Background: During the past decades, increasing attention has been given to elucidating the molecular details of interactions between the pharmacological agents and nucleic acids since the drug–DNA complexation may lead to impairment of DNA replication, strand breaking and mutations. A variety of techniques have been developed to characterize the drug-nucleic acid binding, among which the fluorescence dye displacement assay is one of the most informative approaches. Recently, it was demonstrated that cyanine dyes can be successfully employed for the high throughput screening of the interactions between nucleic acids and drugs. To the best of our knowledge, so far, the potential application of cyanine dyes for the drug-displacement studies remains insufficiently evaluated.

Objectives: The aim of the present study was to investigate the ability of a novel cyanine dye to serve as a competitor for the potential antitumor compounds, lanthanide complexes bearing europium (III) tris-β-diketonate (EC) for the DNA and RNA binding sites.

Materials and methods: Calf thymus DNA, yeast RNA, trimethine cyanine dye and lanthanide complexes bearing europium (III) tris-β-diketonate were used for sample preparation. The fluorescence data were acquired using Perkin-Elmer LS-55 spectrofluorimeter.

Results: Using the fluorescence spectroscopy technique we conducted the displacement reaction trimethine cyanine dye/europium coordination complexes in the presence of double stranded DNA and single-stranded RNA. An increase of the EC concentration in the systems AK3-5/DNA or AK3-5/RNA was followed by a gradual reduction in the AK3-5 fluorescence intensity, indicating that europium (III) tris-β-diketonate compounds can serve as competitors for the trimethine cyanine dye on the nucleic acids. Both the drug chemical structure and the type of nucleic acid proved to control the extent of EC-induced decrease of AK3-5 fluorescence in the presence of the DNA or RNA.

Conclusion: By recruiting the potential antitumor agents europium chelate complexes as the competitive ligands for the cyanine dye for the DNA and RNA binding sites, we found that a novel trimethine compound can be effectively used in the fluorescence drug displacement assays.

KEY WORDS: trimethine cyanine dye; europium coordination complexes; RNA; DNA; antitumor drug; association constant.
Актуальність. Упродовж останніх десятилітів все більше уваги приділяється з’ясуванню молекулярних особливостей взаємодій між фармакологічними агентами та нуклеїновими кислотами, оскільки комплексоутворення між ДНК та лікарськими препаратами може призвести до порушення реплікації ДНК, розриву полінуклеотидних ланцюгів та мутацій. Розроблено різноманітні методи для вивчення зв’язування лікарських засобів з нуклеїновими кислотами, серед яких підхід, що базується на заміщенні флуоресцентного барвника, є одним з найбільш інформативних. Недавно було продемонстровано, що ціанінові барвники можуть успішно застосовуватись для дослідження взаємодій між нуклеїновими кислотами та лікарськими препаратами. Наскільки вам відомо, можливість застосування ціанінових барвників як конкурентних лігандів до цього часу залишається недостатньо вивченою.

Мета роботи. Метою даної роботи було дослідити здатність нового ціанінового барвника виступати конкурентом для потенційних протипухлинних сполук, комплексів ланцюгів, що містять сполуки (ІІІ) трис-β-дикетонат (ККС), за центри зв’язування на ДНК та РНК.

Матеріали і методи. Для приготування зразків використовувались ДНК тимуса телят, дріжджова РНК, триметиновий ціаніновий барвник та комплекси ланцюгів, що містять сполуки (ІІІ) трис-β-дикетонат. Флуоресцентні спектри реєстрували за допомогою Perkin-Elmer LS-55 спектрофлуориметра.

Результати. За допомогою методу флуоресцентної спектроскопії було досліджено заміщення триметинових сполук координаційними комплексами європії при їх зв’язуванні з подвійною спіраллю ДНК та одноланцюговою РНК. Підвищення концентрації координаційних комплексів європії в системі AK3-5/DNK чи AK3-5/RNK супроводжувалося падінням інтенсивності флуоресценції AK3-5, вказуючи на те, що трис-β-дикетонатні комплекси європії (ІІІ) можуть конкурувати з триметиновим ціаніновим барвником за схід зв’язування на нуклеїнових кислотах. Припускається, що як хімічна структура лікарського препарату, так і тип нуклеїнової кислоти впливають на ступінь зменшення флуоресценції AK3-5, викликаного комплексами європії, в присутності ДНК або РНК.

Висновки. З використанням потенційних протипухлинних агентів, целей європії, як конкурентних лігандів для ціанінового барвника при асconsulta європії сполук з ДНК чи РНК, було виявлено, що новий триметиновий барвник AK3-5 може ефективно використовуватись в флуоресцентних реакціях заміщення лікарських препаратів.

КЛЮЧОВІ СЛОВА: триметиновий ціаніновий барвник; координаційні комплекси європії; РНК; ДНК; протипухлинний препарат; константа ассоціації.

НОВИЙ ЦІАНИНОВИЙ КРАСИТЕЛЬ КАК КОНКУРЕНТНЫЙ ЛИГАНД ДЛЯ ИССЛЕДОВАНИЯ ВЗАИМОДЕЙСТВИЙ МЕЖДУ ЛЕКАРСТВЕННЫМИ ПРЕПАРАТАМИ И НУКЛЕИНОВЫМИ КИСЛОТАМИ

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Актуальность. В течение последних десятилетий все большее внимание уделяется выяснению молекулярных особенностей взаимодействий между фармакологическими агентами и нуклеиновыми кислотами, поскольку комплексообразование между ДНК и лекарственными препаратами может приводить к нарушению репликации ДНК, разрывам полинуклеотидных цепей и мутациям. Разработаны различные методы для изучения связывания лекарственных препаратов с нуклеиновыми кислотами, среди которых, подход, который базируется на замещении флуоресцентного красителя, является одним из наиболее информативных. Недавно было продемонстрировано, что цшяновые красители могут успешно применяться для исследования взаимодействий между нуклеиновыми кислотами и лекарственными препаратами. Насколько нам известно, возможность применения цшяновых красителей как конкурентных лигантов к настоящему времени остается недостаточно изученной.

Цель работы. Целью данной работы была оценка возможности применения нового триметинового цшянового красителя в качестве конкурентного лиганда для потенциальных противоангиогенных препаратов, комплексы лантаноидов, содержащие трис-β-дикетонат европия (ІІІ) (ККЕ), за центры связывания на ДНК и РНК.

Материалы и методы. Для приготовления образцов использовали ДНК тимуса телят, дрожжевую РНК, триметиновый цшяновый краситель AK3-5 и комплексы лантаноидов,
throughput analysis of the drug–nucleic acid interactions is of particular importance because fluorescent ligands in drug displacement assay [20]. The application of cyanines for high-

Cyanine dyes, photosensitive compounds possessing two quaternized nitrogen containing heterocyclic structures which are linked through a polymethine bridge, have found numerous applications in a variety of fields as optical imaging agents [1, 2], active ingredients in semiconducting materials [3, 4], laser dyes [5], photographic sensitizers [6, 7], photopolymerization initiators [8, 9], stains and fluorescent labels [10–13], to name only a few. These compounds are of particular interest for biomedical research and diagnostics due to their favorable spectral characteristics, namely, an intensive absorption in a broad spectral region from UV to NIR; a high sensitivity to physicochemical properties of their microenvironment; a capability to convert light energy to electricity, etc. Likewise, due to dual hydrophobic and cationic nature of cyanines, which leads to a strong interaction with polyanionic DNA duplex, cyanines have been mainly employed for sizing and purification of DNA fragments [14, 15], DNA damage detection [16], microarray-based expression analysis [17], DNA sequencing [18], staining of nucleic acids in electrophoresis [19], and as fluorescent ligands in drug displacement assay [20]. The application of cyanines for high-throughput analysis of the drug–nucleic acid interactions is of particular importance because ethidium bromide, a most widespread dye in the fluorescence drug displacement assay, is shown to be carcinogenic, mutagenic, teratogenic and toxic [21]. Since DNA and RNA represent the prime targets for various anticancer drugs and antibiotics [22, 23], the fluorescence drug displacement assay seems to be an especially promising tool in clarifying the mechanisms underlying the interactions between nucleic acids and drugs.

To the best of our knowledge, the cyanine dyes have not yet found a wide application in the drug-displacement studies. To exemplify, Achyuthan et al. [20] have demonstrated that the supramolecular self-assembling cyanine dye may serve as an alternative to the mutagenic ethidium bromide in probing the DNA-drug interactions. To fill this gap, herein we directed our efforts towards assessing the applicability of a novel trimethine cyanine dye as a competitor to the potential antitumor compounds, europium chelates for the DNA and RNA binding sites. The choice of Eu(III) tris-β-diketonato coordination complexes as the model drugs was dictated by the fact that these agents have been reported to exert cytotoxic effect against different tumor cell lines whereas the DNA-intercalating motifs in the structure of europium complexes is a key to the augmentation of their cytotoxicity [23].

MATERIALS AND METHODS

Calf thymus DNA and yeast RNA were from Sigma (Sigma, St. Louis, MO, USA). Trimethine cyanine dye [24] and Eu(III) coordination complexes [23] denoted here as V5, V7, V9 and V10 were synthesized in the University of Sofia, Bulgaria, as described
previously (Fig. 1). All other materials and solvents were commercial products of analytical grade and were used without further purification.

![Chemical structures](image)

Fig.1. Structures of the cyanine dye AK3-5 and europium coordination complexes V5, V7, V9 and V10.

The stock solutions of the cyanine dye and Eu(III) coordination complexes were prepared by dissolving the compounds in dimethyl sulfoxide, while the calf thymus DNA and yeast RNA were dissolved in 5 mM sodium phosphate buffer (pH 7.4). The concentrations of the dye, EC complexes and nucleic acids were determined spectrophotometrically using their molar extinction coefficients: $\varepsilon_{AK3-5}^{\text{V5}} = 1.5 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$, $\varepsilon_{266}^{\text{V7}} = 2.6 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$, $\varepsilon_{266}^{\text{V9}} = 2.6 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$, $\varepsilon_{266}^{\text{V10}} = 3.4 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$, $\varepsilon_{250}^{\text{DNA}} = 6.6 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$ and $\varepsilon_{260}^{\text{RNA}} = 6.9 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$.

The fluorescence data were acquired using Perkin-Elmer LS-55 spectrofluorimeter equipped with 10 mm quartz cuvettes at 20°C. The measurements were carried out in 5 mM sodium phosphate buffer (pH 7.4) within the range 620–800 nm upon excitation at 600 nm. The excitation and emission slit widths were set at 10 nm.

The dye-nucleic acid binding was analyzed using the McGhee-von Hippel formalism for non-cooperative ligand binding to a one-dimensional lattice [25]:

$$
\frac{B}{F} = K_a P \left( \frac{1}{P} \right)^n \left[ \frac{1 - (nB/P)}{1 - (n - 1)(nB/P)} \right]^{n-1},
$$

(1)
where $B$ and $F$ are the concentrations of the bound and free dye, respectively, $P$ is the total phosphate concentration, $K_a$ is the association constant and $n$ represents the site exclusion parameter (i.e. the number of base pairs excluded by the binding of a single ligand molecule). The values of $K_a$ and $n$ were estimated using the nonlinear least-square fitting procedure.

The competition between the cyanine dye and EC complexes for the binding sites on the DNA or RNA matrices was analyzed in terms of the model proposed by Gaugain et al. [26]. In the ternary systems nucleic acid–cyanine dye–EC, the concentration of the bound dye ($B_{\text{dye}}$) was calculated by the numerical solution of the system of two equations:

\[
\frac{B_{\text{dye}}}{(Z_{\text{dye}} - B_{\text{dye}})} = \frac{K_{\text{dye}} [P - n_{\text{dye}} B_{\text{dye}} - n_{\text{drug}} B_{\text{drug}}]^{n_{\text{dye}}}}{[P - (n_{\text{dye}} - 1) B_{\text{dye}} - (n_{\text{drug}} - 1) B_{\text{drug}}]^{n_{\text{dye}} - 1}},
\]

\[
\frac{B_{\text{drug}}}{(Z_{\text{drug}} - B_{\text{drug}})} = \frac{K_{\text{drug}} [P - n_{\text{dye}} B_{\text{dye}} - n_{\text{drug}} B_{\text{drug}}]^{n_{\text{drug}}}}{[P - (n_{\text{dye}} - 1) B_{\text{dye}} - (n_{\text{drug}} - 1) B_{\text{drug}}]^{n_{\text{drug}} - 1}},
\]

where $K_{\text{dye}}$, $n_{\text{dye}}$ are the association constant and stoichiometry of the dye-nucleic binding found in the absence of a drug; $Z_{\text{dye}}$, $Z_{\text{drug}}$ are the total concentrations of the dye and EC, respectively; $K_{\text{drug}}$, $n_{\text{drug}}$ are the association constant and stoichiometry of the drug-nucleic complexation. The experimental dependences of the EC-induced decrease of the dye fluorescence $\Delta I_{\text{exp}}(Z_{\text{drug}})$ were approximated by the following expression:

\[
\Delta I_{\text{calc}} = \left( a_{\text{bound}} - a_{\text{free}} \right) (B_0 - B_{\text{dye}}) = F_{\text{mol}} \left( B_0 - B_{\text{dye}} \right),
\]

where $B_0$ and $B_{\text{dye}}$ are the concentrations of the bound dye in the absence and presence of a drug, respectively, $I_{\text{mol}} = \left( a_{\text{bound}} - a_{\text{free}} \right)$ is a coefficient proportional to the difference of the dye quantum yields in buffer and when bound to macromolecule.

**RESULTS AND DISCUSSION**

In general, to explore the drug-nucleic acid interactions, the fluorescence displacement assays can be carried out in two ways: i) a drug associated with DNA or RNA is then displaced by a dye; and ii) a dye bound to nucleic acid is dislodged by a drug [20]. Reasoning from the ability of the examined cyanine dye to respond to nucleic acid complexation by a marked fluorescence increase, we conducted our measurements using the latter of the above approaches. The first step of the study was aimed at quantifying the binding of the novel cyanine dye AK3-5 to DNA and RNA. More specifically, the following questions have been addressed: i) what are the binding mode and the parameters of the dye association with nucleic acids; ii) is the cyanine dye capable of distinguishing between a double stranded DNA and a single stranded RNA structures. To this end, we performed a series of the fluorimetric titrations of AK3-5 with nucleic acids. The typical fluorescence spectra of this dye in the DNA- and RNA-containing systems are presented in Fig. 2 A. The examined cyanine dye was found to have a negligible fluorescence in the buffer solution, in the absence of nucleic acids. Such a dramatic fluorescence reduction of cyanine dyes is explained by their rapid deactivation from the singlet excited state as a result of rotation around the doublebond joining the benzothiazole and quinoline rings [16]. As seen from Fig. 2 A, the fluorescence intensity drastically increases upon the AK3-5–nucleic acid complexation because of the restricted motional freedom of the dye on the nucleic acid matrix leading to the energy dissipation via fluorescence [16]. It appeared that the enhancement of AK3-5 fluorescence is
significantly stronger on the dye binding to DNA, compared to RNA, whereas the dye emission maximum in the presence of RNA is bathochromically shifted by ~10 nm compared to that of dsDNA. This finding can be used to differentiate between DNA and RNA in solution. Despite a long-standing tradition of the use of cyanine dyes in the nucleic acid studies, only a few cyanines are capable of distinguishing between DNA and RNA [27, 28].

Next, the binding parameters and the underlying binding mechanisms were obtained from the fluorescence titration data. The experimental dependencies of the dye fluorescence increase upon DNA or RNA binding ($\Delta I$) as a function of phosphate concentration are presented in Fig. 2 B and C, respectively. These dependencies were analyzed in terms of the non-cooperative McGhee & von Hippel model (Eq. (1)) [25]. The association constants for the dye-DNA and the dye-RNA complexes were found to be $(5.1 \pm 1.1) \times 10^4$ M$^{-1}$, $(3 \pm 0.6) \times 10^3$ M$^{-1}$, respectively, while the site exclusion parameters were equal to 2 for both systems. Based on the analysis of the available information [12, 14, 16] on the nucleic acid interactions of intercalating agents the following two tendencies can be highlighted: i) the highest possible dye-base pair ratio is 1:2, since, according to the principle of the nearest neighbor exclusion, the binding of one intercalating molecule between the two base pairs hinders the access of the next binding site to another intercalator [12, 14, 16, 29]; ii) the association constants for intercalators do not exceed $10^5$ M$^{-1}$ [12, 14, 16]. Allowing for the fact that the association constants and the neighbor exclusion parameters recovered here for the novel trimethine dye under study in the presence of the double stranded DNA and single stranded RNA are in accordance with those observed for typical intercalators, we assumed that the examined dyes associate with nucleic acids via intercalating binding mode. Importantly, despite the fact that the calculated thermodynamic parameters of the AK3-5 in
the RNA-containing systems are similar to those of typical intercalators, one cannot exclude the possibility of a “partial” intercalation of AK3-5 between the RNA bases [30, 31].

Recently, we demonstrated that the cyanine dye AK3-5 can be employed in the fluorescence displacement assay [29]. Specifically, we have evaluated the ability of the potential pharmacological agents, Eu(III) tris-β-diketonate to coordination complexes to dislodge the AK3-5 dye bound to the double stranded DNA [29]. In a continuation of our interest in fluorescent dyes suitable for the drug displacement analysis on the nucleic acid, herein we directed our efforts towards investigation of the ability of a novel cyanine dye AK3-5 to serve as a competitor for europium coordination complexes for DNA and RNA binding sites. Importantly, for the quantitative analysis of the AK3-5 displacement assays it was reasonable to collect the multiple data sets. Therefore, we performed the competitive binding experiments under conditions of simultaneous varying the concentrations of EC and nucleic acids. The plots of the fluorescence intensity decrease vs EC concentration are presented in Fig. 3.

![Graphs](image)

Fig. 3. The fluorescence intensity decreases of AK3-5-DNA (A-C) and AK3-5-RNA (D) complexes in the presence of the increasing concentrations of europium complexes V5(a), V7(b), V9(c), V10 (d). The concentrations of DNA were 9.3 μM (A), 27.9 μM (B), 46.5 μM (C), respectively. The RNA concentration was 24.5 μM. The concentration of AK3-5 was 0.25 μM.

However, due to a significantly smaller binding affinity of AK3-5 to the single stranded RNA, no significant fluorescence decrease was observed at RNA concentrations lower than 25 μM (Fig. 3 D). As seen in Fig. 3, Eu(III) coordination complexes produced a dose-dependent fluorescence decrease reflecting the competition between the cyanine dye and EC for the binding sites on DNA or RNA. Notably, the magnitude of the fluorescence intensity decrease in the RNA-containing systems, similarly to the DNA-containing systems, was more
pronounced for V5 and V10 compared to other europium complexes under study [29]. Next, the dependencies of the AK3-5 fluorescence decrease with increasing concentration of europium complexes were analyzed in terms of the competition model proposed by Gaugain et al. (Eqs. 2, 3) to obtain the association constants of the drug binding to DNA or RNA [26, 29]. The calculated values of this parameter were estimated to be (1.8±0.4)×10^4 M^{-1} and (5±1.1)×10^5 M^{-1} for V7 and V9, respectively, regardless of the DNA concentration in the tested sample. However, in line with our previous results [29], the values of the binding parameters for the europium complexes V5 and V10 in the DNA-containing system were unrealistic. The above finding most probably stems from: i) the quenching of the AK3-5 fluorescence by V5 and V10, accompanying the competition between the dye and EC for the DNA binding sites; and ii) the limitations of the competition model [26] in describing the complex nature of interactions between europium complexes, AK3-5 and DNA. Moreover, we obtained inappropriate association constants for all EC under study in the presence of single stranded RNA, which, most likely, may be related with the law binding affinity of the europium coordination complexes to the RNA. To the best of our knowledge, the exact mechanisms and EC binding mode to the single stranded nucleic acids are still unclear. Therefore, further studies are needed.

CONCLUSIONS

In conclusion, by recruiting the potential antitumor europium chelate complexes as the competitive ligands for the cyanine dye associating with DNA or RNA, we found that the novel trimethine compound AK3-5 can be effectively used in the fluorescence drug displacement assays. Both the drug chemical structure and the type of nucleic acid seem to control the extent of EC-induced reduction of AK3-5 fluorescence in the presence of the DNA or RNA.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Education and Science of Ukraine (the Young Scientist project № 0117U004966 “Nano- and microsized liophilic and liophilized self-associated systems: application in modern technologies and biomedicine”) and by the President’s of Ukraine grant No 0118U002284 from the State Fund for Fundamental Research of Ukraine “Development of novel anti-amyloid strategies by high-throughput screening of inhibitors of pathological protein aggregation”.

CONFLICT OF INTEREST

The authors report that there is no conflict of interest.

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