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Original article

A novel anti-tumor agent, Ln(III) 2-thioacetate benzothiazole induces anti-angiogenic effect and cell death in cancer cell lines

Belal H.M. Hussein [a], Hassan A. Azab [a], Mona F. El-Azab [b], Abdullah I. El-Falouji [c,*]

[a] Chemistry Department, Faculty of Science, Suez Canal University, Ismailia 41522, Egypt
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Suez Canal University, Ismailia 41522, Egypt
[c] Biotechnology Research Center, Suez Canal University, Ismailia 41522, Egypt

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ABSTRACT

New complexes with a potent DNA-binding anti-tumor agent, europium(III)- and terbium(III)-2-thioacetate benzothiazole were synthesized and characterized. These complexes showed strong binding affinity to calf thymus DNA using fluorometric and electronic absorption spectroscopy. The synthesized complexes resulted in inhibition of proliferation of EAC cells and ascites formation. Their anti-tumor effect was found to be through anti-angiogenic activity as was evident by the reduction of microvessel density and down-regulation of VEGF receptor type-2 (Flk-1). It was found that EAC cells had distinct DNA fragmentation patterns analyzed by capillary electrophoresis in the treated animals. Moreover, the synthesized complexes exhibited significant cytotoxic activity against HepG2 and MCF7 cell lines. Furthermore, complexes showed a potent anti-bacterial activity against two pathogenic bacteria *Escherichia coli* and *Salmonella*.

1. Introduction

Although struggling efforts against cancer have grown tremendously in the last few years, cancer is still the second leading cause of death in economically developed countries following heart disease. 7.1 millions of people died in 2007 of cancer worldwide and it is expected that approximately 12 million cancer patients will die by 2015 [1,2]. Therefore, the discovery and development of novel therapeutic agents for the treatment of cancer have a vital importance. It has been reported that lanthanides, as a group of therapeutic rare earth complexes, manifest anti-tumor activity [3–12]. Hence, lanthanides represent an effective approach for searching novel anti-tumor agents. DNA is one of the most important pharmacological targets of many drugs currently in clinical use. Drugs that bind with genomic DNA or RNA have proven to be effective anti-tumor, antivirus and anti-bacterial therapeutic agents [13–15]. Therefore, the interactions of metal complexes with DNA have been the subject of interest for the development of anticancer drugs [16–18] and effective chemotherapeutic agents for numerous diseases, probes for nucleic acids, DNA-dependent electron transfer reactions, DNA footprinting, and sequence-specific cleaving agents [19–22]. Of these studies, the interaction of fluorescent metal complexes, containing multidentate aromatic ligands, with DNA has gained much attention [23,24].

Benzothiazole are one of sulfur and nitrogen-containing aromatic heterocyclic compounds, which are formed from fusion of the aryl and thiazolyl rings. They have diverse interesting medical and industrial applications [25]. Many studies on the synthesis and anti-tumor activity of benzothiazole derivatives displayed a potent and a selective anti-tumor activity against different types of tumors such as breast, ovarian, lung, renal cell lines, and colon cancer. Moreover, those studies also demonstrated that benzothiazole derivatives have antimicrobial, antitubercular, anti-inflammatory, antirheumatic, and antiglutamate activities [26–34].

In this work, novel Eu (III) and Tb(III) complexes containing ligand of 2-thioacetate benzothiazole were synthesized and characterized. The interaction of europium (III)- or terbium (III)-2-thioacetate benzothiazole with double stranded calf thymus DNA (ct-DNA) was investigated by fluorescence and UV spectroscopic techniques. Furthermore, the anti-tumor and anti-angiogenic effects of these complexes against Ehrlich ascites carcinoma (EAC) cells, cytotoxicity against HepG2 and MCF7 human tumor cell lines and anti-bacterial activity against two pathogenic bacteria were also tested.

* Corresponding author. Tel.: +2 0115990299; fax: +2 064 3200452.
E-mail address: falouji@hotmail.com (A.I. El-Falouji).

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2. Experimental

2.1. Materials and reagents

$TbCl_3.6H_2O$, $EuCl_3.6H_2O$, RPMI-1640 media and calf thymus DNA were purchased from Sigma (St. Louis, MO, USA) and used without further purification. GF-1 DNA Extraction Kit was purchased from *Vivantis*. Primary antibody Flk-1 was purchased from Santa Cruz Biotechnology (CA, USA) and anti-CD31 Class II was purchased from Dako Cytomation (Carpinteria, USA). HANK's balanced salt solution and fetal bovine serum (FBS) were purchased from Invitrogen Inc. (Carlsbad, CA, USA). Double deionized water was used for all experiments. The stock solution of calf thymus deoxyribonucleic acid (ct-DNA) was prepared in Tris–HCl buffer solution at pH 7.4 and stored in refrigerator at 4 °C until used. DNA concentrations were determined by Nano-Drop spectrophotometer at the wavelength of 260 nm, a solution of ct-DNA in the buffer gave a ratio of UV absorbance of about 1.89 at 260 and 280 nm, indicating that the DNA was sufficiently free from protein [35,36].

2.2. Cell lines

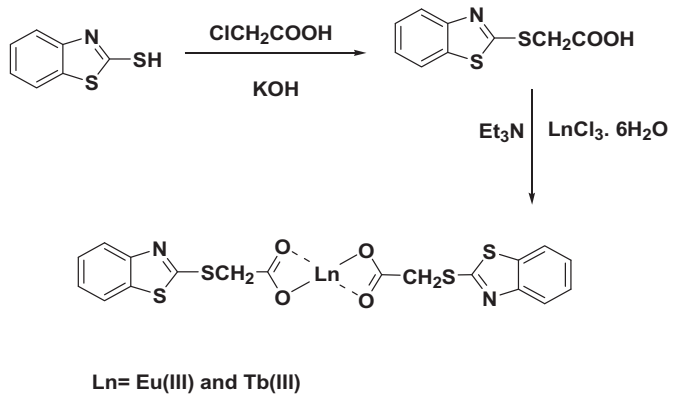
Human liver carcinoma (HepG2), human breast carcinoma (MCF7) and Ehrlich ascites carcinoma (EAC) cell lines were purchased from the Tumor Biology Department, National Cancer Institute, Cairo University (Cairo, Egypt). HepG2 and MCF7 were maintained in RPMI-1640 supplemented with 10% FBS and 1% pen/strep (100 units/ml penicillin and 100 μg/ml streptomycin). The cells were grown at 37 °C in a humidified incubator containing 5% CO_2 in air. Cells were passaged biweekly and used for experiments in the exponential growth phase. EAC is a murine spontaneous breast cancer from which an ascites variant was obtained. The tumor cell line was maintained in our laboratory by serial intraperitoneal (i.p.) passage in female Swiss albino mice at 7–10 day interval. Tumor cells were tested for viability and contamination using trypan blue dye exclusion technique [37].

2.3. Apparatus

Fluorescence spectra were recorded on a Jasco FP-6300 spectrofluorometer with a 150W xenon lamp for excitation. Absorbance spectra were recorded on Jasco FP-6300 attached UV unit using a 1.0 cm path length cell. Nanodrop spectrophotometer model ND 1000 was used for measuring DNA concentrations. Multi-capillary electrophoresis QIAxcel (QIAGEN Germany) system was used for DNA fragmentation analysis. The infrared spectra were obtained in the 4000–400 cm^{-1} region by using Bruker Alpha with KBr discs. Mass spectrometry was carried out by Shimazdu Qp-2010 plus mass analyzer, ^1H and ^{13}C-NMR spectra were recorded on a Joel-400 NMR, elemental analysis was carried out by elementar vario.

2.4. Synthesis and characterization of 2-thioacetic acid benzothiazole

2-Thioacetic acid benzothiazole was prepared according to the method in literature [38,39]. Commercially available 2-mercapto benzothiazole (0.1 mol) was added to a solution of chloroacetic acid (0.1 mol) in aqueous methanol in the presence of KOH as a basic media. The reaction mixture was refluxed for 4 h, then cooled, filtered and acidified with diluted hydrochloric acid. The formed precipitate after filtration was crystallized from aqueous ethanol to give (80%) of the 2-thioacetic acid benzothiazole. The steps of the synthesis of 2-thioacetic benzothiazole were displayed in Scheme 1. The purity of 2-thioacetic acid benzothiazole was confirmed by RPHPLC. Only one sharp peak appears at 300 nm with



Scheme 1. Synthesis of 2-thioacetic acid benzothiazole ligand and Ln(III)-2-thioacetate benzothiazole complexes.

Rt 4.20 min. The mobile phase was prepared by mixing acetonitrile and 25 mM NaH_2PO_4 in a ratio of 50:50 (v/v). The chemical structure of 2-thioacetic acid benzothiazole was confirmed by elemental analysis and spectroscopic methods. 2-Thioacetic acid benzothiazole was obtained as a yellowish white solid. Yield 80%; mp: 145–147 °C; IR (KBr, ν/cm^{-1}) 3435, 1710, 1573, 1031 and 693 cm^{-1}; ^1H NMR ($CDCl_3$): δ (ppm) = 10.57 (br, 1H, COOH),7.20- 7.9 (4H, m, ArH), 4.17 (2H, s, SCH_2; ^{13}C NMR ($CDCl_3$): δ (ppm) = 169.79 (C=O), 151.09 (N=C(S)-S, Cq), 134.87 (N-C,Cq), 126.86 (S-C,Cq), 125.42 (CH Ar), 124.54 (CH Ar), 121.54 (CH Ar), 121.16 (CHAr), 35.8 (S-CH_2); MS (*m*/*z*): 225.29 ($M^{+\cdot}$); Anal. Calcd. for $C_9H_7NO_2S_2$: C, 47.98; H, 3.13; N, 6.22; S, 28.1. Found: C, 49.0; H, 3.40; N, 6.23; S, 28.4.

2.5. Synthesis and characterization of Ln(III) 2- thioacetate benzothiazole

2-Thioacetic acid benzothiazole (1.0 mmol, 0.224 g) was dissolved in ethanol (20 mL) and triethylamine (1.0 mmol, 0.102 g) was then added. The solution was stirred for 15 min at room temperature. $LnCl_3.6H_2O$ (0.5 mmol) dissolved in 20 ml ethanol and then added slowly with vigorous stirring to the 2-thioacetic acid benzothiazole solution. Precipitate appeared immediately after mixing the two solutions. Stirring overnight has been performed to complete precipitation. This product was collected by filtration, purified by washing several times with hot ethanol and dried at 50 °C. The suggested structure of Eu(III)- and Tb(III)-complexes shown in Scheme 1 was confirmed by IR and mass spectroscopic methods and elemental analysis. Yield: 55%; Color: yellowish brown; mp: over 300 °C; IR (KBr, ν/cm^{-1}): 3345, 1732, 1705, 1623, 1558, 1028, 689 and 515 cm^{-1}; MS (*m*/*z*): 780.1 ($M^{+\cdot}$); Anal. Calcd. for$[Eu(TAB)_2Cl.3H_2O].5H_2O$: C, 27.66; H, 3.69; N, 3.58; S, 8.22; Eu(III), 19.48. Found: C, 28.3; H, 3.29; N, 4.09; S, 7.61; Eu(III), 19.2.

Complex $[Tb(TAB)_2Cl.3H_2O].5H_2O$ was synthesized as described above, Yield: 40%; Color: yellowish brown. mp: over 300 °C; IR (KBr, ν/cm^{-1}): 3332, 1709, 1627, 1563, 1453, 1033, 694 and 530 cm^{-1}; MS (*m*/*z*):787($M^{+\cdot}$); Anal. Calcd. for$[Tb(TAB)_2Cl.3H_2O].5H_2O$: C, 27.47; H, 3.85; N, 3.56; S, 8.15; Tb(III), 20.19. Found: C, 26.9; H, 3.99; N, 4.03 S, 8.0; Tb(III), 19.8.

2.6. In vitro anti-tumor activity

The effect of europium (III) and terbium (III)-2-thioacetate benzothiazole complexes on the growth of HepG2 and MCF7 cell lines were evaluated according to the procedure adopted by the National Cancer Institute for *in vitro* anti-tumor drug screening that using Skehan method [40]. Cisplatin was used as a standard to assess the cytotoxicity of complexes. The capabilities of complexes

to arrest the proliferation of tumor cells were evaluated after 48 h of incubation. The results were analyzed by means of cell viability curves and expressed as IC_{50} values. Cells were plated in 96-well plate (10^4 cells/well) for 24 h by using RPMI-1640 media before treatment with the compounds to allow attachment of cell to the wall of plate, different concentrations of the compound under test (1, 5, 10, 25 and 50 μM) were added to the cell monolayer triplicate wells prepared for each individual dose, monolayer cells incubated with the compounds for 48 h at 37 °C and in atmosphere of 5% CO_2. After 48 h, cell were fixed, washed and stained with Sulfo-Rhodamine-B stain, excess stain was washed with acetic acid and attached stain was recovered with Tris–EDTA buffer, color intensity was measured in ELISA reader.

The relation between surviving fraction and complexes concentration were plotted to get the survival curve for breast tumor cell line after the specified time. The molar concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and compared to the reference drug Cisplatin (CAS, 15663-27-1). The surviving fractions were expressed as means ± standard error.

2.7. In vivo anti-tumor activity

2.7.1. Experimental animals

All animal procedures and experimental protocols were approved by the research ethics committee of Suez CanalUniversity and were carried out in accordance with the guide for the care and use of laboratory animals. Swiss albino mice weighing 20–25 g were obtained from the Egyptian organization for biological products and vaccines (Vacsera, Egypt). They were housed under controlled conditioning (25 ± 1 °C constant temperature, 55% relative humidity, and 12 h dark/light cycles), food and water were allowed *ad libitum* throughout the study period.

2.7.2. Induction of solid tumors

EAC cells were suspended in normal saline so that each 100 μl contains 5×10^6 cells. Each mouse was inoculated intradermally (i.d.) at 2 sites bilaterally on the lower ventral side with 100 μl EAC suspension (2.5×10^6 cells) on each site [41].

2.7.3. Induction of ascites

In a parallel experiment, mice were injected intraperitoneally (i.p.) with 100 μl EAC suspension (5×10^6 cells)/mouse to induce ascites. These mice received the same pharmacological treatment as explained below [42–45]. At the end of the experiment, the ascites fluids were collected and divided into two portions, the 1st portion was immediately snap frozen in liquid nitrogen for the DNA extraction and the other portion was used for the determination of EAC cell counts.

2.7.4. Pharmacological treatment

The mice were randomly assigned to 3 main groups, 8 mice each. The first group received vehicle injection (DMSO in PBS) and served as control. Second and third groups were injected daily with 4 mg/kg (i.p.) of europium (III)-2- benzothiazole or terbium (III)-2-benzothiazole complex, respectively. All treatments started 6 days after tumor cells inoculation [42–45]. At the end of the experiment, mice were sacrificed by cervical dislocation then tumors were excised and fixed in 10% neutral buffered formalin.

2.7.5. DNA extraction

DNA was purified from EAC cells using the GF-1 DNA extraction kit (*Vivantis*). DNA fragments were analyzed in the automatic multi-capillary electrophoresis QIAxcel system. Isolated DNA was placed in the instrument sample tray, 10 μl of the DNA samples were automatically injected into the capillary channel and subjected to electrophoresis according to the protocol AM320 (applied separation time: 323 s, method separation time: 320 s, method injection time: 10 s, method separation voltage: 6.0 kV and method injection voltage: 5.0 kV) of the QIAxcel DNA Screening Kit.

2.7.6. Immunohistochemistry

IHC was performed on 5-um paraffin sections using the avidin-biotin-peroxidase method. Sections were deparaffinized in xylene and rehydrated in a graded series of ethanol and rinsed with distilled water. Antigen retrieval was performed by citrate-EDTA antigen retrieval protocol [46]. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 5 min. Slides were incubated overnight at 4 °C with the primary antibody Flk-1 (1:80) or anti-CD31 Class II (1:50) for the determination of VEGF receptor type-2 or microvessel density, respectively. After conjugation with streptavidin–biotin–peroxidase complex, coloring was performed with 3,3′-diaminobenzidine substrate-chromogen and Mayer's hematoxylin was used for counter staining.

2.7.7. Tumor mass

All tumors, 2-discs/animal, were punched out, weighed immediately, and the average weight was calculated. All tumors were then kept in 10% neutral buffered formalin for histological evaluation.

2.8. Quantification of immunohistochemistry

For quantitative analysis, the intensities of immunoreactive parts were measured. Measurements were done using an image analyzer (ImageJ programme). For each slide from all experimental groups, 7 fields were randomly selected; integrated densities (IntDen) of nine random parts within each field were analyzed and the mean was expressed as field IntDen.

2.9. Data analysis

Statistics were calculated with SPSS for windows version 14.0, the mean values obtained in the different groups were compared by one way ANOVA followed by Duncan's multiple range test. All results were expressed as mean values ± SE and significance was defined as $p \leq 0.05$.

2.10. Test of anti-bacterial activity

In vitro anti-bacterial activity of 2-thioacetic acid benzothiazole and its europium (III)- and terbium (III)-2-thioacetate benzothiazole complexes were studied by using the minimum inhibitory concentration (MIC) method [47–49] against two pathogenic strains; *Escherichia coli* and Salmonella *spp*.

All the bacteria were incubated and activated at 37 °C by inoculation into Mueller-Hinton Broth for 24 h. The compounds were dissolved in DMSO and then diluted using Mueller-Hinton Broth. Different serial concentrations of the compounds were used to determine the minimum inhibitory concentration (MIC) ranging from 2500 μM to 1 μM. Test cultures were incubated at 37 °C (24 h). The lowest concentrations of antimicrobial agents that resulted in complete inhibition of growth were represented as MIC (μM).

3. Results and discussion

3.1. Chemistry of the complexes

Analytical data for the synthesized Ln(III)-2-thioacetate benzothiazole complexes are presented in Table 1. The elemental analytical data show that the formulas of the complexes could be $[Ln(TAB)_2Cl.3H_2O].5H_2O$ as displayed in Scheme 1. The complexes

Table 1
Elemental analytical data for the ligand and Ln(III)-complexes.

Complex	C (%) found (calc.)	H (%) found (calc.)	N (%) found (calc.)	S (%) found (calc.)	Ln (%) found (calc.)
2-Thioacetic acid benzothiazole	49.0 (47.98)	3.40 (3.13)	6.23 (6.22)	28.1 (28.4)	–
[Tb $(TAB)_2$Cl.3H_2O].5H_2O	26.9 (27.47)	3.99 (3.85)	4.03 (3.56)	8.0 (8.15)	19.8 (20.19)
[Eu$(TAB)_2$Cl.3H_2O].5H_2O	28.3 (27.71)	3.29 (3.88)	4.09 (3.59)	7.61 (8.22)	19.2 (19.48)

are yellowish brown colored and stable. They are soluble in DMF and DMSO and insoluble in water, ethanol, benzene, diethyl ether and tetrahydrofuran. Because of the insolubility of the complex in suitable solvents we were unsuccessful in growing crystals for single crystal X-ray structural studies.

The FTIR spectrum of the ligand shows a characteristic stretching absorption bands at 3435, 1710, 1573, 1027 and 694 cm^{-1} assigned to the stretching of hydroxyl, carbonyl, C=N of the thiazole ring, C–O of the hydroxyl in the carboxylate group and the stretching of C–S group [39], respectively. On complexation these bands were shifted to a lower or higher frequency region. This shift is probably due to the complexation of the lanthanide (III) ions to the ligand through oxygen of the carbonyl group. Stretching of Ln(III)-oxygen bands of the complexes appeared in low frequency region (515–530 cm^{-1}).

In the mass spectra of the complexes, the molecular ion peaks ($M^{+\cdot}$) are observed at 780.1 and 787 *m/z* which are ascribed to [Eu$(TAB)_2$Cl.3H_2O].5H_2O and [Tb$(TAB)_2$Cl.3H_2O].5H_2O, respectively. The fragment ion peaks observed at 600 and 607 *m/z* are due to Eu$(TAB)_2$ and Tb $(TAB)_2$ respectively.

The luminescence properties of Eu (III) and Tb (III)-complexes were measured at room temperature as shown in Figs. 1 and 2. Owing to the solubility of the complexes, the luminescence spectra of the complexes have been investigated in DMSO. Luminescence spectrum of Eu(III)-2-thioacetate benzothiazole complex shows the characteristic emission bands for Eu (III) ions. The emission band centered at 615 nm ($^5D_0 \rightarrow {}^7F_2$) is obviously higher than the emission band 590 nm ($^5D_0 \rightarrow {}^7F_1$). The luminescence spectrum of Tb(III)-$(TAB)_2$ shows the emission bands at 490, 545, 582, and 618 nm corresponding to the $^5D_4 \rightarrow {}^7F_6$, $^5D_4 \rightarrow {}^7F_5$, $^5D_4 \rightarrow {}^7F_4$, $^5D_4 \rightarrow {}^7F_3$ transition respectively, and the strongest among them is $^5D_4 \rightarrow {}^7F_5$.

3.2. DNA binding activity

Numerous biological experiments have demonstrated that DNA is the primary intracellular target of anticancer drugs due to the interaction between small molecules and DNA, which cause DNA

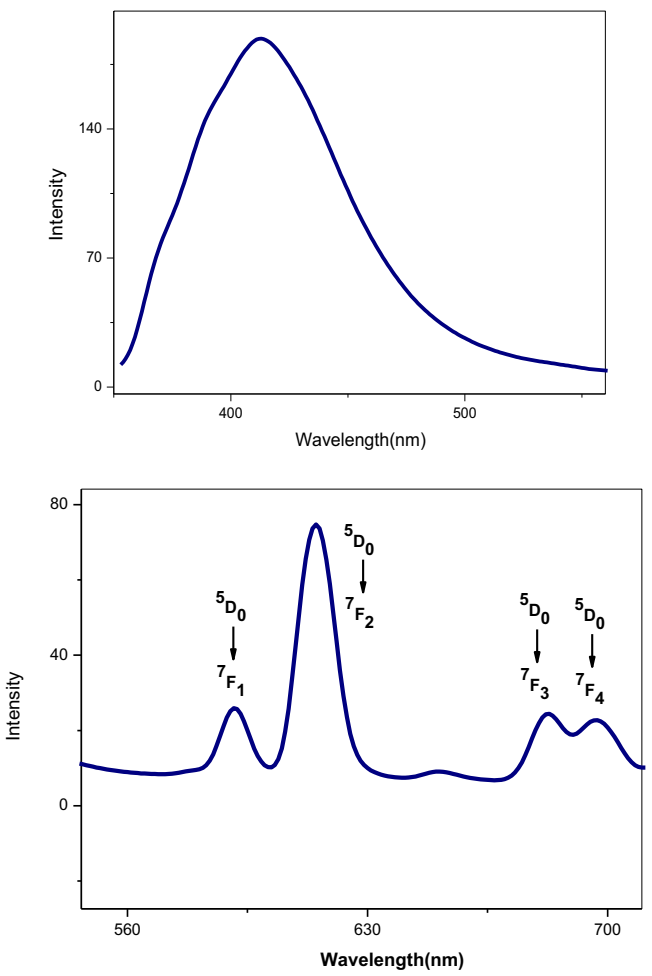


Fig. 1. Fluorescence emission spectra of Tb(III)-complex. Conditions: in DMSO, 25 °C, 1×10^{-3} M, $\lambda_{ex} = 342$ nm, emission and excitation slit width were 5 nm.

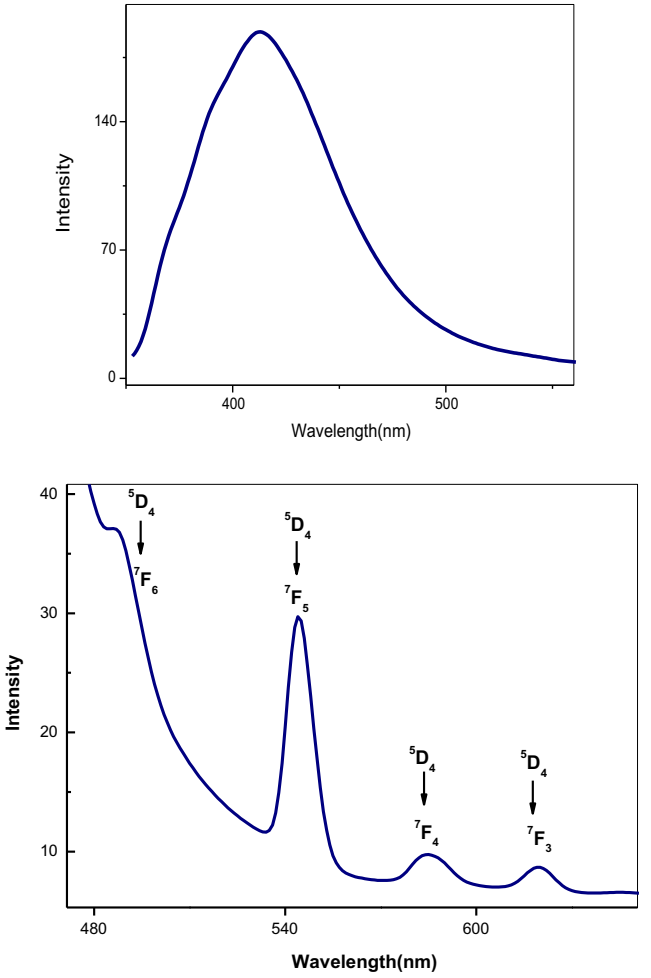


Fig. 2. Fluorescence emission spectra of Eu(III)-complex. Conditions: in DMSO, 25 °C, 1×10^{-3} M, $\lambda_{ex} = 340$ nm, emission and excitation slit width were 5 nm.

damage in cancer cells, blocking the division of cancer cells and resulting in cell death [50,51]. In this work the interactions of Eu(III) or Tb(III)-2-thioacetate benzothiazole complexes with DNA were investigated by fluorescent and UV spectroscopy.

3.2.1. Electronic absorption spectroscopy

Electronic absorption spectroscopy is employed to identify the binding mode of DNA with the metal complex. Three fundamentally different modes of DNA binding by metal complex can be identified: non-specific external association, groove binding and intercalative binding that small molecules intercalate into the base pairs of nucleic acids [52,53].

The electronic absorption spectra of the Eu (III) and Tb(III)-complexes with or without ct-DNA are shown in Fig. 3. The electronic absorption spectra of complexes consist of three resolved bands at about 281, 290 and 300 nm. The high energy absorption band appeared in the spectra of respective complexes are assigned to π-π* transitions. Upon the addition of DNA, the above bands corresponding to complexes showed significant hypochromism accompanied with slight red shift from 381 to 383 nm. These results are similar to those reported earlier for various intercalations [52], suggesting that the Ln(III)-complexes showed strong binding to DNA via an intercalative mode.

The binding constants (K) of the complexes with DNA can be calculated from the absorption titration experiments according to following equation [35]

$$\frac{1}{\Delta\varepsilon_a} = \frac{1}{\Delta\varepsilon} + \frac{1}{\Delta\varepsilon K[\mathrm{DNA}]} \quad (1)$$

where $\Delta\varepsilon_a = (\varepsilon_a - \varepsilon_f)$ and $\Delta\varepsilon = (\varepsilon_b - \varepsilon_f)$. ε_a, ε_f and ε_b are the apparent extinction coefficients of complex, the extinction coefficient for the free complex, and the extinction coefficient for the complex in the bound form, respectively. The double reciprocal plots of $1/\Delta\varepsilon_a$ versus 1/[DNA] were linear (Fig. 4) and the binding constants were calculated from the ratio of the slope to the y-intercept. The binding constants obtained for Eu(III) and Tb(III)-complexes to DNA are 2.55×10^5 and 9.72×10^4 M^{-1}, respectively. These binding constant values compare well with that obtained for the intercalation of complex into the DNA molecules [54–57].

3.2.2. Luminescence titration with ct-DNA

The interaction of Eu(III)- and Tb(III)-2-thioacetate benzothiazole with DNA in Tris–HCl buffer at pH 7.4 were investigated by fluorescence measurement as shown in Figs. 5 and 6. The effect of DNA concentrations on the fluorescence intensity of Ln (III) exhibited a pronounced change in emission intensity. The fluorescence

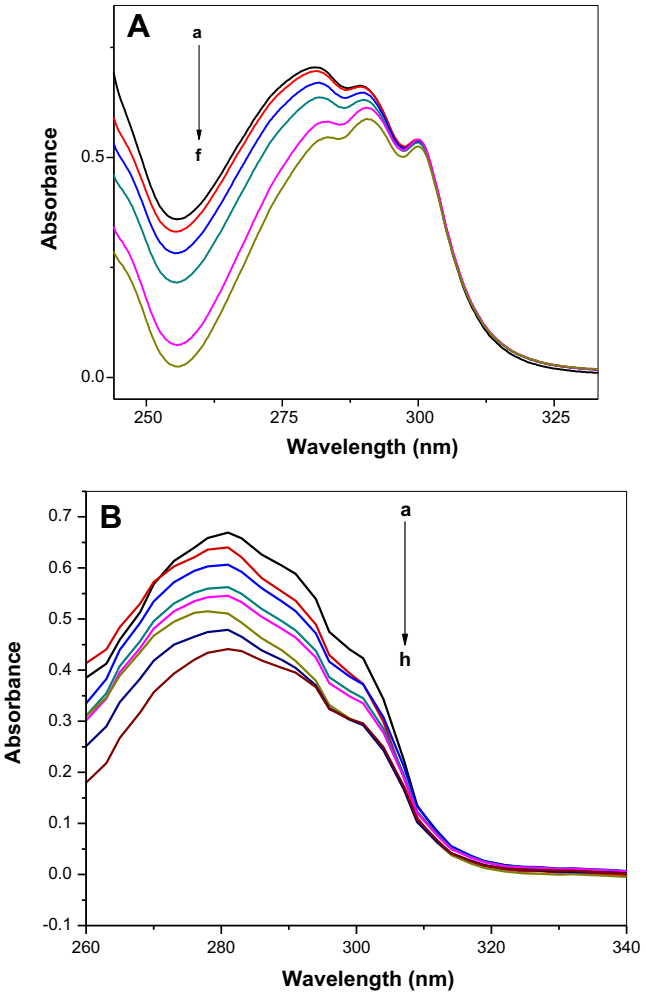


Fig. 3. Electronic absorption spectra of Eu(III)-complex (A) and Tb(III)-complex (B) in the absence and the presence of increasing amounts of ct-DNA. Arrows show the absorbance changes upon increasing ct-DNA concentration.

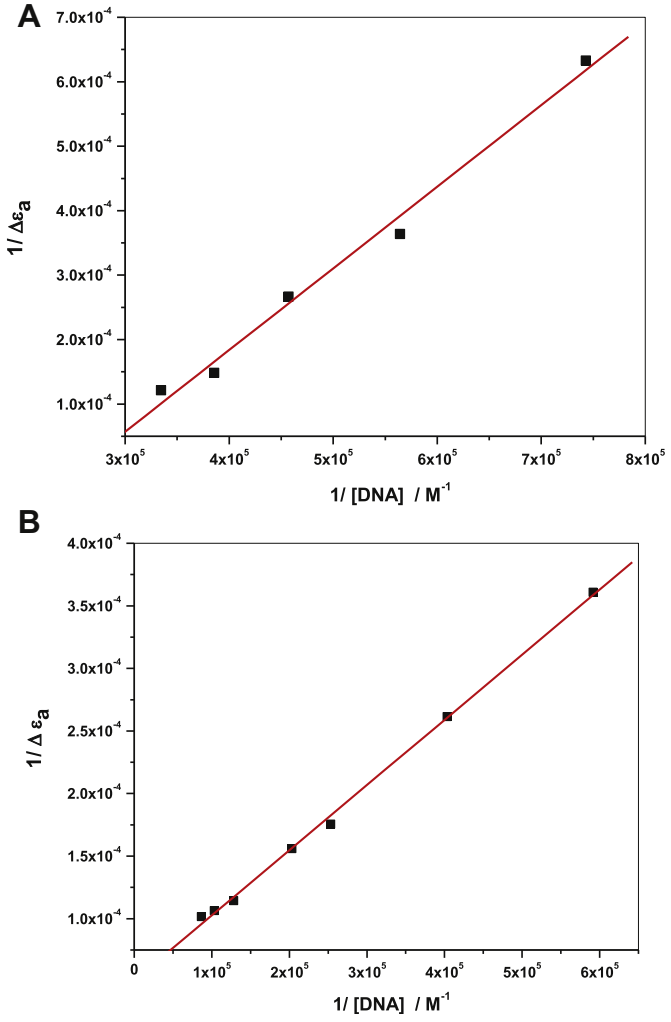


Fig. 4. Double reciprocal plots for the effect of ct-DNA concentration on the absorption spectra of Eu(III)-complex (A) and Tb(III)-complex (B) at room temperature.

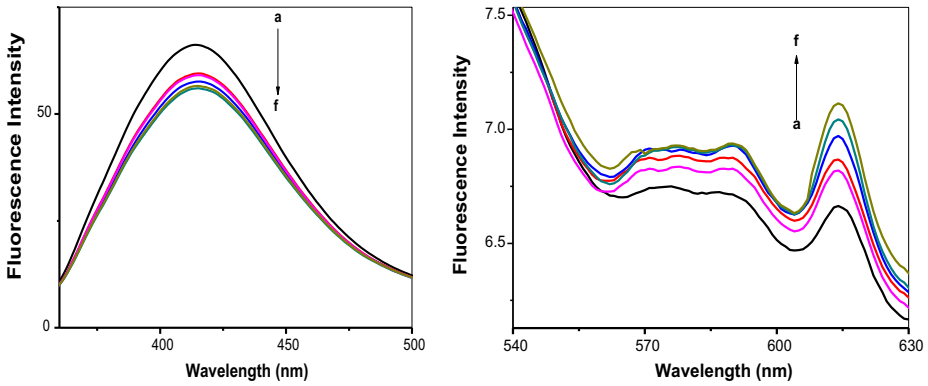


Fig. 5. Fluorescence emission spectra of Eu(III)-complex in the absence and in the presence of DNA. Conditions: in Tris–HCl buffer pH 7.4, 25 °C, Eu(III)-complex at 2×10^{-5} M, $\lambda_{ex} = 340$ nm, emission and excitation slit width were 5 nm.

intensity of Ln(III) in the complexes enhanced with increasing DNA concentration, while the fluorescence intensity at 415 in the region of ligand decreases with increasing of DNA concentration due to the interaction of DNA with Ln(III)-complexes.

In order to compare quantitatively the binding strength of the complexes, the binding constants of them with ct-DNA were obtained by monitoring the changes in the fluorescence intensities of Eu(III)- and Tb(III)-complexes at 614 and 545 nm, respectively, with increasing concentration of DNA using the following equation [58,59]:

$$\log\left[\frac{F - F_0}{F}\right] = \log K + n \log[DNA] \quad (2)$$

Where K and n are the binding constant and the molar ratio, respectively. [DNA] is the free concentration of DNA. The Plot of log [$(F-F_0)/F$] versus log [DNA] at room temperature gave a straight line (Fig. 7). The slope of such curve is equal to n while the intercept is equal to log K. The values of n approximately equal to 1, indicating that there is one binding site in Eu(III) and Tb(III)-complexes for DNA. Where the magnitudes of binding constants (K) were calculated to be 2.296×10^5 M^{-1} and 8.616×10^4 M^{-1} corresponding to Eu(III) and Tb(III)-complexes, respectively. These values are very comparable to that calculated from the absorption titration with correlation coefficient of 0.99. It was observed that the negative signs for free energy ΔG (30.58 and 28.16 kcal/mol for Eu(III) and Tb(III)-complexes, respectively) mean that the interaction process of Ln(III)-complex and DNA is spontaneous. The binding constants of the formed complexes (K) between the Ln(III)-complex and DNA are essentially of the same order of magnitude as that of the well-known intercalators [53–57].The values of binding constant revealed that the Eu(III)-complex is strongly bound with ct-DNA than Tb(III)-complex.

3.3. In vivo anti-tumor activity

3.3.1. Anti-angiogenic activity

To investigate the effect of our compounds on the tumor angiogenesis, we evaluated the expression of CD31 and VEGF receptor type-2 (Flk-1). CD31 is commonly used as an endothelial cell marker to highlight tumor blood vessels reflecting the degree of angiogenesis [41,60–63]. EAC-control group showed the highest number of microvessel density on the fourteenth day post-inoculation. Groups treated with Eu(III)- or Tb(III)-2-thioacetate benzothiazole showed a significant reduction ($P \leq 0.05$) in microvessel density on the fourteenth day compared to the control group (Fig. 8). These results indicate that Eu(III)- and Tb(III)-2-thioacetic acid benzothiazole have anti-angiogenic activity against EACs (Fig. 9).

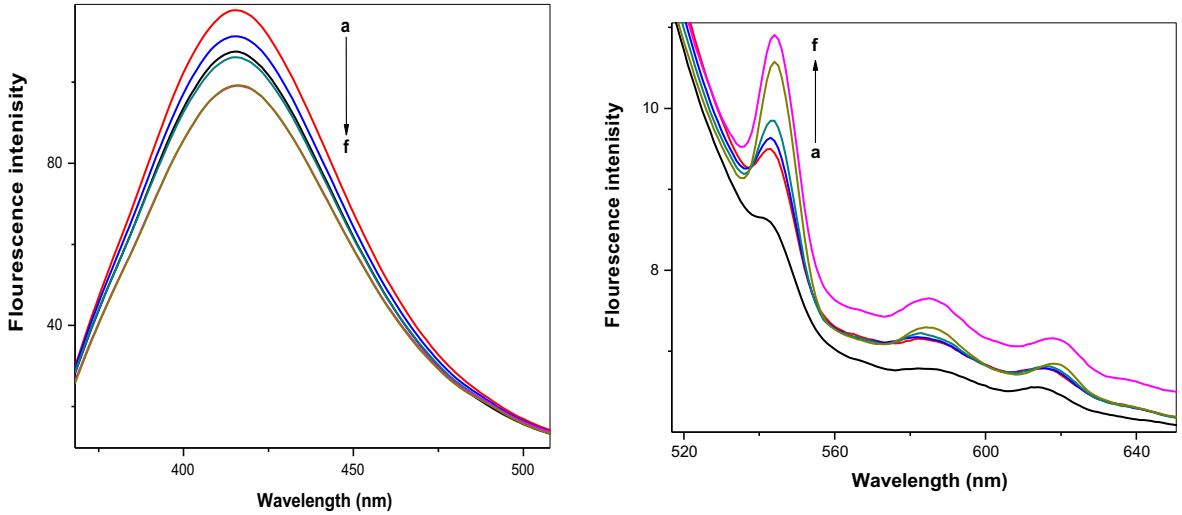


Fig. 6. Fluorescence emission spectra of Tb(III)-complex in the absence and in the presence of ct-DNA. Conditions: in Tris–HCl buffer pH 7.4, 25 °C, Tb(III)- complex at 2×10^{-5} M, $\lambda_{ex} = 342$ nm, emission and excitation slit width were 5 nm.

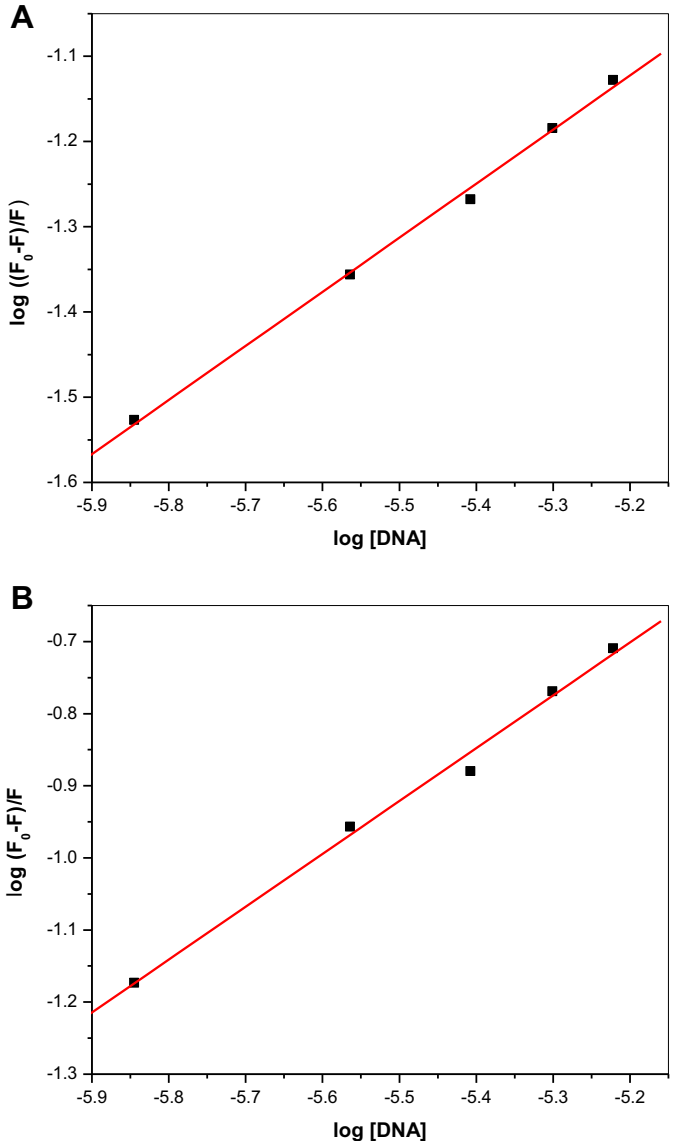


Fig. 7. Double logarithm plots for the effect of ct-DNA concentration on the fluorescence intensity of Eu(III)-complex (A) and Tb(III)-complex (B) at room temperature.

VEGF and its receptors have been implicated as key components in the vascularization of a tumor [41,64]. But, most of the VEGF angiogenic properties are mediated by interaction with VEGF receptor type-2 (Flk-1) [65,66]. Studies show that high VEGF expression along with high VEGF receptor expression is associated with increased microvessel density, poor clinical prognosis, and increased metastasis in many cancers [41,67,68]. The loss of VEGF expression in a tumor causes a dramatic decrease in vascular density and vascular permeability and increases tumor cell apoptosis [69].

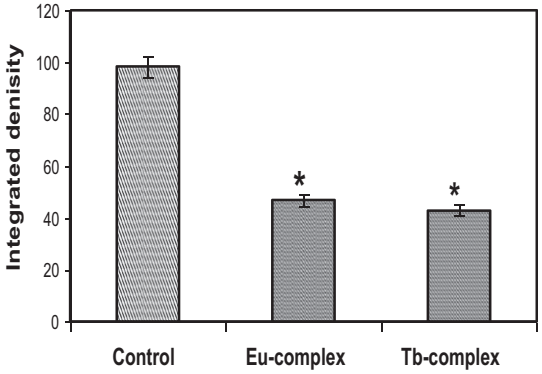


Fig. 9. Statistical analysis of the Eu(III)- and Tb(III)-2-thioacetate benzothiazole complexes treatment on CD31 assessed immunohistochemically on day 14 post-implantation of EAC cells in Swiss albino mice.

Strong intra-tumoral expression of Flk-1 was observed in all EACs in the control group (Fig. 10). Flk-1-rich tumors were detected in untreated EACs while the groups treated with Eu(III)- or Tb(III)-2-thioacetate benzothiazole complex showed poor expression of the receptor with significant reduction ($P \leq 0.05$) in the integrated density as compared to the untreated control (Fig. 11).

3.3.2. Tumor mass and number of EAC cells

To determine the *in vivo* effect on tumor cell growth, mouse mammary carcinoma cell line EAC was treated with complexes europium(III)- and terbium(III)-2-thioacetate benzothiazole (4 mg/kg body weight/ip), daily for total of seven doses during its growth period, starting at the day 6 after inoculation of tumor cells. Solid tumor mass and number of EAC cells were evaluated at the end of treatment. In control EAC-bearing mice, there was an increase in solid tumor mass and number of EAC cells. A rise in solid tumor weight of about 165 mg over a period of 14 days of tumor growth was observed. However, when treated with the [Europium(III)-2-thioacetate benzothiazole] and [Terbium(III)-2-thioacetate benzothiazole] complexes, about 39% and 52% decrease in the solid tumor mass was observed, respectively, indicating the anti-tumor effect of [Europium(III)-2-thioacetate benzothiazole] and [Terbium(III)-2-thioacetate benzothiazole] complexes as shown in Fig. 12.

Similarly, single treatment with Eu(III)- or Tb(III)-2-thioacetate benzothiazole complexes produced a significant reduction

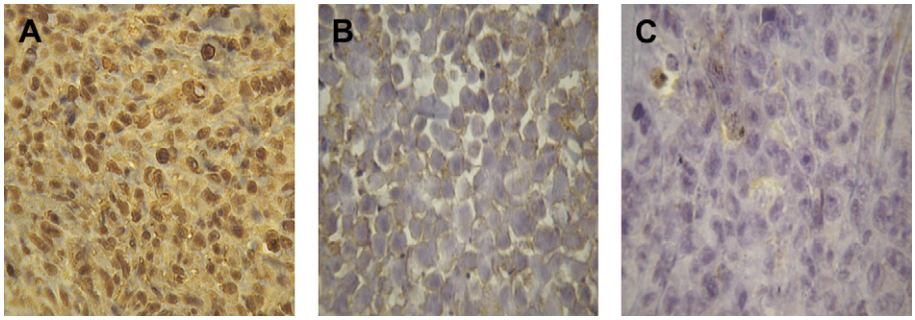


Fig. 8. Effect of treatment of Eu(III)- and Tb(III)-2-thioacetate benzothiazole complexes on CD31 assessed immunohistochemically on day 14 post-inoculation of EAC cells in Swiss albino mice (A) control; (B) Eu(III)-2- thioacetate benzothiazole treatment; (C) Tb(III)-2- thioacetate benzothiazole treatment.

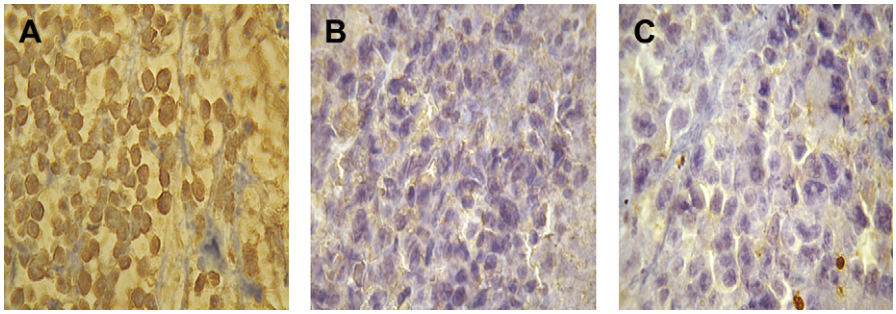


Fig. 10. Effect of treatment of Eu(III)- and Tb(III)-2-thioacetate benzothiazole complexes on VEGF assessed immunohistochemically on day 14 post-inoculation of EAC cells in Swiss albino mice (A) control; (B) Eu(III)-2-thioacetate benzothiazole treatment; (C) Tb(III)-2-thioacetate benzothiazole.

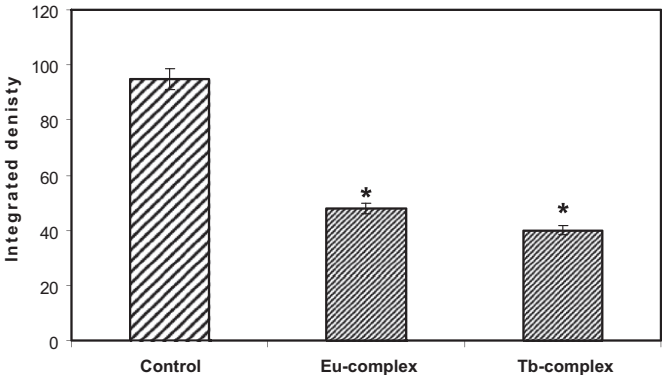


Fig. 11. Statistical analysis of Eu(III)- and Tb(III)-2-thioacetate benzothiazole complexes treatment on VEGF assessed immunohistochemically on day 14 post-inoculation of EAC cells in Swiss albino mice.

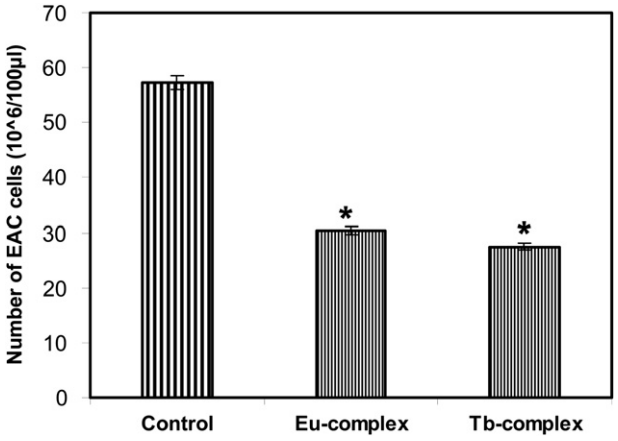


Fig. 13. Statistical analysis of the Eu(III)- and Tb(III)-2-thioacetate benzothiazole complexes treatment on number of Ehrlich ascites carcinoma cells (or EAC).

($P \leq 0.05$) in number of EAC cells in ascetic fluid on day 14 post-inoculation as compared to the control animals (Fig. 13).

3.3.3. DNA fragmentation assay

Activation of programmed cell death (apoptosis) in cancer cells offers novel and potentially useful approaches to improve patient responses to conventional chemotherapy. The most dramatic biochemical feature of apoptosis is DNA fragmentation as a result of cleavage of the cell's DNA at intervals of 160–240 base pairs (bp) which are the size of oligonucleosomes [70–72].

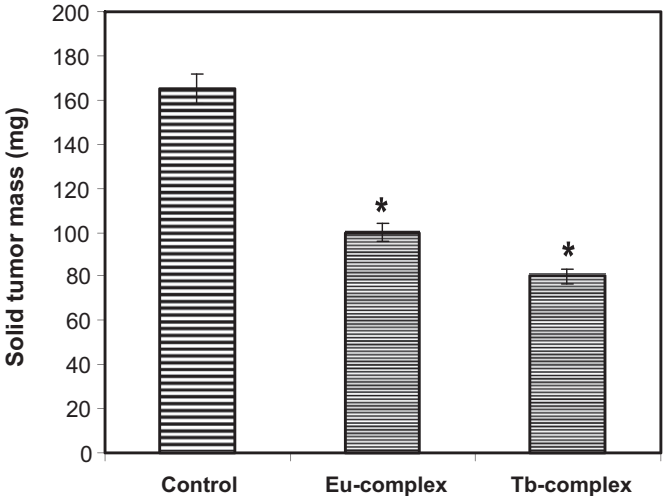


Fig. 12. Statistical analysis of the Eu(III)- and Tb(III)-2-thioacetate benzothiazole complexes treatment on solid tumor mass.

An attempt was made to find the mechanism of inhibition of proliferation of EAC cells by Eu(III)- and Tb(III)-2-thioacetate benzothiazole complexes. As expected, DNA fragments were observed in EAC cells after treatment with Eu(III)- and Tb(III)-2-thioacetic acid benzothiazole complexes (Fig. 14. lane B & C) and no fragmentation was observed in untreated EAC cells (Fig. 14. lane A). This indicates that there is apoptotic role of Eu(III) and Tb(III)-2-thioacetate benzothiazole complexes in EAC cells.

The oligonucleosomes fragments can be observed as a DNA ladder on agarose gel electrophoresis. Limited information about DNA fragmentation has been obtained by traditional agarose gel electrophoresis. Therefore, the capillary electrophoresis has been conducted to analysis the DNA fragments. Capillary electrophoresis (CE) has been widely applied to DNA separation and characterization of cell apoptosis based on the determination of very small DNA fragmentation from 15 bp with a size difference of 5 bp [73,74].

Figs. 15 and 16 show the data of DNA fragments extracted from EAC cells treated by Eu(III)- and Tb(III)-2-thioacetic acid benzothiazole complexes for 7 days. Many peaks appeared for DNA treated with Eu(III)-complex as shown in Fig. 15. By comparing the migration time of the peaks with those of the standard DNA ladder marker 50–800 bp, peaks 1–5 have sizes of about 40, 69, 89, 108 and 121 bp which can be regarded as cleaved DNA fragments of short length. Peaks 6–9 have approximately sizes of 151, 183, 203 and 219 bp, respectively, corresponding to one to four nucleosomes.

Fig. 16 displays the data for DNA treated with Tb(III)-complex, by comparing with the reference marker peaks 1–5 which have sizes

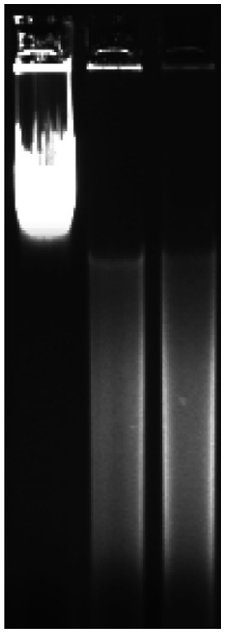


Fig. 14. Effect of Eu(III)- and Tb(III)-2-thioacetate benzothiazole complexes on genomic DNA of EAC cells: EAC cells treated with or without Eu(III), Tb(III)-2-thioacetate benzothiazole complexes *in vivo* were harvested and DNA was isolated; Lane 1: DNA of EAC untreated, Lane 2: DNA of EAC treated with Eu(III)-2-thioacetate benzothiazole complexes Lane 3: DNA of EAC treated with Tb(III)-2-thioacetate benzothiazole complexes.

of about 47, 72, 91, 112 and 125 bp which can be regarded as cleaved DNA fragments of short length. Peaks 6–11 have approximately sizes of 152, 170, 192, 210, 231 and 317 bp, respectively, corresponding to one to eight nucleosomes.

The biochemical character of apoptosis is the cleavage of genomic DNA into 160–240 bp fragments [70,71]. Therefore peaks 6–9 for DNA fragments treated with Eu(III)-complex and peaks 6–11 for DNA fragments treated with Tb(III)-complex can be regarded as typical apoptotic DNA fragments, suggesting that the cells undergo apoptosis after they have treated with Eu(III)- and Tb(III)-2- thioacetate benzothiazole complexes for 7 days.

3.4. In vitro anti-tumor activity

HepG2 and MCF7 cell lines were selected for preliminary studies on the *in vitro* anti-tumor activity of the [Eu(III)-2-thioacetate benzothiazole] and [Tb(III)-2-thioacetate benzothiazole] complexes. Our choice was based on the fact that hepatocellular carcinoma (HCC) being one of the most common and lethal types in Eastern Mediterranean Region (EMR) especially in Egypt and breast cancer which represents the most common cancer among women in Egypt, representing 35.1% of total cancer cases [75].

Cisplatin, which is one of the most effective metallo-anticancer agents, was used as the reference drug in this study. The relationship between surviving fraction and drug concentration was plotted to obtain the survival curve of HepG2 and MCF7 cell lines. The response parameter calculated was the IC_{50} value, which corresponds to the concentration required for 50% inhibition of cell viability.

As shown in Fig. 17, [Eu(III)-2-thioacetate benzothiazole] and [Tb(III)-2-thioacetate benzothiazole] complexes inhibited the growth MCF7 cell line with IC_{50} values of 5.1 ± 0.2 and 4.5 ± 0.15 μM, respectively. Complexesed show better cytotoxicity which is about 2 times the strength of cisplatin (IC_{50} values of 9.0 ± 0.28 μM).

Fig. 18 displays the effect of [Eu(III)-2-thioacetate benzothiazole] and [Tb(III)-2- thioacetate benzothiazole] complexes on HepG2 cell growth. The antiproliferation effect of the [Eu(III)- and Tb(III)-2-thioacetate benzothiazole] complexes revealed that [Eu(III)-complex] IC_{50} = 25 ± 0.17 μM and [Tb(III)-complex] IC_{50} = 35 ± 0.2 μM.

Table 2 shows the *in vitro* cytotoxic activity of the synthesized compounds. It can be seen that Tb(III)-complex was the most potent compound in this screening, and exhibited a higher cytotoxic activity (IC_{50} = 4.5 μM) against MCF7 when compared with the reference drug cisplatin (IC_{50} = 9 μM), followed by Eu(III)-complex with IC_{50} = 5.1 μM. On the otherhand, Eu(III)- and Tb(III)-complexes showed IC_{50} values of 25 μM and 35 μM, respectively, which are lower than that of cisplatin (IC_{50} = 12 ± 0.88 μM), indicating lower activity against HepG2 cell line.

3.5. Antimicrobial activity

There is a need for new antimicrobial agents and one of the attractive strategies in this field is to develop nucleic acid targeting agents that inhibit the pathogenic gene to replicate and transcript. The central dogma of all living organisms (prokaryotic and Eukaryotic) is based on replication of DNA, transcription to produce mRNA and translation to synthesis proteins. Inhibition of DNA transcription and replication would restrict protein synthesis, or replication, and could induce cell death. On the basis of a previously observed correlation between the antimicrobial activity and DNA binding strength of some molecules [76–81], the Eu(III)- and Tb(III)-complexes were screened for anti-bacterial activity against Escherichia coli and Salmonella by the minimum inhibitory concentration (MIC) method [47–49].

The results showed that the antimicrobial activity of the complexes (Eu(III)- or Tb(III)-2-thioacetate benzothiazole) against the Escherichia coli and Salmonella is much higher than 2-thioacetic acid benzothiazole. The results of our study showed

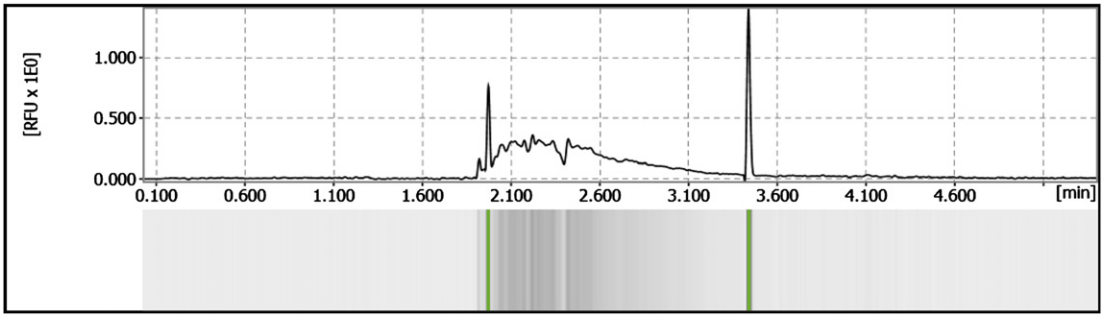


Fig. 15. Capillary electropherogram of DNA extracted from EAC cells after treatment with Eu(III)-2-thioacetate benzothiazole complex *in vivo*.

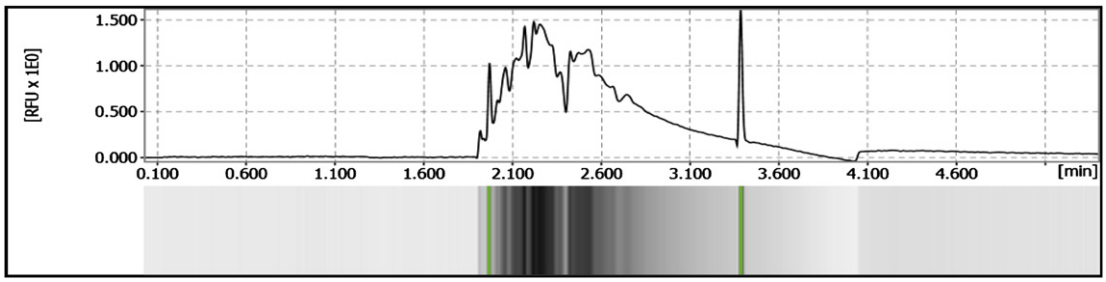


Fig. 16. Capillary electropherogram of DNA extracted from EAC cells after treatment with Tb(III)-2-thioacetate benzothiazole complex *in vivo*.

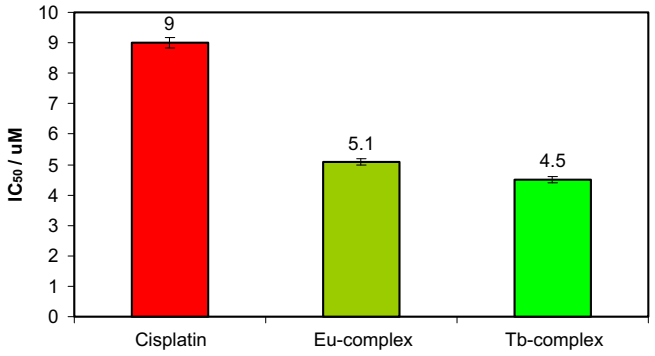


Fig. 17. IC_{50} values for Eu(III)- and Tb(III)-complexes against HepG2 (μM). IC50 values are presented as the mean ± SE obtained from at least three independent experiments.

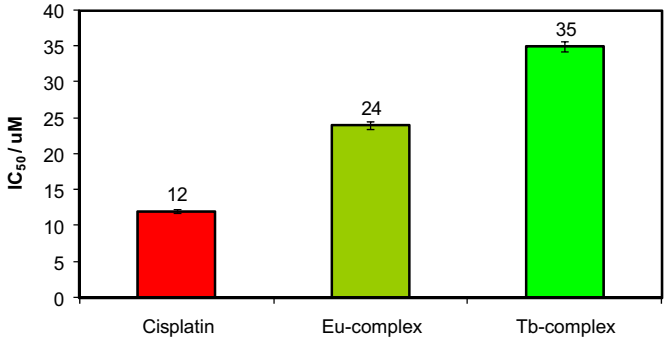


Fig. 18. IC_{50} values for Eu(III)- and Tb(III)-complexes against MCF7 (μM). IC50 values are presented as the mean ± SE obtained from at least three independent experiments.

that in the case of Escherichia coli, the Eu(III)- or Tb(III) complexes exhibited MIC = 120 and 130 μM, respectively, which are more potent than the ligand MIC = 440 μM. Furthermore, the Eu(III)- or Tb(III) complexes exhibited MIC = 140 and 110 μM, respectively, in the case of Salmonella, which are more potent active than ligand MIC = 500 μM. These data indicate that our new complexes are more potent bactericides than the ligand. The anti-bacterial mechanism was hypothesized that the benzothiazole complexes has an effect on the two pathogenic bacteria cell proliferation.

Table 2
In vitro anticancer screening of the synthesized Ln(III) complexes against human breast cell line (MCF7) and human liver cell line (HepG2).

Compound	MCF7 IC_{50} μM	HepG2 IC_{50} μM
Tb(III)-complex	4.5 ± 0.15 μM	35 ± 0.2 μM
Eu(III)-complex	5.1 ± 0.2 μM	25 ± 0.17 μM
Cisplatin	9 ± 0.28 μM	12 ± 0.88 μM

4. Conclusion

Two new trivalent lanthanide complexes have been synthesized and characterized using various spectroscopic methods which revealed that the ligand forms mononuclear Eu(III)- and Tb(III)-complexes with 1:2 stoichiometry between lanthanum ions and ligand. The newly synthesized complexes were evaluated for DNA binding, antimicrobial activity and cytotoxicity studies. We conclude from the current study that there is a strong interaction between Eu(III)- or Tb(III)-2-thioacetate benzothiazole complexes and DNA by absorption and fluorescence spectral techniques which is due to the intercalation into the hydrophobic state of the ds-DNA with binding constants ranging from 10^3-10^5 M^{-1}.

Furthermore the positive results presented in this work regarding the anti-bacterial and anti-tumor activity of Eu(III) and Tb(III)-2-thioacetate benzothiazole complexes confirm our preliminary hypothesis of strong interaction proposed early in this paper.

In addition, our novel compounds proved to exert an anti-angiogenic effect as it was evident by the reduction of micro-vessel density through, at least in part, the inhibition of VEGFR type 2. The apoptotic study indicates that the two complexes can effectively induce the apoptosis of EAC cells by formation inter-nucleosomal DNA ladder. Moreover Eu(III)- and Tb(III)-2-thioacetic benzothiazole acid complexes exhibited significant anticancer activity, when compared to cisplatin as a reference drug. The achieved results suggest that these synthetic compounds have a strong therapeutic potential as anti-tumor and anti-bacterial agents.

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