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SYNTHESIS, CHARACTERIZATION AND EVALUATION OF THE ANTIMICROBIAL AND CYTOTOXIC ACTIVITY OF TWO NOVEL COMPLEXES OF Gd(III) WITH PIROXICAM AND MELOXICAM

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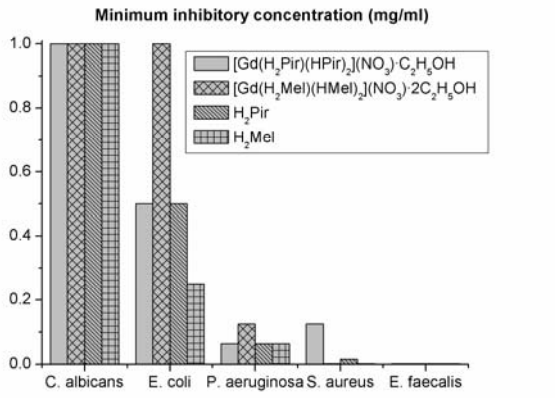
Two novel complexes of Gd(III) were synthesized using piroxicam (H_2Pir) and meloxicam (H_2Mel) as ligands. Their characterizations were accomplished through FT-IR, UV-VIS, TG/DTG, elemental analysis, magnetic measurements and molar conductivity. The obtained complexes and the free ligands were evaluated for their microbicidal, anti-biofilm and influence on the viability and cellular cycle of human adenocarcinoma cells (HT-29) using *in vitro* qualitative and quantitative assays. The results of the biological evaluation revealed the multifunctionality of the obtained compounds and their potential for different biomedical applications. At low concentrations, inferior to 0.063 mg mL^{-1}, $[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$ (**1**) and $[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$ (**2**) complexes proved to be active only against the Gram-positive cocci planktonic cells, while **1** exhibited also good anti-*Candida albicans* biofilm activity. At high concentrations the tested compounds induced cytotoxic, pro-apoptotic and cellular cycle modulatory effects on the HT-29 cells, being thus promising for the further development of novel anti-proliferative agents.



INTRODUCTION

In the last decades, lanthanide complexes attracted increasing interest in coordination and bioinorganic chemistry due to their interesting structures, and also for their photo-physical, magnetic and biological properties. Due to their special electronic configuration, a variety of lanthanide complexes have been proven to be very good antibacterial, anti-inflammatory, antiviral, anticoagulant and antitumor agents.[1-6] Among them, some of those containing Gd(III) are widely used in biomedical analysis as magnetic resonance imaging (MRI) contrast agents both for its high paramagnetism and favorable properties in terms of electronic relaxation.[7] It is well known that the

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first contrast agent approved for clinical use was Gd-DTPA (Magnevist®, Schering AG, Germany) that, in more than 10 years of clinical experimentation, has been administered to more than 20 million patients.[7]

Mainly used for their analgesic, antipyretic and anti-inflammatory properties, the nonsteroidal anti-inflammatory drugs (NSAIDs) piroxicam and meloxicam (Scheme 1) are among the most consumed drugs over the world. Their antibacterial and *in vitro* antitumor effects towards various cancer cell lines have been demonstrated in several studies.[8-13] Beside their great potential in biomedical chemistry these molecules proved to be versatile ligands toward *d*-block metal ions.[14-22] Only very few studies about the interaction of lanthanide ions with ligands belonging to oxicam family were found in literature.[23-25]

In the present study we report on the synthesis, characterization and biological (antimicrobial and antitumor activity) evaluation of two new Gd(III) complexes containing piroxicam and meloxicam as ligands. The *in vitro* antimicrobial activity of the complexes has been evaluated against Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*), Gram-positive (*Staphylococcus aureus, Enterococcus faecalis*) bacterial and fungal strains (*Candida albicans*), both in planktonic and sessile cells, while their cytotoxicity was evaluated using the HT-29 cell line.

EXPERIMENTAL

Materials and methods

$Gd(NO_3)_3 \cdot 6H_2O$ (Strem Chemicals, France), piroxicam (Boehringer-Ingelheim, Germany), meloxicam (Alfa Aesar, Germany), triethylamine (Sigma-Aldrich, Germany), absolute ethanol (Chimreactiv, Romania) were used without further purification.

Elemental analysis were performed on a Perkin Elmer CHNS/O Analyzer 2400 Series II. Molar conductivity of 10^{-3} M solutions in DMSO was measured at 28 °C, on a Mettler Toledo SevenGo Duo SG23 conductivity meter. Infrared spectra were recorded on a Jasco FTIR 4100 spectrophotometer in wavenumber region 4000 – 400 cm^{-1} using KBr disks. Absorption spectra were recorded at room temperature with a JASCO V-670 spectrophotometer. The photoluminescence measurements were carried out at room temperature using a JASCO FP 6500 spectrofluorometer. The magnetic susceptibilities were measured on solid samples using the Faraday method. Thermal measurements were performed on a Netzch STA 449 F1 Jupiter Simultaneous Thermal Analyzer apparatus, in air, with a heating rate of 10 °C/min, from room temperature to 1100 °C.

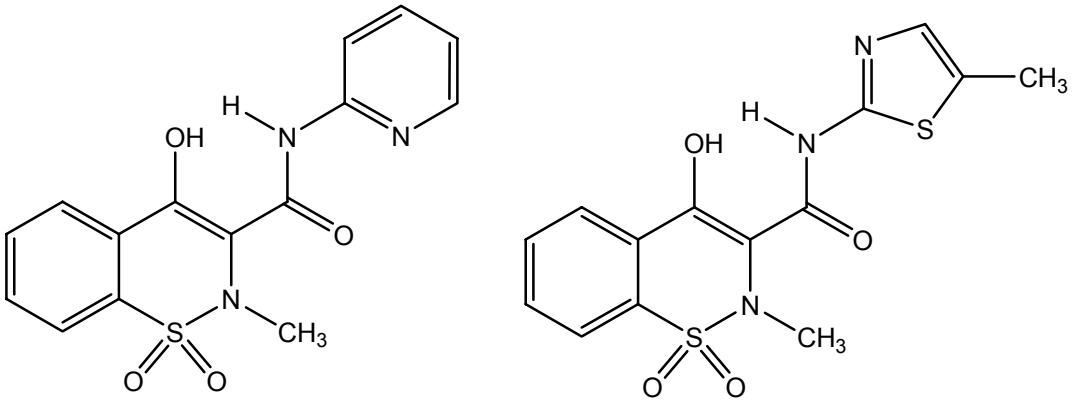
Synthesis of the complexes

The complexes were prepared according to the following procedure: a hot ethanolic solution of $Gd(NO_3)_3 \cdot 6H_2O$ (10 mL, 0.66 mmol) was added to a hot ethanolic solution of deprotonated oxicam (20 mL, 2 mmol). The mixture was refluxed under stirring for approximately 5 h. The resulting solution was concentrated using a rotary evaporator and the product was precipitated by adding diethyl ether and kept at 4 °C for few hours to complete the precipitation. The precipitate was filtered, washed three times with diethyl ether and dried in a dessicator under P_4O_{10} at room temperature.

The deprotonation of the oxicam ligands (piroxicam and meloxicam) was carried out by the addition of an equimolar amount of triethylamine to the ethanolic solution of oxicam.

Antimicrobial activity assays

The antimicrobial activity of the obtained compounds was assayed on Gram-negative (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853), and Gram-positive (*Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 29212) bacterial and fungal strains (*Candida albicans* ATCC 10231).



Scheme 1 – Structural formulas of piroxicam (H_2Pir) and meloxicam (H_2Mel).

Microbial suspensions of 1.5 x 10^8 CFU mL^{-1} (0.5 McFarland density) obtained from 15 to 18 h bacterial cultures developed on solid media were used. The compounds were suspended in DMSO to prepare a stock solution of 10 mg mL^{-1} concentration. The quantitative assay of the antimicrobial activity was performed by liquid medium microdilution method in 96 multi-well plates. Two-fold serial dilutions of the compounds solutions (ranging between 1000 μg and 2 μg mL^{-1}) were performed in a 200 μL volume of broth, and each was well seeded with 50 μL microbial inoculum. Culture positive controls (wells containing culture medium seeded with the microbial inoculum) were used. The influence of the DMSO solvent was also quantified in a series of wells containing DMSO, diluted accordingly with the dilution scheme. The plates were incubated for 24 h at 37 °C, and the minimal inhibitory concentration (MIC) values were considered as the lowest concentration of the tested compound that inhibited the growth of the microbial overnight cultures, as compared to the positive control, revealed by a decreased value of absorbance at 600 nm (Apollo LB 911 ELISA reader).[26]

For the evaluation of the influence of the tested suspensions on the ability of microbial strains to colonize the inert substratum, a microtiter plate method was used. The microplates used for the MIC assay were emptied and washed three times by phosphate buffered saline. The biofilm formed on the plastic wells wall was fixed for 5 min with cold methanol, coloured for 15 min by violet crystal solution and resuspended with a 33% acetic acid solution. The cellular density was measured by reading the optical density of the coloured solution at 490 nm. The minimal biofilm eradication concentration (MBEC) values were considered as the lowest concentration of the tested compound that inhibited the development of biofilm on the plate wells.[27,28]

Cytotoxicity assay

HT-29 (human colorectal adenocarcinoma) cell line was cultivated in RPMI 1640 (Gibco, NY, SUA) supplemented with 10% heat-inactivated bovine serum and penicillin/streptomycin at 37 °C with 5% CO_2. The adherent cells were detached, centrifuged, suspended in fresh medium, counted by trypan blue exclusion and adjusted to 5 x 10^4 cells mL^{-1} and then, were cultivated in 6-well plate and treated for 24 h with 100 μg mL^{-1} compound. After the treatment period, cells were taken from the substrate, fixed in 70 % cold ethanol for at least 30 min at -20 °C, washed twice in phosphate-buffered saline (PBS) and, then incubated 15 min at 37 °C with RNAse A (100 μg mL^{-1}), and 1 h with propidium iodide (100 μg mL^{-1}). After staining of cells with propidium iodide the acquisition was done using Epics Beckman Coulter flow cytometer. Data were analyzed using FlowJo software and expressed as fractions of cells in the different cell cycle phases.[29]

RESULTS AND DISCUSSION

Both complexes have been separated as crystalline yellow powders. The results of the elemental analysis, molar conductivity data and magnetic moments are listed in Table 1. The molar conductivity values indicated a 1:1 electrolyte nature of both coordination compounds[30], confirming the presence of the nitrate ions in the outer coordination sphere.

IR spectral studies

The IR spectra of the complexes were recorded in the region 4000 - 400 cm^{-1} and compared with those of the free ligands. By examining the IR spectra of the Gd(III) complexes it can be noticed that the strong and sharp band at 3340 (piroxicam) / 3290 cm^{-1} (meloxicam) attributed to the O-H enol group of the free ligand is markedly attenuated, supporting the partial deprotonation of this group. The band characteristic of the N-H amide group vibration at 3396 (**1**) / 3390 cm^{-1} (**2**) in complexes seems to be slightly intensified as compared with the free ligands, indicating the possible involvement of the hydrogen atom of this group in the formation of intramolecular N-H···acceptor bond with the deprotonated enolic oxygen. The characteristic amide II vibration of free piroxicam at 1529 cm^{-1} is shifted to 1505 cm^{-1} by coordination to Gd(III), while in the corresponding meloxicamato complex **2** this vibration is shifted from 1530 to 1515 cm^{-1}. The presence of the nitrate anions in the formula of the complexes is confirmed by the appearance of a new very sharp band at 1384 cm^{-1}, characteristic for this group.[31] The bands located at 1352 and 1182 cm^{-1} that are attributable to the antisymmetric and symmetric stretching vibrations of the SO_2 group remain unaltered upon complex formation, suggesting that these groups are not involved in coordination. In the absence of crystallographic data it was impossible to attribute the structural formula of the complexes. The molecular formula of the resulting complexes have been proposed on the basis of the analytical and spectroscopic data (Table 1).

Electronic spectra

The electronic spectra of the Gd(III) complexes are presented in Fig. 1. Both spectra exhibit a broad intense band between 200 – 500 nm that overlaps the band corresponding to the $\pi \rightarrow \pi^*$ transition of the ligand and the less intense and sharp bands characteristic to the transitions within the $4f^7$ configuration of the Gd^{3+} ions.[32] Gd has f^7 configuration with $^8S_{7/2}$ as the ground state and the absorption spectrum lies in the UV region and these transitions correspond to components of ^{6}PIDG multiplets[32].

Table 1

Elemental analysis and physical properties of the obtained compounds

Compound	Elemental chemical analysis % Found (Calculated)				μ_{eff} (BM)	Molar conductivity ($\mu S\ cm^{-1}$)
	C	H	N	S		
$[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$ (**1**)	44.55 (44.80)	4.05 (3.42)	10.37 (11.13)	6.74 (7.63)	7.66	76.90
$[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$ (**2**)	40.75 (40.48)	3.12 (3.59)	11.01 (10.26)	14.98 (14.01)	7.17	76.60

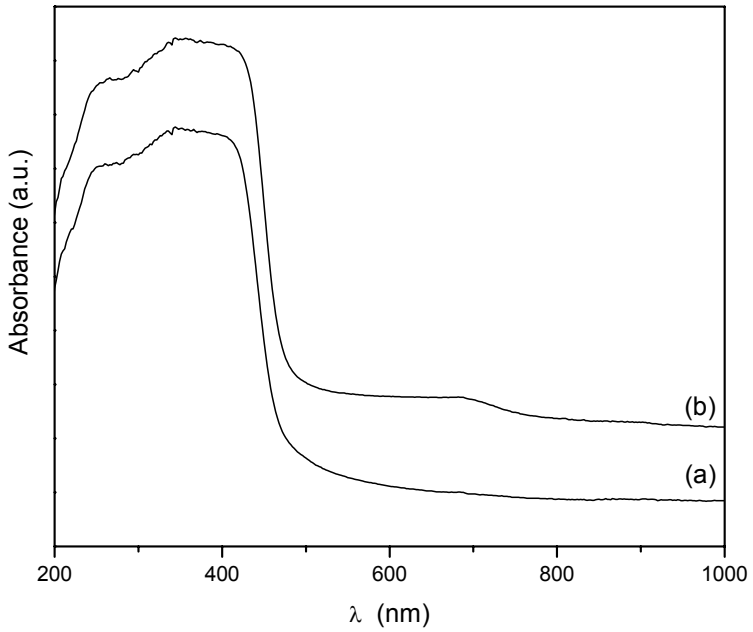


Fig. 1 – UV-Vis spectra of: a) $[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$ (**1**); b) $[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$ (**2**).

The magnetic properties of the complexes have been investigated at room temperature. The effective magnetic moments found μ = 7.66 BM for **1** and μ = 7.17 BM for **2**, are slightly lower than the calculated one (μ = 7.937 BM) corresponding to Gd^{3+} ion ($4f^7$ configuration), ground state ($^8S_{7/2}$),

$\mu \approx g_J\sqrt{J(J+1)}$,

$g_J = 1 + \frac{J(J+1)+S(S+1)-L(L+1)}{2J(J+1)}$, with $g_J = 2$, where J is the total angular momentum, g_J the Landé factor, S the total spin angular momentum and L the total orbital angular momentum, respectively, of the ground multiplet.[33]

Emission spectra

The photoluminescent properties of the complexes in solid state at room temperature have been investigated. The emission spectra of the complexes and ligands are depicted in Fig. 2. Both compounds exhibit luminescence with peak maxima at 493 nm for **1** and 495 nm for **2**, when the excitation wavelength is 350 nm. These bands are attributed to the intraligand $^1(\pi^*\text{-}\pi)$ fluorescent emission.

Thermal analysis

The thermo-gravimetric analysis for the metal complexes was carried out from ambient temperature up to 1100 °C in static air. Thermal analysis curves (TG/DSC) for both compounds are given in Fig. 3. The first step of the decomposition process occurring in the temperature range 60 - 180 °C corresponds to the loss of one ethanol molecule for the compound **1** (mass loss of 3.40%) or two ethanol molecules for **2** (mass loss of 6.63%). From the DSC curves the loss of ethanol molecules appears as endothermic peaks with peak

temperatures at 103 and 170 °C for **1** and 105 and 163 °C for **2**, respectively. After the first step, the decomposition of the complexes undergo in a series of overlapping reactions. The decomposition process associated with the complete pyrolisis of the organic parts and the oxidation of metal through carbonate and oxocarbonate into its oxide (Gd_2O_3) occurs progressively in four steps in the temperature range 180 – 1100 °C. The chemical changes accompanying the thermal decomposition are observed as exo- or endothermic peaks on the DSC curves.

Antimicrobial activity assays

In this study we have evaluated the antimicrobial activity of novel compounds against a wide array of planktonic and adherent bacterial and fungal strains, including four ESKAPE pathogens, namely *E. coli, S. aureus, P. aeruginosa* and *E. faecalis,* capable of "escaping" from the biocidal action of antibiotics due to multiple resistance mechanisms.[34] The results of the quantitative assay of the microbicidal activity of the tested compounds are summarized in Table 2.

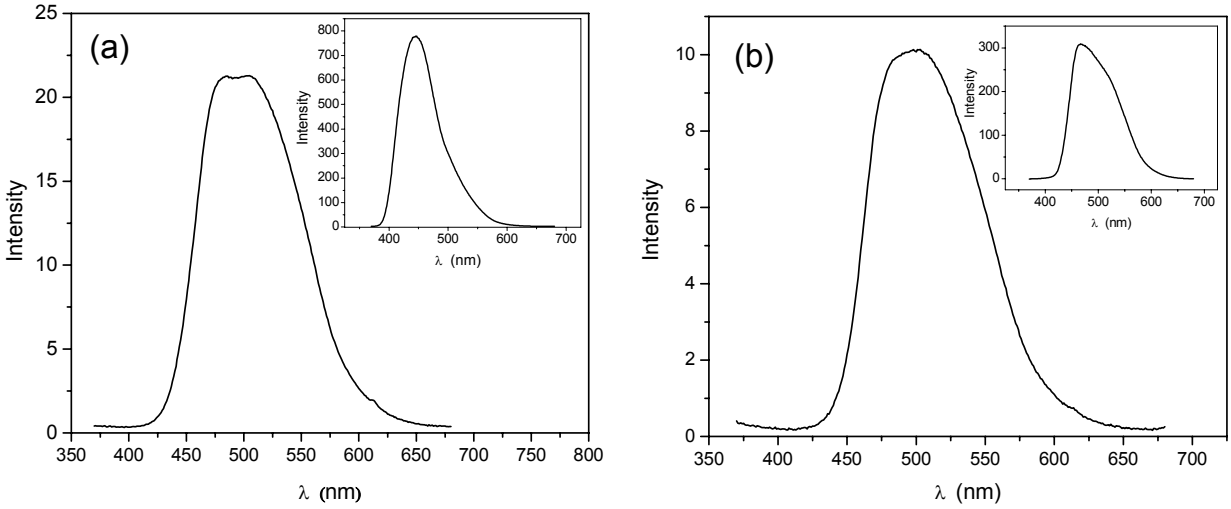


Fig. 2 – Emision spectra of: a) $[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$ (**1**);
b) $[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$ (**2**) (λ_{exc} = 350 nm) (The inset shows the emission spectra of the ligands).

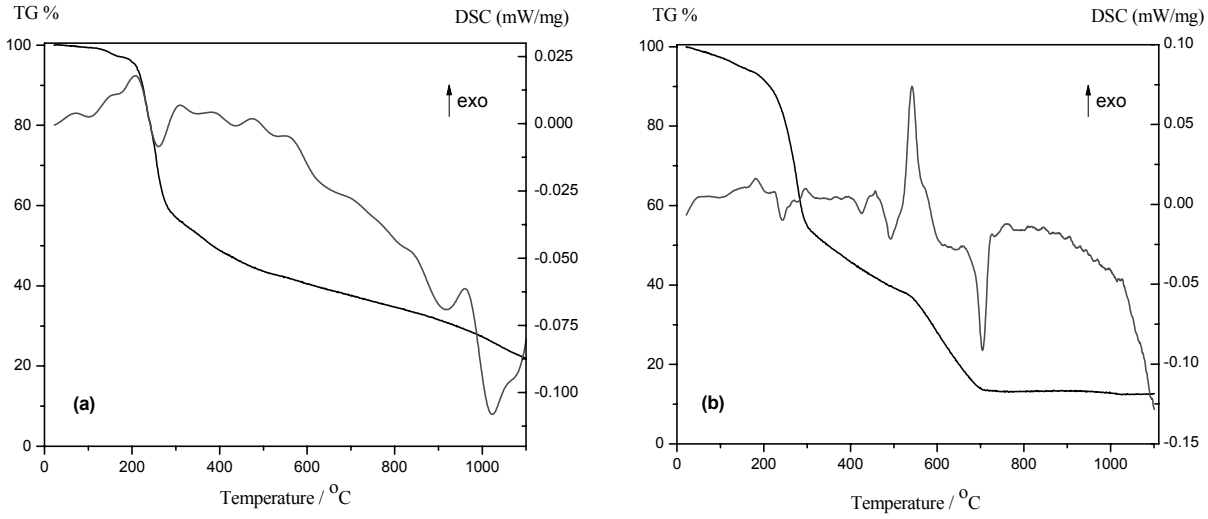


Fig. 3 – TG/DSC curves of the complexes: a) $[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$ (**1**);
b) $[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$ (**2**).

Table 2

The MIC (mg mL^{-1}) values of the tested compounds against the tested microbial strains

Compound	*Gram-positive cocci*		*Gram-negative bacilli*		
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*	*C. albicans*
$[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$	0.125	0.002	0.500	0.063	>1.000
$[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$	0.002	0.002	1.000	0.125	>1.000
H_2Pir	0.016	0.002	0.500	0.063	>1.000
H_2Mel	0.002	0.002	0.250	0.063	>1.000
DMSO	1.000	0.031	1.000	0.063	>1.000

Note: dark grey – intensive antimicrobial activity with low MIC values (<0.063 mg/mL)

A good antimicrobial activity was considered for MIC values lower than 125 mg mL^{-1}, a moderate one for MIC of 0.125-0.250 mg mL^{-1} and a low activity at MIC values higher than 250 mg mL^{-1}.[28,35] All tested compounds exhibited a better antimicrobial activity against the Gram-positive bacterial strains as compared to the Gram-negative ones, as revealed by the very low MIC values ranging from 0.002 to 0.031 mg mL^{-1} for the Gram-positive strains *versus* 0.063 mg mL^{-1} for the Gram-negative one. The most susceptible strain to all tested complexes was *E. faecalis,* while the best antimicrobial properties were expressed by H_2Pir and H_2Mel. The $[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$ (**1**) and $[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$ (**2**) complexes exhibited a narrow antimicrobial spectrum, being active only against one of two of the tested Gram-positive cocci strains. None of the tested compounds did exhibit antimicrobial activity against the tested fungal strain.

The investigation of the anti-biofilm activity of the obtained compounds revealed that the MBEC values were, as expected, much higher than the MIC ones, taking into account the increased resistance of biofilm embedded bacteria to antimicrobials and other limitative stress factors. Concerning the anti-biofilm spectrum, a different behaviour of the tested compounds as compared to their microbicidal properties (Table 3) it is to be noticed. H_2Pir and H_2Mel, followed by $[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$ inhibited the ability of *C. albicans* to form biofilms, despite their weak activity against planktonic floating cells evaluated by the MIC assay.

Cytotoxicity studies

A dose-dependent cytotoxic activity was noticed using the HT-29 cell line. At the lowest tested concentration (0.031 mg mL^{-1}) none of the tested compounds proved to be cytotoxic (Table 4), the percentages of the viable cells being 100%. At 0.063 mg mL^{-1}, three of the compounds (H_2Pir, H_2Mel and $[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$) induced a decrease of the percentage of viable cells below 80%. Starting with the concentration of 0.125 mg mL^{-1} all tested compounds exhibited different degrees of cytotoxicity on the HT-29 cells, decreasing the percent of viabile cells from 60 to 20%.

All tested compounds slighltly decreased the number of cells found in G1 and increased the percentage of cells found in the S phase. For all tested compounds an apoptosis peak was recorded on the flow cytometry histograms obtained for the HT-29 cells treated with the respective compounds for 48 h at 100 μg mL^{-1} (Fig. 4). This pro-apoptotic effect exhibited towards the HT-29 tumoral cells highlights the potential of these compounds for the development of novel anti-proliferative agents.

Table 3

MBEC (mg mL^{-1}) values of the compounds against the tested microbial strains

Compound	*Gram-positive cocci*		*Gram-negative bacilli*		
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*	*C. albicans*
$[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$	>1.000	>1.000	>1.000	>1.000	0.063
$[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$	>1.000	>1.000	>1.000	>1.000	>1.000
H_2Pir	>1.000	>1.000	>1.000	>1.000	0.002
H_2Mel	>1.000	>1.000	>1.000	>1.000	0.002
DMSO	>1.000	>1.000	>1.000	>1.000	1.000

Note: dark grey – intensive antibiofilm activity with low MBEC values (<0.063 mg/mL)

Table 4

Percentages of viable HT-29 cells, after treatment with different concentrations of the tested compounds

Compound	**Percentage of viable cells**					
	Concentration (mg mL^{-1})					
	1.000	0.500	0.250	0.125	0.063	0.031
H_2Pir	19.81	42.11	22.89	39.08	62.98	100.00
H_2Mel	42.12	27.36	30.86	49.43	64.05	100.00
$[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$	26.64	42.12	48.49	58.82	84.78	100.00
$[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$	22.85	26.73	34.92	40.56	65.39	100.00

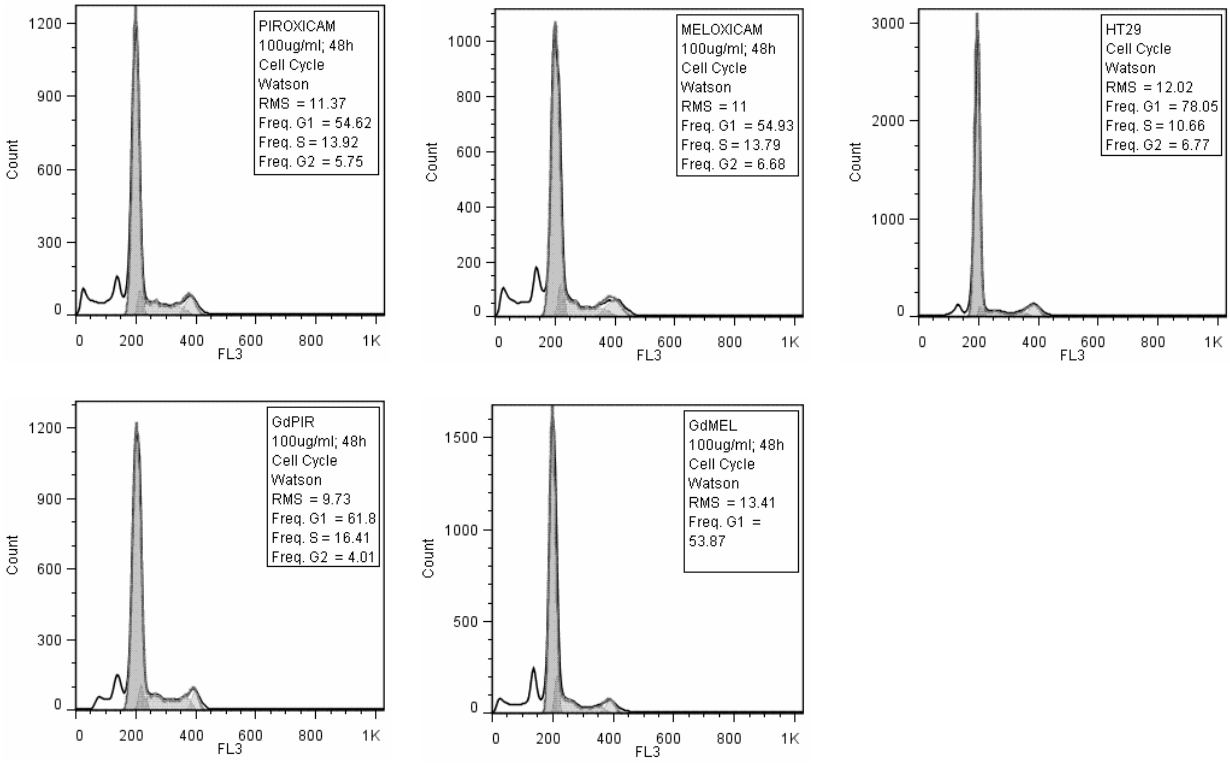


Fig. 4 – Flow cytometry histograms and percentages of HT-29 cells found in different cell cycle phases, after treatment with the different concentrations of the tested compounds.

CONCLUSIONS

In summary, two novel mononuclear Gd(III) complexes, $[Gd(H_2Pir)(HPir)_2](NO_3)\cdot C_2H_5OH$ (**1**) and $[Gd(H_2Mel)(HMel)_2](NO_3)\cdot 2C_2H_5OH$ (**2**), were synthesized and characterized by elemental analysis, FTIR, electronic and luminescence spectra, TG/DTG, molar conductivity and magnetic measurements. The results of the biological evaluation revealed the multifunctionality of these compounds and their potential for different biomedical applications. Thus, at low concentrations, inferior to 0.063 mg mL^{-1} the obtained complexes could be used for the design and development of novel biocompatible antimicrobial platforms, while at higher concentrations, the obtained complexes seem to be promising for the further development of novel anti-proliferative agents, inducing both cytotoxicity, apoptosis and changes in the tumor cell cycle dynamics.

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