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Original article

New zirconium (IV) complexes of coumarins with cytotoxic activity

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Abstract

Complexes of zirconium (IV) with some bis-coumarin ligands have been synthesized. The zirconium (IV) complexes with bis-coumarins were characterized by different physicochemical methods—elemental analysis, IR-, and ^{1}H-NMR-spectroscopies and mass spectral data. The spectral data of zirconium (IV) complexes were interpreted on the basis of comparison with the spectra of the free ligands. The results of the ligands and their complexes, based on spectral data are informative and useful for suggestion of the metal–ligand binding mode. Cytotoxic screening by MTT assay was carried out. In the present study we performed comparative evaluation of the cytotoxic effects of the three newly synthesized zirconium complexes against the acute myeloid leukemia derived HL-60 and the chronic myeloid leukemia LAMA-84. The preliminary cytotoxicity screening program revealed that the investigated zirconium complexes induced 50% inhibition of the cell viability of HL-60 and LAMA-84 cells at micromolar concentrations and thus could be considered as biologically active. Independently of the tumor test system evaluated the complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane proved superior to the remaining agents with respect to the IC_{50} values obtained. The complexes of both the other coumarins evaluated proved to be less potent than the corresponding free ligands, as evidenced by the IC_{50} values obtained. Thus the zirconium complexes with coumarin ligands represent a novel class of antiproliferative agents, which deserve further attention in search of anticancer lead compounds.

Keywords: Bis-coumarins; Zirconium (IV) complexes; IR- and NMR-spectra; Cytotoxic activity

1. Introduction

3,3′-Methylenebis[4-hydroxycoumarin] (dicumarol), the main part of investigated ligands, is a naturally occurring anticoagulant derived from coumarin, which is obtained from sweet clover (*Melilotus alba*) [1,2]. Coumarin, the parent molecule of dicumarol, and a variety of coumarin compounds have demonstrated numerous antitumor and antiproliferative effects. Coumarin compounds have been shown to inhibit proliferation of particular human malignant cell lines in vitro [3–8], as well as affecting tumor activity against several in vivo tumor types [1,9–13]. In clinical trials, these compounds have also been demonstrated to have some activity against prostate cancer, malignant melanoma, and metastatic renal cell carcinoma [14–16]. Additionally, dicumarol studies have found decreased metastases in animal models [17]. Recently it was proved that dicumarol appears to induce oxidative stress and pancreatic cancer cell cytotoxicity, as well as apoptosis in a time-dependent and dose-dependent manner [18]. Intratumoral injections of dicumarol slowed tumor growth and extended survival. Although such coumarin compounds as dicumarol have been used in cancer therapy, little is known about the mechanism of action of these drugs.

A number of coumarins have been investigated for complexing ability. A recent review summarizes advances in the field of cytotoxic properties of coumarins and their coordination complexes [9]. A lot of different coordination compounds and the mechanism of cytotoxic action have been discussed with regard to the development of new antitumor agents. It was found that in some cases the metal complexes of coumarins obtained revealed higher biological activity than their ligands [19–29].

As a result from our earlier work the cytotoxic profile of some new metal complexes of coumarin derivatives against different human tumor cell lines was proved [19–29]. The pro-

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mising results, concerning their significant cytotoxic activity, prompted us to search for new transition metal complexes with coumarin derivatives. The previous data from the literature which are in accordance with our investigations give our reason to suppose that complexes of coumarins with zirconium (IV) could present interesting metalorganic compounds with antitumor activity.

Metallocene-diacido complexes containing transition metals, such as titanium, vanadium, niobium, zirconium, and molybdenum, exhibit variable antitumor activity for a wide spectrum of murine and human tumors with reduced toxicity when compared with cisplatin [30–34].

Unfortunately, little is known about the complexing ability of zirconium (IV) with coumarins. Zirconium complexes of mendiaxon, warfarin, coumachlor, and niffcoumar have been synthesized by us recently [19]. Cytotoxic screening by MTT assay was carried out and cytotoxic activity against human promyelocytic leukemic HL-60 cells was proved.

A survey of the literature reveals that no work has been done on the reactions of zirconium (IV) with bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane, bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane and bis (4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane. It was, therefore, considered worthwhile to study the complexation and in the first place the objective of this study was to determine whether the new complexes were active as cytotoxic agents.

In the present study we perform investigation of the coordination ability of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane, bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane in complexation reaction with zirconium (IV). The obtained zirconium (IV) complexes with these coumarin ligands were characterized by elemental analysis, physicochemical methods, mass-, NMR- and IR-spectroscopy. The complicated vibrational spectra of zirconium (IV) complexes were interpreted on the basis of comparison with the vibrational spectra of the free ligands. The most sensitive to coordination modes of the ligands have been assigned and discussed.

In a systematic effort aimed at identifying new cytotoxic agents with potent activity against cancer cells, we examined the cytotoxic effects of zirconium (IV) complexes with coumarins on the human cancer cell lines, chronic myeloid leukemia LAMA-84 and the acute promyelocyte leukemia HL-60. We observed that zirconium (IV) possess a cytotoxic activity [19] and literature data show that the coumarins have also these properties. That is why our synthesis of complexes of Zr (IV) is taken into consideration with cytotoxic screening and further pharmacological study.

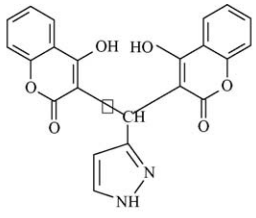
Our results presented herein provide evidence that zirconium (IV) complexes with these coumarin ligands possess cytotoxic activity in human chronic myeloid leukemia LAMA-84 and the acute promyelocyte leukemia HL-60 cells. To our knowledge, this is the first report on the antitumor effects of such kind of zirconium (IV) complexes against human cancer cells.

2. Chemistry

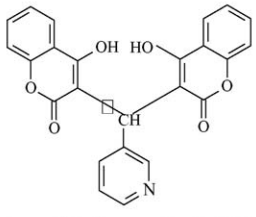
The compounds used for preparing the solutions were Merck products, p.a. grade: $ZrCl_4$. Bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane, bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane were used for the preparation of metal complexes as ligands (Scheme 1).

The complexes of zirconium (IV) with bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (H_2L1), bis (4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (H_2L2) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (H_2L3) were synthesized by reaction of zirconium (IV) salt and the ligand, in amounts equal to metal/ligand molar ratio of 1:1. The complexes were prepared by adding an aqueous solution of zirconium (IV) salt to an aqueous solution of the ligand subsequently raising the pH of the mixture gradually to ca. 5.0 by adding dilute solution of sodium hydroxide. The reaction mixtures were stirred with an electromagnetic stirrer at 25 °C for 1 hour. At the moment of mixing of the solutions, precipitates were obtained. The precipitates were filtered, washed several times with water and dried in a desiccator to constant weight.

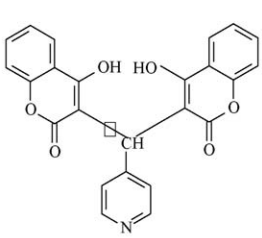
The complexes were insoluble in water, slightly soluble in methanol and ethanol and good soluble in DMSO.



H_2L1= bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (H_2L1)



H_2L2= bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (H_2L2)



H_2L3= bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (H_2L3)

Scheme 1. Structures of the ligands.

3. Pharmacology

In the present study we investigated the cytotoxic effects of the three newly synthesized zirconium complexes, the corresponding free ligands and the zirconium salt $ZrCl_4$ against the human chronic myeloid leukemia LAMA-84 and the acute promyelocyte leukemia HL-60 cells using the standard MTT-dye reduction assay for cell viability.

4. Results and discussion

4.1. Chemistry

The complexes were characterized by elemental analysis. The metal ion was determined after mineralization. The water content in the complexes was determined by Karl Fisher analysis. The formation of the complexes was confirmed by IR-spectroscopy, ^{1}H-NMR-spectroscopy and mass spectral data.

Table 1 shows the data of the elemental analysis of the complexes serving as a basis for the determination of their empirical formulae. The elemental analysis data of the Zr (IV) complexes obtained are in agreement with the presented formulas.

The suggested formulas were further confirmed by mass spectral fragmentation analysis. As it is seen from Table 2, the first peaks in the Zr (IV) complexes spectra (although with low intensity) correspond to the mass-weight of the complex formation and the next ones to that of the ligands. The results thus obtained are in agreement with metal/ligand ratio 1:1 in the investigated complexes. The data of mass spectral fragmentation of the ligands and of the complexes are presented in Table 2.

Table 1
Elemental analysis data for Zr (IV) complexes with bis-coumarins

		Found/calculated				
Complex	Yields (%)	% C	% H	% N	% H_2O	% Zr
$Zr(L1)(OH)_2{\cdot}3H_2O$	73	45.15	3.13	4.63	9.44	15.36
		45.59	3.45	4.83	9.33	15.72
$Zr(L2)(OH)_2{\cdot}3H_2O$	84	48.38	3.09	2.27	8.85	15.05
		48.81	3.55	2.37	9.15	15.42
$Zr(L3)(OH)_2{\cdot}4H_2O$	89	46.92	3.35	2.08	11.35	14.58
		47.36	3.78	2.30	11.84	14.97

L_1: $C_{22}H_{12}N_2O_6^{2-}$; L_2: $C_{24}H_{13}NO_6^{2-}$; L_3: $C_{24}H_{13}NO_6^{2-}$.

Table 2
Mass spectral data of bis-coumarins and their Zr (IV) complexes

Ligand	*m/z*	(%)	Complex	*m/z*	(%)
$H_2L1=C_{22}H_{14}N_2O_6$	402	7	$Zr(L1)(OH)_2{\cdot}3H_2O$	579	1
	395	2		490	3
	252	7		460	5
	162	30		402	2
	120	28		307	80
	92	38		176	100
$H_2L2=C_{24}H_{15}NO_6$	413	7	$Zr(L2)(OH)_2{\cdot}3H_2O$	590	1
	395	2		460	2
	252	30		436	42
	162	62		410	23
	120	74		307	55
	92	86		176	40
$H_2L3=C_{24}H_{15}NO_6$	413	0	$Zr(L3)(OH)_2{\cdot}4H_2O$	608	1
	252	18		490	2
	250	50		460	5
	162	62		410	42
	120	74		307	48
	92	86		176	100

4.2. IR spectra of the complexes

The mode of bonding of the ligands to Zr (IV) was elucidated by recording the IR spectra of the complexes as compared with this of the free ligands.

IR-spectra of the compounds were recorded on solid state in Nujol in the range 3800–400 cm^{-1}. The data of the IR spectra of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (H_2L1), bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (H_2L2) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (H_2L3) and of the zirconium complexes with these ligands are presented in Table 3.

A broad band, characteristic of ν_{OH} of coordinated water was observed in the range 3300–3450 cm^{-1} in the spectra of the complexes. A comparison of the infrared spectra of the ligands and of the respective complexes reveals the disappearance of absorption bands associated with the stretching and deformation OH of the phenolic groups, indicating the loss of phenolic protons on complexation, thus forming a metal–oxygen bonds which appear as bands in the far IR region. The $\nu_{C=O}$ bands exhibit shifts of 20–30 cm^{-1} to lower wavenumber values on complexation which may be taken as suggestion for the participation of the C=O groups in coordination. The C–C and C–O stretch and the C–O–C band are all shifted in the complexes. Similar frequency shifts are observed for the other complexes and are attributed to complexation of the positive ion with the carbonyl oxygen [35]. IR-spectra of the compounds were recorded on solid state in Nujol in the range 700–220 cm^{-1}. The spectra of the complexes showed new bands in comparison with these of the free ligands and these bands have been assigned to the rocking, waggling and metal–oxygen stretching vibrations.

4.3. ^{1}H-NMR spectra of the ligands and their Zr (IV) complexes

Metal ion coordination with ligand by means of the deprotonated hydroxyl groups was shown owing to data of ^{1}H-NMR spectra.

Proton spectra of the compounds recorded at 250 MHz in DMSO-d_6, confirmed the formation of the complex. The typical chemical shifts of the ^{1}H-NMR spectra in DMSO-d_6 are presented in Table 4. As it is seen from Table 4 and Fig. 1, different chemical shifts were observed in the complexes and these changes were attributed to coordination of the ligands to Zr (IV).

Due to electron transfer from the hydroxyl and carbonyl oxygen atoms to Zr (IV), a difference in chemical shifts was observed for the neighboring protons and they confirmed the

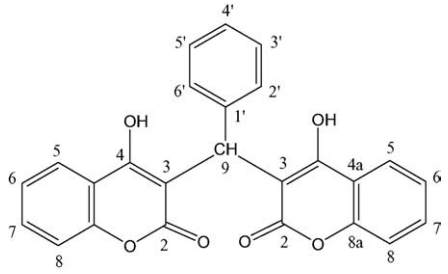
Table 3
Selected experimental IR frequencies of the ligands and their Zr (IV) complexes (cm^{-1})

Compound	νOH/H_2O	ν(C=O)	ν(C=C)	ν(Py)	ν(Ar)	δ(COH)		ν(C–O)	
$H_2L1=C_{22}H_{14}N_2O_6$	3139m	1669s	1610s	1620	1496m	1360m		1187m	770
	3070m	1635s	1539s	1559		1300m		1150m	748
				1507				1110s	
				1417				1044m	
$Zr(L1)(OH)_2{\cdot}3H_2O$	3377br	1652sh	1545s	1622	1436m	–		1196w	759
		1609s		1558				1150w	
				1506				1109m	
				1412				1062w	
$H_2L2=C_{24}H_{15}NO_6$	3070m	1687s	1610s	1620	1491m		1350m	1176m	807
	3051m	1614s	1536s	1560			1329m	1120m	753
				1506				1104s	
				1409				1050m	
$Zr(L2)(OH)_2{\cdot}3H_2O$	3418br	1674sh	1520s	1616	1460m		–	1181w	760
		1600s		1557				1120w	
				1506				1109m	
				1417				1069w	
$H_2L3=C_{24}H_{15}NO_6$	3180m	1699s	1610s	1620	1498m		1340m	1181m	770
	3120m	1635s	1538s	1558			1315m	1155m	750
				1520				1107s	
				1405				1037m	
$Zr(L3)(OH)_2{\cdot}4H_2O$	3434br	1674sh	1545s	1622	1460m		–	1180w	760
		1600s		1557				1149w	
				1515				1109m	
				1410				1075w	

[a] br: broad; s: strong; m-medium; sh: shoulder; w: weak.

Table 4
^{1}H-NMR spectral shifts, δ (ppm) of the ligands and their Zr (IV) complexes (250 MHz, DMSO-d_6)

Compound	δ (ppm)		
	H_5–H_8	H_9	$H_{2'}$–$H_{6'}$
$H_2L1=C_{22}H_{14}N_2O_6$	7.23–7.55	6.36	7.83–8.15
$Zr(L1)(OH)_2{\cdot}3H_2O$	7.19–7.78	6.05	8.03–8.18
$H_2L2=C_{24}H_{15}NO_6$	7.22–7.57	6.42	7.79–8.70
$Zr(L2)(OH)_2{\cdot}3H_2O$	7.25–7.63	6.36	7.98–8.50
$H_2L3=C_{24}H_{15}NO_6$	7.22–7.58	6.46	7.80–8.68
$Zr(L3)(OH)_2{\cdot}4H_2O$	7.25–7.52	6.35	7.83–8.49



expected coordination of the ligand through both deprotonated hydroxyl groups.

On the basis of the results thus obtained, it was suggested that the ligands act as tetradentate ones in the Zr (IV) complex formation.

4.4. Pharmacology

4.4.1. In vitro cytotoxicity

The cytotoxic effects of the three newly synthesized zirconium complexes of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (Z-1), bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (Z-2) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (Z-3) against the human chronic myeloid leukemia LAMA-84 and the acute promyelocyte leukemia HL-60 cells were determined using the standard MTT-dye reduction assay for cell viability. The cytotoxic potential of the free ligands, as well as of the zirconium chloride was evaluated as well. The retrieved MTT-formazan absorption values are summarized in Tables 5–8.

The 72 h exposure of both cell lines with the tested compounds resulted in a concentration-dependent reduction of cell viability as assessed by the MTT-dye reduction assay, which enabled the construction of concentration–response curves (Figs. 2–15). In addition the corresponding IC_{50} values were derived in order to allow a quantitative merit for assessment of the relative potencies of the agents under investigation (Table 9).

All of the novel zirconium complexes exerted profound cytotoxic activity upon the human promyelocyte leukemia HL-60, inducing practically total eradication of the malignant cell population at concentrations above 300 μM (Figs. 2–4). In terms of relative potency the zirconium complexes of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane, complex Z-1 (IC_{50} value of 55.4 μM) proved to be superior to the other two complexes, which induced half-maximal inhibition of the malignant cell viability at approximately twofold higher concentrations (Table 9). Interestingly the free ligands were also found to be cytotoxic and in case of L_2 and L_3 they actually outclassed the corresponding complexes (Figs. 5 and 6). The ligand of the most potent complex, however exerted only marginal effects, and thus the complexation of L_1 proved beneficial in terms of antileukemic activity in vitro (Fig. 7).

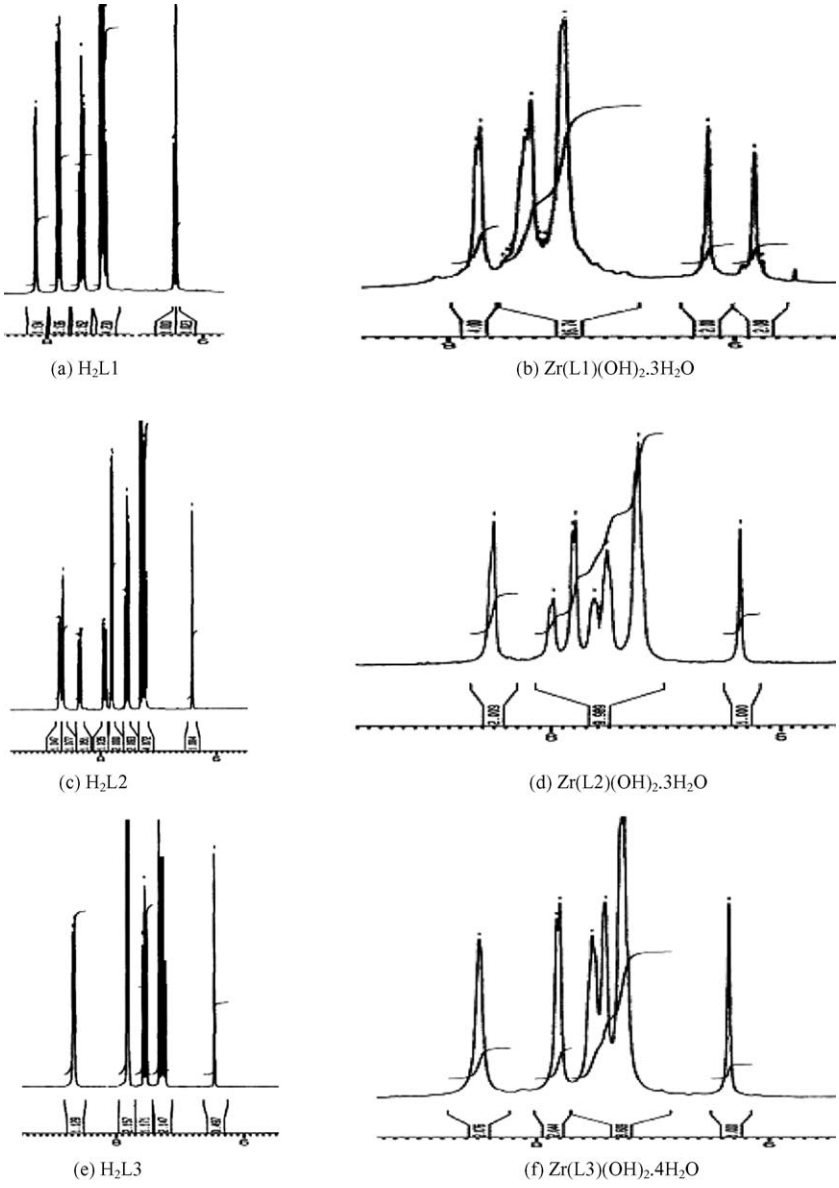


Fig. 1. NMR spectra of the ligands and their complexes.

The metal salt also exerted cytotoxic effects with an IC_{50} value of 177.2 μM (Fig. 8).

Table 5
Spectrophotometrical data from the MTT assay concerning the cytotoxic effects of the newly synthesized zirconium complexes of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (Z-1), bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (Z-2) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (Z-3) on HL-60 leukemic cells

Concentration (μM)	MTT-formazan absorption (580 nm)		
	Z-1	Z2	Z-3
0	0.867 ± 0.030	0.867 ± 0.030	0.867 ± 0.030
100	0.067 ± 0.006	0.478 ± 0.016	0.559 ± 0.049
200	0.013 ± 0.019	0.048 ± 0.019	0.072 ± 0.014
300	0.00	0.040 ± 0.0240	0.044 ± 0.010
400	0.00	0.021 ± 0.005	0.027 ± 0.009
500	0.00	0.006 ± 0.003	0.023 ± 0.008

Each value represents the arithmetic mean ± standard deviation of at least six independent experiments.

The evaluation of the cytotoxic activity of the novel zirconium compounds and the coumarin ligands in LAMA-84 cells revealed that generally this cell line was more responsive to these agents than the acute leukemia HL-60 (Figs. 9–11). Thus the zirconium complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane, complex Z-1, again outclassed the remaining two agents in terms of relative potency, while its ligand showed a low cytotoxic potential. The complex compound proved to exert superior maximal efficiency inducing practically total eradication of LAMA-84 cells at the highest concentration.

In both Z-2 and Z-3 the complexation again proved to be detrimental for the cytotoxic effects, since both free ligands were more potent on molar basis than the corresponding coordination compounds (Table 9). The zirconium salt exerted the weakest cytotoxic effect upon LAMA-84 with an IC_{50} value of 151.29 μM (Fig. 15).

Table 6
Spectrophotometrical data from the MTT assay concerning the cytotoxic effects of ZrC_4 and the bis-coumarin ligands bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (L_1) bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (L_2) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (L_3) on HL-60 leukemic cells

Concentration (μM)	MTT-formazan absorption (580 nm)			
	$ZrCl_4$	L_1	L_2	L_3
0	0.589 ± 0.043	0.589 ± 0.043	0.589 ± 0.043	0.589 ± 0.043
100	0.426 ± 0.036	0.482 ± 0.031	0.245 ± 0.012	0.200 ± 0.022
200	0.254 ± 0.028	0.362 ± 0.029	0.167 ± 0.019	0.155 ± 0.025
300	0.230 ± 0.035	0.123 ± 0.022	0.066 ± 0.035	0.071 ± 0.003
400	0.168 ± 0.024	0.073 ± 0.016	0.034 ± 0.018	0.019 ± 0.007
500	20.55 ± 0.009	0.0039 ± 0.019	0.011 ± 0.010	0.00

Each value represents the arithmetic mean ± standard deviation of at least six independent experiments.

Table 7
Spectrophotometrical data from the MTT assay concerning the cytotoxic effects of the newly synthesized zirconium complexes of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (Z-1), bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (Z-2) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (Z-3) on LAMA-84 leukemic cells

Concentration (μm)	MTT-formazan absorption (580 nm)		
	Z-1	Z-2	Z-3
0	0.965 ± 0.030	0.965 ± 0.030	0.965 ± 0.030
100	0.206 ± 0.030	0.584 ± 0.041	0.727 ± 0.024
200	0.074 ± 0.018	0.137 ± 0.017	0.110 ± 0.008
300	0.039 ± 0.009	0.107 ± 0.007	0.121 ± 0.023
400	0.028 ± 0.018	0.092 ± 0.007	0.109 ± 0.028
500	0.007 ± 0.006	0.085 ± 0.011	0.024 ± 0.015

Each value represents the arithmetic mean ± standard deviation of at least six independent experiments.

Table 8
Spectrophotometrical data from the MTT assay concerning the cytotoxic effects of ZrC_4 and the bis-coumarin ligands bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (L_1) bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (L_2) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (L_3) on LAMA-84 leukemic cells

Concentration (μM)	MTT-formazan absorption (580 nm)			
	$ZrCl_4$	L_1	L_2	L_3
0	1.012 ± 0.061	1.012 ± 0.061	1.012 ± 0.061	1.012 ± 0.061
100	0.653 ± 0.029	0.655 ± 0.057	0.487 ± 0.038	0.378 ± 0.024
200	0.382 ± 0.028	0.109 ± 0.051	0.00	0.00
300	0.189 ± 0.027	0.00	0.00	0.00
400	0.019 ± 0.022	0.00	0.00	0.00
500	0.00	0.00	0.00	0.00

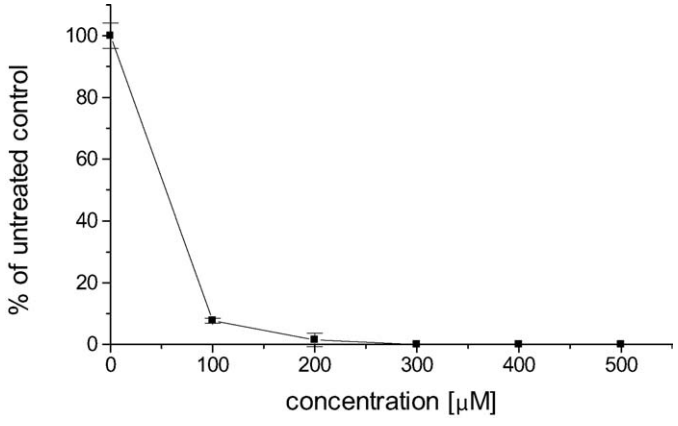


Fig. 2. Cytotoxic effects of the tested zirconium complex Z-1 against the human promyelocyte leukemia HL-60 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least six independent experiments.

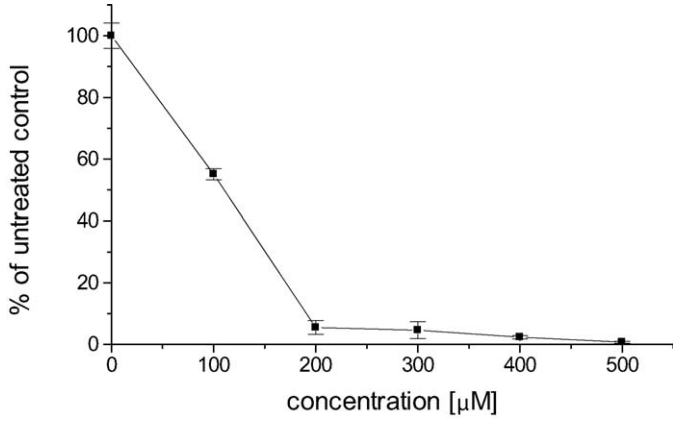


Fig. 3. Cytotoxic effects of the tested zirconium complex Z-2 against the human promyelocyte leukemia HL-60 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least six independent experiments.

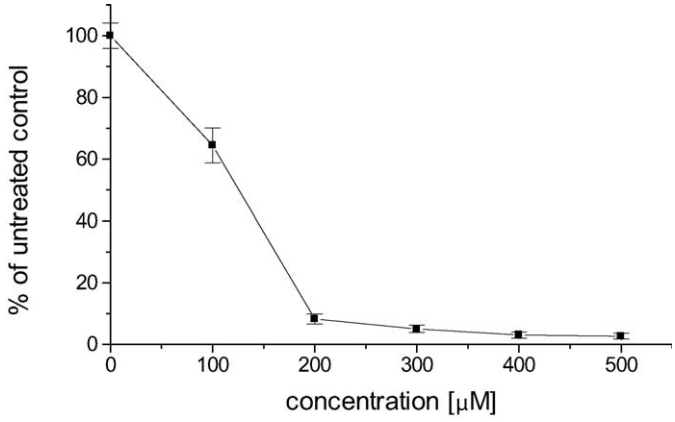


Fig. 4. Cytotoxic effects of the tested zirconium complex Z-3 against the human promyelocyte leukemia HL-60 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least six independent experiments.

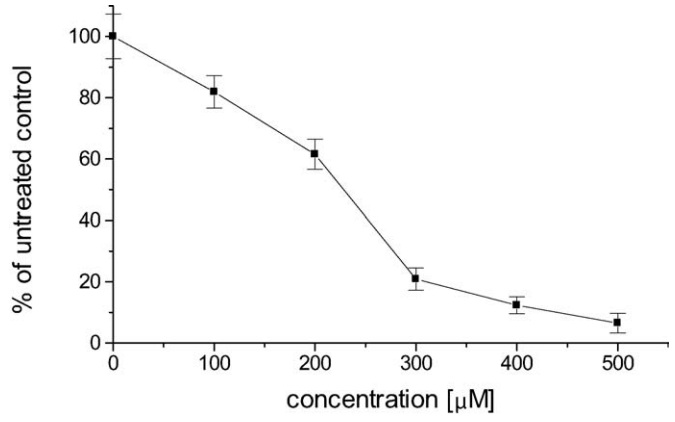


Fig. 5. Cytotoxic effects of L_1 against the acute myeloid leukemia HL-60 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least four independent experiments.

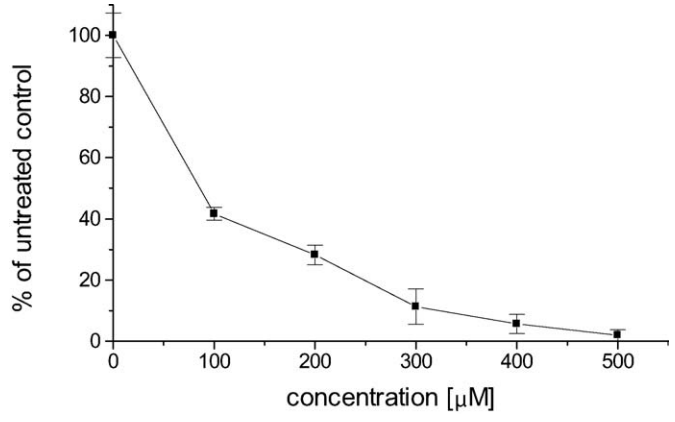


Fig. 6. Cytotoxic effects of L_2 against the acute myeloid leukemia HL-60 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least four independent experiments.

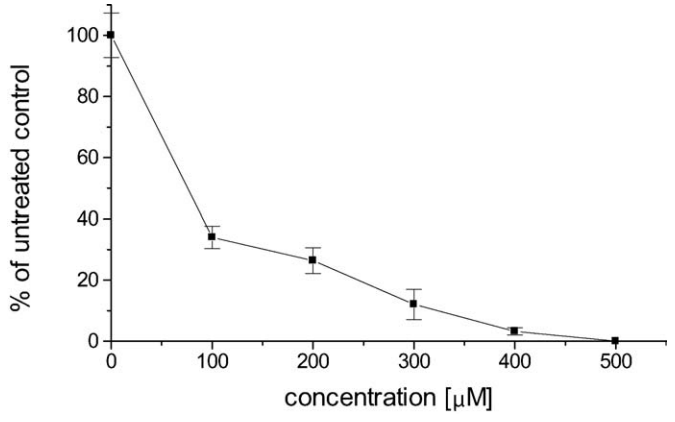


Fig. 7. Cytotoxic effects of L_3 against the acute myeloid leukemia HL-60 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least four independent experiments.

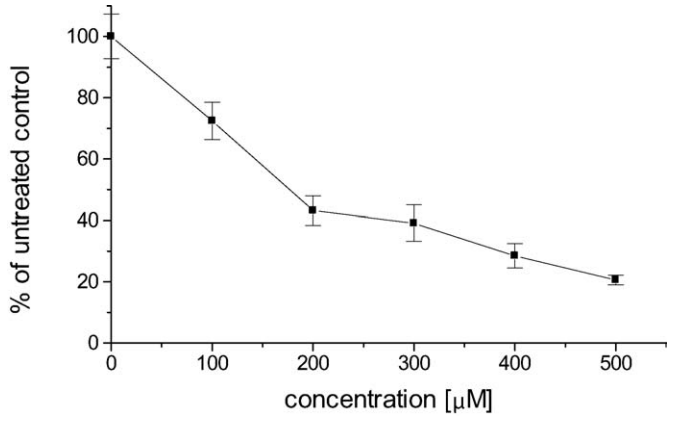


Fig. 8. Cytotoxic effects of $ZrCl_4$ against the acute myeloid leukemia HL-60 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least four independent experiments.

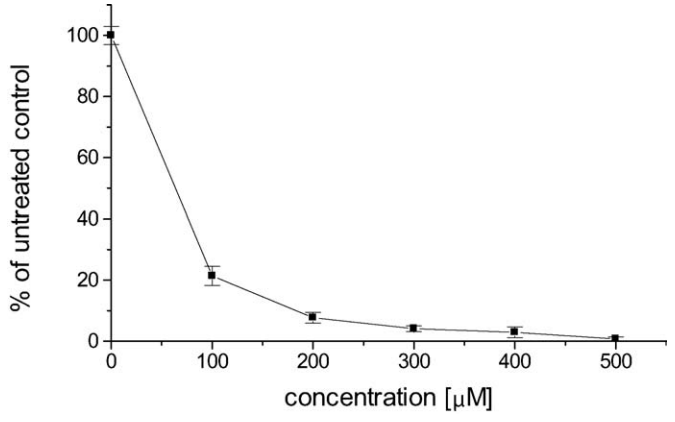


Fig. 9. Cytotoxic effects of the tested zirconium complex Z-1 against the chronic myeloid leukemia LAMA-84 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least six independent experiments.

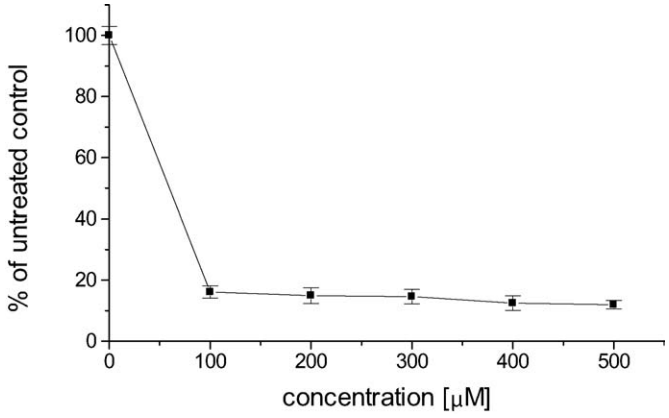


Fig. 10. Cytotoxic effects of the tested zirconium complex Z-2 against the chronic myeloid leukemia LAMA-84 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least six independent experiments.

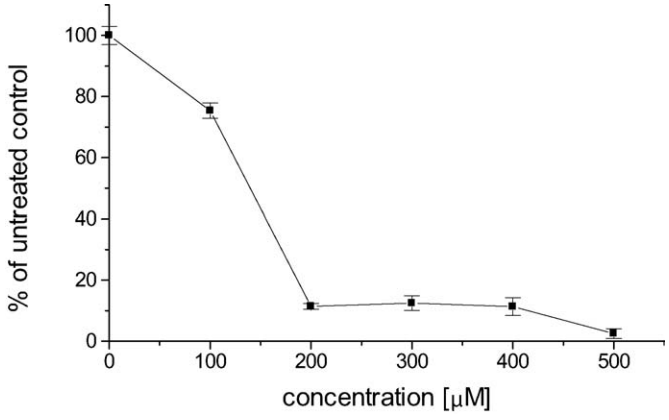


Fig. 11. Cytotoxic effects of the tested zirconium complex Z-3 against the chronic myeloid leukemia LAMA-84 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least six independent experiments.

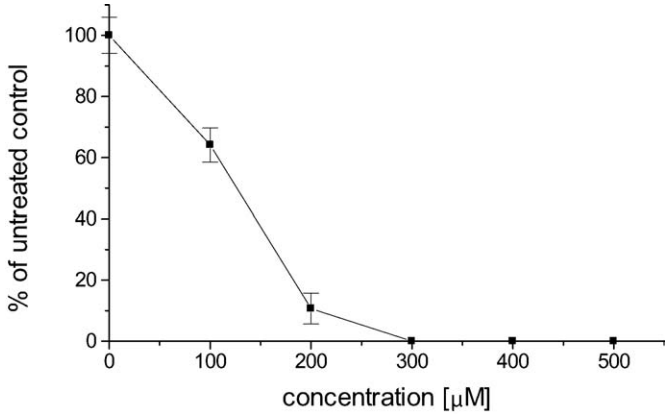


Fig. 12. Cytotoxic effects of L_1 against the chronic myeloid leukemia LAMA-84 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least four independent experiments.

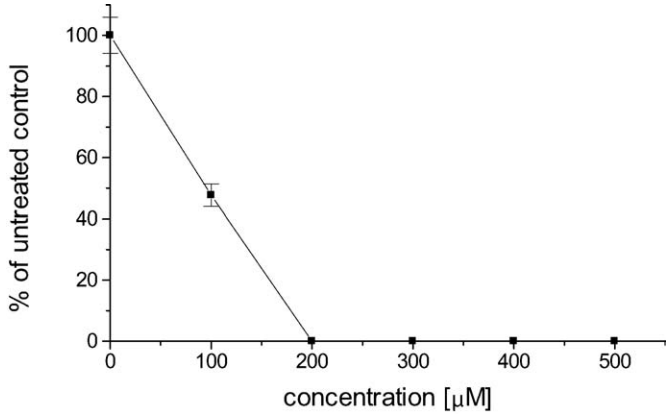


Fig. 13. Cytotoxic effects of L_2 against the chronic myeloid leukemia LAMA-84 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least four independent experiments.

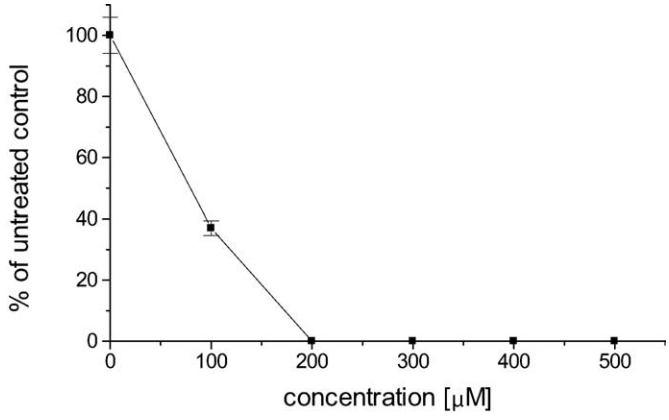


Fig. 14. Cytotoxic effects of L_2 against the chronic myeloid leukemia LAMA-84 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least four independent experiments.

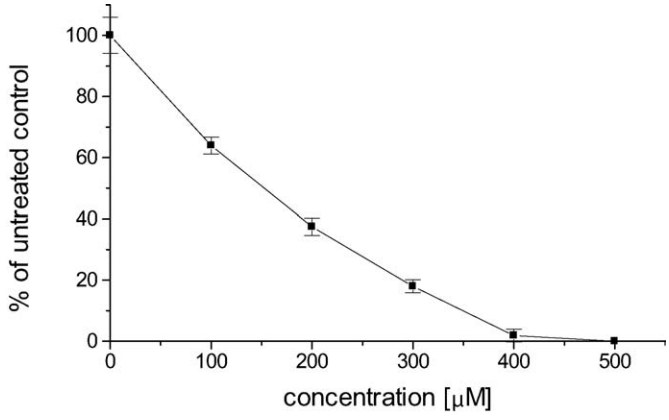


Fig. 15. Cytotoxic effects of $ZrCl_4$ against the chronic myeloid leukemia LAMA-84 after 72 h exposure as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean ± standard deviation of at least four independent experiments.

Table 9
Relative potency of the compounds after 72 h exposure (MTT-dye reduction assay)

Compound	IC_{50} value (μM)	
	LAMA-84	HL-60
$ZrCl_4$	151.29	177.2
L_1	126.04	255.5
L_2	92.84	85.8
L_3	78.96	75.5
Z-1	63.7	54.4
Z-2	122.6	110.1
Z-3	139.7	125.6

5. Conclusions

The coordination ability of the ligands has been proved in complexation reaction with zirconium (IV) ion. The elemental analysis and mass spectral data confirmed the compositions of the compounds. ^{1}H-NMR- and IR-spectral analysis of the ligands and their Zr (IV) complexes confirmed the suggested coordination of the ligands through the hydroxyl groups.

The preliminary cytotoxicity screening program revealed that the investigated zirconium complexes induced 50% inhibition of the cell viability of HL-60 and LAMA-84 cells at micromolar concentrations and thus could be considered as biologically active. Thus the zirconium complexes with coumarin ligands represent a novel class of antiproliferative agents, which deserve further attention in search of anticancer lead compounds. Independently of the tumor test system evaluated the complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane proved superior to the remaining agents with respect to the IC_{50} values obtained. The complexes of both the other coumarins evaluated proved to be less potent than the corresponding free ligands, as evidenced by the IC_{50} values obtained. According to our expectations the complexes of zirconium (IV) possess a cytotoxic activity and their in vitro effects are clearly expressed. These results confirmed our previous observations on the cytotoxicity of zirconium (IV) complexes.

6. Experimental protocols

6.1. Chemistry

The carbon, hydrogen and nitrogen contents of the compounds were determined by elemental analysis.

The water content was determined by Metrohn Herizall E55 Karl Fisher Titrator.

IR spectra (Nujol) were recorded on an IR-spectrometer FTIR-8101M Shimadzu (3800–400 cm^{-1}) and on a IR-spectrometer Perkin–Elmer GX Auto image system (700–200 cm^{-1}).

^{1}H-NMR spectra were recorded at room temperature on Brucker WP 250 (250 MHz) spectrometer in DMSO-d_6. Chemical shifts are given in ppm.

Mass spectra were recorded on a Jeol JMS D 300 double focusing mass spectrometer coupled to a JMA 2000 data system. The compounds were introduced by direct inlet probe, heated from 50 to 400 °C at a rate of 100 °C min^{-1}. The ionization current was 300 mA, the accelerating voltage 3 kV and the chamber temperature 150 °C.

6.1.1. General method of synthesis

The complexes of zirconium (IV) with bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (H_2L1), bis (4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (H_2L2) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (H_2L3) were synthesized by reaction of zirconium (IV) salt and the ligand, in amounts equal to metal/ligand molar ratio of 1:1. The complexes were prepared by adding an aqueous solution of zirconium (IV) salt to an aqueous solution of the ligand subsequently raising the pH of the mixture gradually to ca. 5.0 by adding dilute solution of sodium hydroxide. The reaction mixtures were stirred with an electromagnetic stirrer at 25 °C for 1 hour. At the moment of mixing of the solutions, precipitates were obtained. The precipitates were filtered, washed several times with water and dried in a desiccator to constant weight.

6.2. Pharmacology

6.2.1. Cell culture maintenance, drug solutions and treatment

The cytotoxic activity of the tested zirconium complexes was evaluated against the human chronic myeloid leukemia LAMA-84 and the acute promyelocyte leukemia HL-60 cells. LAMA-84 cells are distinguished by lower responsiveness to cytotoxic drugs because of the strong expression of the fusion oncoprotein BCR-ABL, a constitutive non-receptor tyrosin-kynase, which renders the cells less responsive to pro-apoptotic stimuli. The cell lines exploited herein were supplied from the German Collection of Microorganisms and Cell Cultures. They were maintained as suspension-type cultures in a controlled environment (RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine, at 37 °C in a 'Heraeus' incubator with 5% CO_2 humidified atmosphere). In order to maintain the cells in log phase cellular suspension aliquots were re-fed with fresh RPMI-1640 medium two or three times per week. The stock solutions of the tested compounds were freshly prepared in DMSO and consequently diluted in RPMI-1640. At the final dilutions obtained the concentration of the solvent never exceeded 0.5%.

6.2.2. Cell viability determination (MTT assay)

The MTT-dye reduction assay was carried out as described by Mosmann [36] with some modifications. Briefly, 100 μl aliquots of cell suspension (1×10^5 cells per ml) were seeded in 96-well microplates. Following 24 h incubation at 37 °C the cells were exposed either to the newly isolated lignan or to etoposide for 72 h. After the incubation period MTT solution (10 mg ml^{-1} in PBS) was added (10 μl per well) and the plates were further incubated for 4 h at 37 °C. Thereafter the formazan crystals formed were dissolved through addition of 100 μl per well 5% formic acid in 2-propanol (Merck) and the absorption of the samples was measured with an ELISA reader (Uniscan Titertec) at 580 nm. Hundred microliters of RPMI 1640

medium (Sigma), 10 μl MTT stock and 100 μl 5% formic acid in 2-propanol served as a blank solution. The results were expressed as survival fraction (% of untreated control).

6.2.3. Statistics

The data processing included the Student's *t*-test with $P \leq 0.05$ taken as significance level, using Microsoft EXCEL for PC.

Acknowledgements

Financial support from Medical Science Council at the Medical University, Sofia through Project No. 21/2003 is acknowledged. The ligands were synthesized by Doc. I. Manolov and were published by us recently [26].

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