The present study was undertaken to assess the applicability of the novel trimethine cyanine dye AK3-5 as a competitive ligand for the antitumor agents, Eu(III) coordination complexes (EC), in the DNA-containing systems, using the displacement assay as an analytical instrument. The analysis of fluorescence spectra revealed a strong association of AK3-5 with nucleic acids, with the strength of interaction being higher for the double stranded DNA, compared to the single-stranded RNA. The binding parameters of the cyanine dye have been determined in terms of the McGhee & von Hippel neighbouring site-exclusion model and a classical Langmuir model. The AK3-5 association constant in the presence of DNA was found to be equal to $5.1 \times 10^4 \text{ M}^{-1}$, which is consistent with those of the well-known DNA intercalators. In turn, the binding of the cyanine to the RNA was characterized by a significantly lower association constant ($\sim 3.4 \times 10^3 \text{ M}^{-1}$) indicating either the external or "partially intercalated" binding mode. The addition of the europium complexes to the AK3-5-DNA system was followed by the fluorescence intensity decrease, with a magnitude of this effect being dependent on the EC structure. The observed fluorescence decrease of AK3-5 in the presence of europium complexes V7 and V9 points to the competition between the cyanine dye and antitumor drugs for the DNA binding sites. The dependencies of the AK3-5 fluorescence decrease upon theeuropium complex concentration were analyzed in terms of the Langmuir adsorption model, giving the values of the drug association constant equal to $5.4 \times 10^4 \text{ M}^{-1}$ and $3.9 \times 10^5 \text{ M}^{-1}$ for the europium complexes V7 and V9, respectively. A more pronounced decrease of the AK3-5 fluorescence in the presence of V5 and V10 was interpreted in terms of the drug-induced quenching of the dye fluorescence, accompanying the competition between AK3-5 and Eu(III) complexes for the DNA binding sites. Cumulatively, the results presented here strongly suggest that AK3-5 can be effectively used in the nucleic acid binding assays and in the dye-drug displacement assays.

**KEYWORDS:** trimethine cyanine dye, europium coordination complexes, antitumor drug, DNA, association constant

Over the past decade significant research efforts have been devoted to the investigation of the interaction between drugs and nucleic acids, since DNA was found to serve as a prime target for various antitumor drugs and antibiotics [1-3]. A variety of powerful techniques, including an agarose gel based assay, circular dichroism, mass spectrometry, differential scanning calorimetry, surface plasmon resonance, electrophoresis, high-performance or thin-layer chromatography, Raman, fluorescence and absorption spectroscopy, are currently used to monitor the drug-nucleic acid interactions [10,11]. The applicability of this class of fluorophores is based on the fact that they display a high affinity towards evaluating the DNA- and RNA-binding ability of the novel trimethine cyanine dye with an emphasis on its use in the drug-displacement studies. More specifically, the aims of the present study were: i) to investigate the sensitivity of the novel trimethine cyanine, referred to here as AK3-5, to the double stranded DNA and single stranded RNA; ii) to estimate the parameters of the cyanine association with nucleic acids; iii) to analyze the binding mode of the novel dye;
iv) to assess the applicability of AK3-5 as a competitive ligand for pharmacological agents, represented here by the novel antitumor compounds, europium coordination complexes.

EXPERIMENTAL SECTION

Materials

Calf thymus DNA and yeast RNA were from Sigma (Sigma, St. Louis, MO, USA). Trimethine cyanine dye AK3-5 [21] and Eu(III) coordination complexes (Fig.1) referred to here as V7, V9 and V10 were synthesized in the University of Sofia, Bulgaria, as described previously [2]. All other materials and solvents were commercial products of analytical grade and were used without further purification.

Preparation of working solutions

The stock solutions of AK3-5 and Eu(III) coordination complexes were prepared in dimethyl sulfoxide. The concentrations of the compounds were determined spectrophotometrically using their molar extinction coefficients ($\varepsilon$) at absorption maxima ($\lambda_{abs}$), which are presented in Table 1. The solutions of calf thymus DNA and yeast RNA were prepared in 5 mM sodium phosphate buffer (pH 7.4) at room temperature with occasional stirring to ensure the formation of a homogenous solution. The concentrations of DNA and RNA solutions were determined using their molar absorptivities $\varepsilon_{260} = 6.6 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$ and $\varepsilon_{260} = 6.9 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$, respectively.

Table 1

| compound | R1 | R2 | $\varepsilon$, M$^{-1}$ cm$^{-1}$ | $\lambda_{abs}$, nm |
|----------|----|----|-------------------------------|------------------|
| V5       | C$\equiv$H$\equiv$ | CH$_3$ | 2.6×10$^4$ | 266 |
| V7       | C$\equiv$H$\equiv$ | CF$_3$ | 2.6×10$^4$ | 266 |
| V9       | C$\equiv$H$\equiv$O | CF$_3$ | 3.4×10$^4$ | 271 |
| V10      | C$\equiv$H$\equiv$ | C$\equiv$H$\equiv$ | 3.4×10$^4$ | 167 |
| AK3-5    | C$\equiv$H$\equiv$ | C$\equiv$H$\equiv$ | 1.5×10$^5$ | 631 |

Fig.1. Structures of the AK3-5 and EC

Spectroscopic measurements

The steady-state fluorescence spectra were recorded with LS-55 spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfield, UK) at 20°C using 10 mm path-length quartz cuvettes. To measure the fluorescence spectra of the AK3-5-DNA/RNA complexes, the appropriate amounts of the nucleic acid stock solution were added to the dye in 5 mM sodium phosphate buffer, pH 7.4. The fluorescence spectra of AK3-5 were recorded in the range 620 - 800 nm upon excitation at 600 nm. The fluorimetric titrations were carried out by keeping the dye concentration constant at varying the DNA and RNA concentrations. In the case of the dye displacement studies, a solution containing AK3-5 and DNA was titrated with the Eu(III) complexes.

Quantitative analysis of the dye-nucleic acid interactions

The thermodynamic analysis of the cyanine-nucleic acid interactions was performed in terms of the McGhee & von Hippel excluded site model allowing the calculation of the binding constant and stoichiometry [22]:

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

where $B$ and $F$ are the concentrations of the bound and free dye, respectively, $P$ is the DNA (RNA) phosphate concentration, $K_a$ denotes 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.

Quantitative analysis of the AK3-5/EC competitive binding

In order to analyse the competitive binding of AK3-5 and EC to the nucleic acids a simplified model based on the one-site Langmuir adsorption model was employed [23,24]. Assuming that the AK3-5 fluorescence response is proportional to the amount of the DNA-bound fluorophore, $B$, the DNA-induced change in the probe fluorescence intensity $\Delta F$ at the fluorescence maximum can be written as:

$$
\Delta F = F - F_0 = \alpha_{\text{bound}} B + \alpha_{\text{free}} (Z - B) - \alpha_{\text{free}} Z = (\alpha_{\text{bound}} - \alpha_{\text{free}}) B + F_{\text{mol}} B,
$$

where $F_0$ is the fluorescence of the free dye, $\alpha_{\text{bound}}$ is the binding constant, and $\alpha_{\text{free}}$ is the free dye constant.
where $F_0$ and $F$ are the fluorescence intensities of the dye in a buffer solution and in the presence of DNA, respectively; $F_{\text{mol}}$ is a coefficient proportional to the difference of the dye quantum yields in buffer and when bound to a macromolecule; $\alpha_{\text{bound}}$ and $\alpha_{\text{free}}$ are the molar fluorescences of the bound and free dye, respectively.

Given that the number of phosphates bound to one dye molecule is $n$, the association constant ($K_a$) can be represented as:

$$K_a = \frac{B}{(Z-B)(P/n-B)} = \frac{B}{F(P/n-B)}, \quad (3)$$

where $P$ and $Z$ are total phosphate and total dye concentrations, respectively.

The $F_{\text{mol}}$ parameter was calculated from the fluorimetric titration of the dye with the DNA or RNA. Specifically, at high DNA/RNA concentrations, when $P/n \gg B$, from the combination of eqns (2) and (3) one obtains:

$$\frac{1}{\Delta F} = \frac{1}{BF_{\text{mol}}} = \frac{1}{K_aPZF_{\text{mol}}/n} + \frac{1}{ZF_{\text{mol}}}, \quad (4)$$

$$F_{\text{mol}} = 1/aZ, \quad (5)$$

where $a$ is the y-intercept of the linear fit of the plot $1/\Delta F(1/P)$ [23].

The parameters $K_a$ and $n$ were estimated from the fluorimetric titration of the DNA by the dye. In this case, when $Z \gg B$, a combination of the eqns (2) and (3) gives:

$$\frac{1}{\Delta F} = \frac{1}{BF_{\text{mol}}} = \frac{1}{K_aPZF_{\text{mol}}/n} + \frac{1}{PF_{\text{mol}}/n}, \quad (6)$$

$$n = bPF_{\text{mol}}, \quad (7)$$

$K_a = -c, \quad (8)$

where $b$ and $c$ are y- and x-intercepts of the linear fit of the plot $1/\Delta F(1/Z)$. When the drug binds to the AK3-5-DNA complexes, the dye fluorescence in the absence ($F_0$) and presence ($F_{\text{corr}}$) of the drug can be written as:

$$F_0 = B_0\alpha_{\text{bound}} + (Z_0 - B_0)\alpha_{\text{free}},$$

$$F_{\text{corr}} = B_{\text{bound}} + (Z_0 - B_0)\alpha_{\text{free}}, \quad (10)$$

where $B_0$ and $B_{\text{free}}$ are the concentrations of the DNA-bound dye in the absence and presence of a drug, respectively.

From the eqns. (9) and (10) one obtains:

$$\Delta F_{\text{calc}} = F_0 - F_{\text{corr}} = (B_0 - B_0)(\alpha_{\text{bound}} - \alpha_{\text{free}}). \quad (11)$$

The association constants of the dye ($K_{\text{dye}}$) and drug ($K_{\text{drug}}$) binding to the DNA were recovered from the numerical solution of the set of equations under the following conditions: $B_{\text{dye}} \leq Z_{0\text{dye}}$; $B_{\text{drug}} \leq Z_{0\text{drug}}$; $B_{\text{dye}} > 0$; $B_{\text{drug}} > 0$; $B_{\text{dye}} < N - B_{\text{drug}}$ (the total number of binding sites $N = P/n$ is greater than the sum of concentrations of bound dye and drug):

$$\frac{B_{\text{dye}}}{(Z_{0\text{dye}} - B_{\text{dye}})(N - B_{\text{dye}} - B_{\text{drug}})} = K_{\text{dye}}, \quad (12)$$

$$\frac{B_{\text{drug}}}{(Z_{0\text{drug}} - B_{\text{drug}})(N - B_{\text{dye}} - B_{\text{drug}})} = K_{\text{drug}}, \quad (13)$$

where $B_{\text{dye}}$ and $B_{\text{drug}}$ are the concentrations of the DNA-bound dye and drug, respectively.

**Molecular docking**

An interactive molecular graphics program, Hex 8.0.0 was used to study the interaction between the examined europium complexes and the double stranded DNA. The program performs docking using the spherical polar Fourier correlatives with the inputs of ligand and receptor in PDB format. The structure of the B-DNA dodecamer d(CGCGAATTCGCG)$_2$ (PDB ID: 1BNA) was downloaded from the Protein Data Bank (http://www.rcsb.org/pdb). The parameters used for molecular docking include: FFT mode – 3D, correlation type – shape only, grid dimension – 0.6, ligand range – 180, receptor range – 180, distance range – 40, and twist range – 360. The docked complexes were visualized by the Visual Molecular Dynamics (VMD) software.
RESULTS AND DISCUSSION

At the first step of the study the AK3-5-nucleic acid binding was characterized using the fluorescence spectroscopy technique. The spectra of AK3-5 in the presence of the double stranded DNA or single stranded RNA are presented in Fig.2. The AK3-5 was found to have a negligible fluorescence in a buffer solution with the emission maximum at 640 nm. In turn, the fluorescence intensity drastically increased upon the dye transfer from the aqueous phase to the dsDNA or RNA environment, with the magnitude of this effect being more pronounced for the dye-DNA complex. Moreover, AK3-5 binding to the RNA caused a 10 nm bathochromic shift in the emission maxima position, whereas no significant change of this parameter was observed in the dye-DNA system. The difference in the position of the fluorescence maxima in the presence of nucleic acids, as well as different affinities for the RNA and DNA were also reported previously for other dyes of cyanine family [25], and can be used to distinguish between the double and single stranded nucleic acids in solution. Moreover, the 10 nm shift of the AK3-5 fluorescence maximum position in the presence of the RNA with respect to that in the DNA-containing system probably reflects a more polar environment of the dye in a single stranded nucleic acid. This finding is suggestive of the different binding modes of AK3-5 to the single and double stranded nucleic acids.

It is well-known that cyanine dyes can interact with the nucleic acids in the three basic modes: i) electrostatic attraction between the cationic dyes and the anionic phosphodiester groups of DNA/RNA backbone; ii) intercalation between adjacent base pairs, iii) minor groove binding. Intercalation is typically observed for the cationic molecules (with the positive charge preferably located on the ring system) possessing a planar aromatic structure, while the minor groove binders should have at least limited flexibility to be able to adjust to the groove [12]. The structural and physicochemical properties of a fluorophore, a nucleic acid sequence, as well as the phosphate to dye ratio (P/D) were found to determine the molecular mechanism of cyanine complexation with nucleic acid [26,27].

In order to gain further insight into the binding mode between the AK3-5 and nucleic acids, as well as to calculate the parameters characterizing the stability of the cyanine-nucleic acid complexes, the experimental dependencies of the dye fluorescence increase (ΔI) on the DNA/RNA concentration (insets in Fig. 2) were analyzed in terms of the non-cooperative McGhee & von Hippel model (Eq. (1)) [22]. The results obtained are summarized in Table 2.

The thermodynamic parameters of the AK3-5 binding to nucleic acid

| Nucleic acid | $K_a \times 10^4 \text{ M}^{-1}$ | $n$ | $F_{\text{max}}, \mu\text{M}^{-1}$ |
|--------------|-------------------------------|----|---------------------------------|
| DNA          | 5.1±0.9                       | 2  | 264.3±52.8                     |
| RNA          | 0.34±0.06                     | 2  | 142.6±29.6                     |

The association constant for the AK3-5-DNA complex was found to be $5.1 \times 10^4 \text{ M}^{-1}$. This value is identical to the association constant observed for the classical intercalating dye, acridine orange [28]. The association constants for other fluorophores possessing intercalating binding mode such as ethidium bromide ($\sim 1.5 \times 10^5 \text{ M}^{-1}$) [29], EvaGreen
Competitive Binding of Novel Cyanine Dye AK3-5 and Europium Coordination Complexes...

Notably, the magnitude of the fluorescence intensity decrease was more pronounced for V5 and V10 complexes in comparison to V7 and V9. The most probable explanation for this observation is the different binding affinities of the europium complexes. Similarly, the metal complexes of Cu, Zn and Ni have been reported to possess the distinct DNA binding affinities [5]. Interestingly, V5 and V10 are more bulky in comparison to V7 and V9. So we cannot exclude the possibility of the EC-induced conformational changes in the double stranded DNA. A good wealth of reports indicates that the drug binding to the right-handed B-DNA can produce its conversion to the left-handed Z-DNA [11].

Pursuing a comprehensive picture of the competition between AK3-5 and Eu(III) compounds for the DNA binding sites, our experimental strategy involved collecting the multiple data sets. More specifically, the AK3-5 fluorescence intensity decrease was measured as a function of EC concentration upon simultaneous varying the DNA concentrations. The plots of the fluorescence intensity decrease vs EC concentration are presented in Fig.5.

The above dependencies were analyzed in terms of the simplified competition model (Eqs. 2-13) to calculate the association constants of the drug ($K_{drug}$) binding to the DNA in the presence of AK3-5. The calculated $K_{drug}$ values were equal to $5.4 \times 10^4 \text{M}^{-1}$ and $3.9 \times 10^5 \text{M}^{-1}$ for V7 and V9, respectively. Moreover, the association constants of the drug were found to be independent of the DNA concentration in the tested sample.
Fig. 4. Competitive displacement assays between the AK3-5 and V5(A), V7(B), V9(C) and V10 (D) in the presence of DNA. Dye concentration was 0.25 μM. DNA concentration was 9.3 μM.

Fig. 5. The fluorescence intensity decreases of AK3-5-DNA complexes in the presence of the increasing concentrations of V7 (A), V9 (B), V10 (C) and V5 (D). The concentrations of the DNA were (a) 9.3 μM, (b) 27.9 μM, (c) 37.6 μM, (d) 46.5 μM, respectively.

However, we failed to obtain the realistic value of the association constant $K_{asso}$ for the europium complexes V5 and V10. Moreover, the theoretically calculated $\Delta F$ values (eq. 11) were significantly greater than those obtained from the experiment. Most likely, the competition between AK3/5 and Eu(III) complexes for the DNA binding sites is not the only reason for the observed fluorescence decrease in the presence of V5 and V10. In this respect, it seems of interest to draw attention to the shapes of the dependencies of $\Delta F$ on the EC concentration: being linear for the V7 and V9, it is almost hyperbolic for V5 and V10. The most probable reason for such a behavior is that besides the competition...
between AK3/5 and EC for the DNA binding sites, V5 and V10 are capable of quenching the AK3-5 fluorescence. A more hydrophobic nature of V5 and V10 in comparison to V7 and V9 seems to account for a pronounced drop in the AK3-5 fluorescence intensity in the combined AK3-5/DNA/EC systems [33].

CONCLUSIONS
In conclusion, the present study was focused on the possible application of the novel trimethine cyanine dye AK3-5 in the displacement assay using the potential antitumor agents, europium coordination complexes as competitive ligands. The AK3-5-DNA/RNA binding studies provided evidence for the strong association of the fluorophore to nucleic acids, with the binding affinity being higher for the double-stranded DNA, in comparison with the single-stranded RNA. Upon addition of increasing concentrations of europium complexes, a gradual reduction in the fluorescence intensity of the dye was observed, indicating that EC are capable of displacing the AK3-5 from the DNA helix. The magnitude of the fluorescence intensity decrease was found to be more pronounced for V5 and V10 compared to V7 and V9. The observed effects were interpreted in terms of the different binding affinities of the europium complexes to the DNA. The assumption was made, that a more pronounced fluorescence intensity decrease of AK3-5 fluorescence intensity in the presence of V5 and V10 results from the EC ability to quench the AK3-5 fluorescence, along with the competition between AK3/5 and Eu(III) complexes for the DNA binding sites. Cumulatively, the results presented here strongly suggest that AK3-5 can be effectively used for the sensitive detection of the nucleic acids, as well as for the drug displacement assays.

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In conclusion, the present study was focused on the possible application of the novel trimethine cyanine dye AK3-5 in the displacement assay using the potential antitumor agents, europium coordination complexes as competitive ligands. The AK3-5-DNA/RNA binding studies provided evidence for the strong association of the fluorophore to nucleic acids, with the binding affinity being higher for the double-stranded DNA, in comparison with the single-stranded RNA. Upon addition of increasing concentrations of europium complexes, a gradual reduction in the fluorescence intensity of the dye was observed, indicating that EC are capable of displacing the AK3-5 from the DNA helix. The magnitude of the fluorescence intensity decrease was found to be more pronounced for V5 and V10 compared to V7 and V9. The observed effects were interpreted in terms of the different binding affinities of the europium complexes to the DNA. The assumption was made, that a more pronounced fluorescence intensity decrease of AK3-5 fluorescence intensity in the presence of V5 and V10 results from the EC ability to quench the AK3-5 fluorescence, along with the competition between AK3/5 and Eu(III) complexes for the DNA binding sites. Cumulatively, the results presented here strongly suggest that AK3-5 can be effectively used for the sensitive detection of the nucleic acids, as well as for the drug displacement assays.
Конкурентне зв'язування нового ціанінового барвника АК3-5 та координаційних комплексів європію з ДНК

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У даній роботі проведено оцінку можливості використання нового триметинового барвника АК3-5 в якості конкурентного ліганду для протипухлинних препаратів, координаційних комплексів європію (ККЄ), у системах, що містять ДНК. Аналіз спектрів флуоресценції показав високу спорідненість АК3-5 до нуклеїнових кислот, причому ступінь взаємодії з ДНК залежала від структури ККЄ. Зменшення флуоресценції АК3-5, що спостерігалось у присутності комплексів європію V7 і V9, найімовірніше, вказує на конкуренцію між ціаніновим барвником і протипухлинними препаратами за сайти зв'язування з ДНК. Концентраційні залежності змін інтенсивності флуоресценції були проаналізовані в рамках моделі Ленгмюра, що дозволило отримати константи зв'язування 5.4×10⁴ M⁻¹ та 3.9×10⁵ M⁻¹ для комплексів європію V7 і V9, відповідно. Більш виражене падіння інтенсивності флуоресценції триметинового красителя в присутності V5 і V10, імовірно, свідчить про більш високий степень взаємодії з ДНК.

Ключові слова: Триметиновий ціаніновий барвник, координаційні комплекси європію, протипухлинний препарат, ДНК, константа асоціації