy change (ΔH), binding constant (K_s) and stoichiometry (N). The value of ΔG was calculated from the binding constant ($\Delta G = -RT\ln K_s$) and the reaction entropy change was calculated from the binding enthalpy and Gibbs energy ($\Delta S = (\Delta H - \Delta G)/T$).

2.2. Biological assays

2.2.1. Subculturing of cells

Adherent cell lines (**MCF-7**, **H460**, **HEK293** and **HeLa**) growing as monolayers on the surface of T-25 flasks (Sigma) were used. Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich) containing amino acids and vitamins, as well as additional supplementary components were used for cell growth. The primary cell culture media spent was removed and discarded from the culture flask. Cells were rinsed with 5 ml of the pre-warmed phosphate-buffered saline (PBS) to remove protease inhibitors, and 0.1% trypsin solution to detach the cells from the substrate. Flask was further placed back in an incubator at 37 °C. The progress of the enzyme treatment was checked with an inverted phase contrast microscope. As the cells have rounded up and detach from the surface, 5 ml of growth medium was added to the cell suspension and cells were vigorously washed. Suspended cells were further centrifuged (100×*g* for 5 min) and trypsin containing medium was replaced with the fresh one. Cells were counted using Neubauer chamber under inverted phase contrast microscope. Considering the required dilutions, the amount of the cells in fresh medium was transferred to new flasks.

2.2.2. Anti-proliferative activity evaluation by MTT assay

This test was performed with MCF-7 and H460 cells. Tested compounds, (AK-A1, AK-A2, AK-A3, AK-A4 and AK-A7) were prepared as stock solutions (4 × 10^{-2} M moldm^{-3}) in dimethylsulfoxide (DMSO), and working solutions (10^{-4}-10^{-8} moldm^{-3}) were prepared in DMEM medium accordingly. Cells were seeded in a 96 micro well flat bottom plates at the concentration 1 × 10^4 cells/l and incubated overnight allowing them to attach to the plate surface. 72 h after incubation with tested compounds, growth medium was discarded and 5 mgml^{-1} of MTT was added. After 4 h incubation at 37 °C water insoluble MTT-formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm on a microplate reader (Multiskan EX, ThermoFisher Scientific). Control cells were grown under the same conditions. The experiments were performed in tetraplicates. The IC_{50} value, defined as compound concentration leading to cellular viability reduction by 50%, was calculated and used as a comparison parameter.

2.2.3. Flow cytometry analysis of the ***A1-7*** dyes cell accumulation kinetics

The cells (1 × 10^6/ml) were incubated in 1 ml culture medium containing respective dyes for indicated amount of time in the dark at 37 °C. Flow cytometry was carried out on a Becton Dickinson FACSCalibur model equipped with a 488 nm Argon laser and a 635 nm red diode laser. The collected data was analysed using BD CellQuest or FlowJo software version 7.2.5 for Microsoft (TreeStar, San Carlos, CA, USA). The cells were gated and discriminated from

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