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Short communication

New cerium(III) complexes of coumarins – Synthesis, characterization and cytotoxicity evaluation

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Abstract

Complexes of cerium(III) with bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane, bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-yl-methane and bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-4-yl-methane were synthesized by reaction of cerium(III) salt and the ligands, in amounts equal to metal–ligand molar ratio of 1:2. The cerium(III) complexes with bis-coumarins were characterized by different physicochemical methods – elemental analysis, IR-, Raman-, ^{1}H NMR- and ^{13}C NMR-spectroscopy and mass-spectral data. The spectral data of cerium(III) complexes were interpreted on the basis of comparison with the spectra of the free ligands. This analysis showed that in the Ce(III) complexes the ligands coordinated to the metal ion through both deprotonated hydroxyl groups. On the basis of the ν(C=O) red shift observed, participation of the carbonyl groups in the coordination to the metal ion was also suggested. 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 cerium complexes against the acute myeloid leukemia derived HL-60 and the chronic myeloid leukemia (CML)-derived BV-173. In addition the cytotoxic effects of Ce(III) complex with bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane were evaluated on the SKW-3 cells. In order to elucidate some of the mechanistic aspects of the observed cytotoxic effects we evaluated the ability of this complex to trigger programmed cell death (apoptosis by means of agarose gel electrophoretic analysis of DNA, isolated from the cytosolic fraction of treated SKW-3 cells). In addition, microscopic morphological evaluation of the treated cells was carried out in order to establish morphological features indicative of programmed cell death.

Keywords: Coumarins; Ce(III) complexes; IR-, Raman- and NMR-spectra; Cytotoxic activity; DNA

1. Introduction

Coumarins, both naturally occurring as well as synthetic derivatives, have found widespread applications as anticoagulant, spasmolytic and bacteriostatic agents [1–4]. A series of coumarin derivatives were recently synthesized and investigated as to their structure and their anticoagulant and cytotoxic activity [5–9]. A number of coumarins have been investigated for complexing ability [10,11].

A recent review summarizes advances in the field of cytotoxic properties of coumarins and their coordination complexes [12]. A lot of different coordination compounds and the mechanism of cytotoxic action have been discussed with regard to the development of new antitumor agents.

A broad array of medicinal applications of metal complexes of coumarins has been investigated. It was found that in some cases the metal complexes obtained revealed higher biological activity than their ligands [13–15]. The rare earth complexes of hydroxycoumarin derivatives are also subjects of increasing interest in bioinorganic and coordination chemistry [16,17]. Recently, some interesting lanthanide complexes of coumarin derivatives like bis(4-hydroxy-3-coumarinyl)-acetic acid [18], *N*,*N*′-bis(8-aceto-7-hydroxy-4-methylcoumarin)-ethylenediamine [19,20] and coumarin-3-carboxylic acid [21] have been reported.

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Lanthanides (III) show an antitumor activity [22]. Furthermore, literature data show that the coumarins also have these properties. As a result from our earlier work the cytotoxic profile of some new complexes of coumarin derivatives with lanthanides against different human tumor cell lines was proved [13–15,23–30]. The promising results, concerning their significant cytotoxic activity, prompted us to search for new lanthanide 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 cerium could present interesting metalorganic compounds with antitumor activity.

Little is known about the complexing ability of cerium(III) with coumarins. A survey of literature reveals that no work has been done on the reactions of cerium(III) with bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane, bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-yl-methane and bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-4-yl-methane and their cytotoxic profile. 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-2*H*-chromen-3-yl)-pyridin-2-yl-methane, bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-yl-methane and bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-4-yl-methane in complexation reaction with cerium(III). The obtained Ce(III) complexes with these coumarin ligands were characterized by elemental analysis, physicochemical methods, mass-, NMR-, IR- and Raman-spectroscopy. The complicated vibrational spectra of cerium(III) complexes were interpreted on the basis of comparison with the vibrational spectra of the free ligands. The most sensitive modes of the ligands have been assigned and discussed.

We observed that Ce(III) possesses a cytotoxic activity and literature data show that the coumarins also have these properties. That is why our synthesis of complexes of Ce(III) is taken into consideration for cytotoxic screening and further pharmacological study.

2. Chemistry

The compounds used for preparing the solutions were Merck products, p.a. grade: $Ce(NO_3)_3 \cdot 6H_2O$. Bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane, bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-yl-methane and bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-4-yl-methane were used as ligands for the preparation of metal complexes (Scheme 1). These ligands were synthesized and reported by us earlier [27,29]. The complexes of cerium(III) with bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane (H_2L1), bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-yl-methane (H_2L2) and bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-4-yl-methane (H_2L3) were synthesized by reaction of cerium(III) salt and the ligands. The complexes were insoluble in water, slightly soluble in methanol and ethanol and well soluble in DMSO.

3. Pharmacology

In the present study we investigated the cytotoxic effects of the three newly synthesized cerium complexes against the human leukemic cell lines HL-60 (human promuelocytic leukemia) and BV-173 (pre-B cell lymphoma) using the standard MTT–dye reduction assay for cell viability.

In order to elucidate some of the mechanistic aspects of the observed cytotoxic effects we evaluated the ability of the cerium complex of bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane to trigger programmed cell death (apoptosis by means of agarose gel electrophoretic analysis of DNA, isolated from the cytosolic fraction of treated SKW-3 cells). In addition, microscopic morphological evaluation of the treated cells was carried out in order to establish morphological features indicative of programmed cell death.

4. Results and discussion

4.1. Chemistry

The complexes were characterized by elemental analysis. The metal ion was determined after mineralisation. The water content in the complexes was determined by Karl Fisher analysis. The formation of the complexes was confirmed by IR and Raman spectroscopy, ^1H and ^{13}C NMR spectroscopy and mass-spectral data.

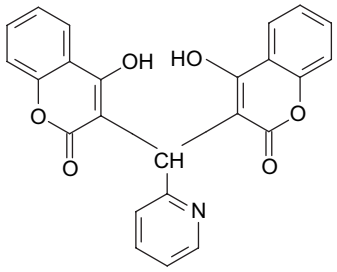
Table 1 shows the data of the elemental analysis of the complexes serving as a basis for the determination of their empirical formulas. The elemental analysis data of the Ce(III) complexes obtained are in agreement with the presented formulas.

The suggested formulas were further confirmed by mass-spectral fragmentation analysis. As it is seen from Table 2, the first peaks in the Ce(III) 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.

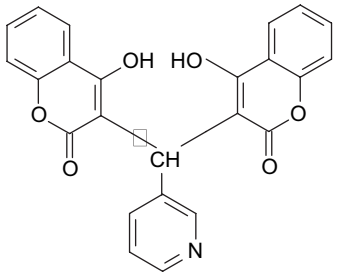
4.2. Vibrational analysis of the ligands and their Ce(III) complexes

The mode of bonding of the ligands to Ce(III) was elucidated by recording the IR and Raman spectra of the complexes as compared with those of the free ligands. The data of the IR and Raman spectra of bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane (H_2L1), bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-yl-methane (H_2L2) and bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-4-yl-methane (H_2L3) and of the cerium complexes with these ligands are presented in Tables 3, 4 and 5, respectively.

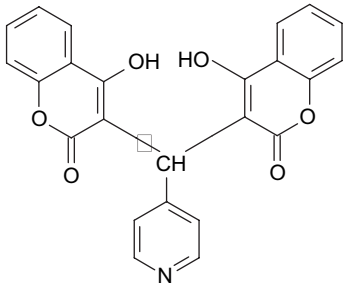
According to the synthesis procedure, the ligands were first deprotonated and the corresponding dianionic species were the active ligand forms in the reaction with Ce(III) ions. Our previous calculations of molecular electrostatic potential of



H_2L1 = bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane



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



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

Scheme 1. Structures of the ligands.

dianionic bis-coumarins suggested that among all the possible reactive sites (pyridine *N*, carbonylic *O* and hydroxylic *O* atoms), both hydroxylic oxygens and carbonylic oxygens revealed the most negative molecular electrostatic potential values and thus they appeared to be the most preferred sites for electrophilic attack, in particular for reaction with Ln(III) ions [30]. Thus, it is expected that the coordination of the ligands to Ce(III) ions is realized through both the carbonylic oxygens and both the hydroxylic oxygens of the ligands. This suggestion will be checked below on the basis of a detailed and comparative vibrational study of the ligands and their Ce(III) complexes.

Selected experimental IR and Raman wavenumbers of the three ligands and of their Ce(III) complexes are given in Tables 3, 4 and 5, respectively. The last column in Tables 3–5 shows the approximate description of the normal modes. A survey of the last column shows that many vibrations are complex and involve strongly coupled motions.

In general, the Raman and IR spectra of Ce(III) complexes with the ligands studied are very similar. The ligands are isomers and similar vibrational behavior of the dicoumarin fragment has to be expected. Pyridine-ring modes, however, are affected by the acceptor (methylene) substituent in *o*-, *m*- and *p*-positions. Thus, the characteristic modes of *o*-, *m*- and *p*-pyridine substituent could be used to characterize and distinguish the isomers, which could be considered as 1:4, 1:3 and 1:2 di-substituted benzene with one donor (N) and one acceptor (alkyl, CHR_2) substituent. At the same time, the coumarin ring vibrations (four adjacent ring hydrogen atoms) ought to resemble the vibrational behavior of 1:2 di-substituted benzene. Both the IR and Raman spectra were considered for full description of the vibrational behavior of the ligands. Below, we discuss characteristic vibrational modes of the ligands that change upon the complexation with Ce(III) ions.

4.2.1. O–H stretching modes, ν(OH)

According to our previous calculations, the weak (to medium) bands at 3124 and 2877 cm^{-1} for H_2L1, at 3061 and 2919 cm^{-1} for H_2L2 and at 3141 and 2887 cm^{-1} for H_2L3 are assigned to the O–H stretching modes [25]. In the vibrational spectra of the Ce(III) complexes with the respective ligands, the bands corresponding to the ν(OH) modes are not

Table 1
Elemental analysis data for Ce(III) complexes with bis-coumarins

Complex	Found/calculated				
	% C	% H	% N	% H_2O	% Ce
Ce(L1)(OH)·H_2O	49.55, 49.14	3.12, 2.73	2.77, 2.39	3.44, 3.07	23.56, 23.89
Ce(L2)(OH)·H_2O	48.88, 49.14	2.89, 2.73	2.75, 2.39	3.15, 3.07	24.05, 23.89
Ce(L3)(OH)·H_2O	49.29, 49.14	3.15, 2.73	2.28, 2.39	3.35, 3.07	23.57, 23.89

$L_1 = C_{24}H_{13}NO_6^{2-}$, $L_2 = C_{24}H_{13}NO_6^{2-}$, $L_3 = C_{24}H_{13}NO_6^{2-}$.

detected and this finding confirms their assignment and the suggestion that the deprotonated forms of the ligands participate in the complexes studied.

4.2.2. Vibrational modes of the carbonylic C=O groups

The most informative of the metal–ligand binding mode in the Ce(III) complexes studied is the behavior of ν(C=O) mode. According to our DFT calculations [25], the bands in the 1700–1670 cm^{-1} region were assigned to the asymmetric and symmetric stretching vibrations of the carbonylic group. In the ligand IR spectra the ν(C=O) mode appears at 1697 cm^{-1} for H_2L1, at 1687 cm^{-1} for H_2L2, whereas in H_2L3 the band is split into four components, Tables 3–5. It should be mentioned that due to the intramolecular C=O···H hydrogen bond, this mode appears at lower wavenumbers in these ligands in comparison with the free C=O vibration (at 1814 cm^{-1}) in the 4-hydroxycoumarin. The negative shift is at about 117–128 cm^{-1}.

In the IR spectra of Ce(III) complexes the bands due to ν(C=O) mode appear as a shoulder at 1653 cm^{-1}, Tables 3–5. Hence, as compared to the ligands, in Ce(III) complexes, the observed ν(C=O) is shifted by ~40 cm^{-1} to lower wavenumbers (~1690 cm^{-1} → 1653 cm^{-1}), indicating explicitly the coordination of the carbonylic oxygens to the Ce(III) ions.

Table 2
Mass-spectral data of bis-coumarins and their Ce(III) complexes

Ligand	*m/z*	(%)	Complex	*m/z*	(%)
$H_2L1 = C_{24}H_{15}NO_6$	413	7	Ce(L_1)(OH)·H_2O	589	1
	395	2		490	3
	252	7		460	5
	162	30		410	2
	120	28		307	40
	92	38		176	100
$H_2L2 = C_{24}H_{15}NO_6$	413	7	Ce(L_2)(OH)·H_2O	589	1
	395	2		490	2
	252	30		460	7
	162	62		410	3
	120	74		307	95
	92	86		176	100
$H_2L3 = C_{24}H_{15}NO_6$	413	0	Ce(L_3)(OH)·H_2O	590	1
	252	18		490	2
	250	50		460	5
	162	62		410	2
	120	74		307	88
	92	86		176	100

The coordination of Ce(III) to the carbonylic oxygens affected also the IR bands due to the lactone ν(C–O) modes. In the ligands, the COH groups are involved in intramolecular H-bonds and the stretching ν(C–O), in-plane deformation, $\delta(COH)_{ip}$ and out-of-plane deformation, $\delta(COH)_{op}$ modes of the ligands should be affected. According to the calculations [25], both in-plane and out-of-plane δ(COH) modes are shifted to higher wavenumbers due to the intramolecular H-bonds in comparison with those of 4-hydroxycoumarin. As expected the bands assigned to the COH bending modes were not observed in the vibrational spectra of the Ce(III) complexes since the ligands participate with their deprotonated forms.

4.2.3. Ring stretching modes, ν(CC)

The ν(CC) stretching vibrations of the coumarin and pyridine rings are expected to appear in the 1650–1400 cm^{-1} range. These modes are slightly affected by coordination of the ligands to the Ce(III) ions (Tables 3–5). Only the bands at 1539 and 1531 cm^{-1} in the ligands spectra were shifted to the lower frequency at 1508 cm^{-1} in the IR spectra of the Ce(III) complexes due to Ce(III)–O interaction and π-conjugation in the coumarin ring after the deprotonation. The increasing IR intensities of these bands in the complexes are also in agreement with our suggestion. The bands due to the CH bending modes and to the ring deformations are slightly changed in the vibrational spectra of Ce(III) complexes.

4.2.4. Ce–O stretching and O–Ce–O bending modes

The analysis of the calculated frequencies [25] showed that the weak bands observed around 570 and 540 cm^{-1} in the vibrational spectra of Ce(III) complexes are due to the Ce–O stretching modes. The bending O–Ce–O modes were predicted to appear around 400 cm^{-1}. As seen from the vibrational spectra of the studied complexes (Tables 3–5) the stretching and bending modes, including Ce, are weak and thus they are not characteristic and informative. Generally, in most of the known lanthanide complexes with coumarins the Ln(III)–ligand bonding is strongly ionic with very small donor–acceptor character and supposes weak and not informative bands [31]. IR spectra of the compounds were also recorded on solid state in Nujol in the range 700–220 cm^{-1}. The spectra of the complexes showed new bands in comparison with those of the free ligands, which have been assigned to the rocking, waggling and metal–oxygen stretching vibrations.

On the basis of vibrational analysis done we suggest binding of the ligands to the Ce(III) ions through the carbonylic and the deprotonated hydroxylic oxygens.

Table 3
Selected experimental (IR and Raman) wavenumbers of bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane (H_2L1)and its Ce(III) complex

H_2L1		Ce complex		Approximate description
ν_{IR}	ν_{Ra}	ν_{IR}	ν_{Ra}	
3441		3390br		$\nu(OH)_w$
3124m				$\nu(OH)$
2877m				$\nu(OH)$
2846w		2724vw		$\nu(CH)_{met}$
1697vs	1695m	1653sh		$\nu(C{=}O)_{as}$
1690sh				$\nu(C{=}O)_s$
1639s	1637w			$\nu(CC)$
1623s		1621s	1607vs	$\nu(CC)$
1610vs	1608s		1520w	$\nu(CC)$
1594s		1599s		$\nu(CC)$
1570s				$\nu(CC)_{py}$
1560s	1562w		1565w	$\nu(CC)_{py} + \nu(CN)_{py}$
1539m,d		1511vs		$\nu(CC) + \delta(COH)_{ip}$
1533m				$\nu(CC) + \delta(COH)_{ip}$
1488m	1482s		1477s	$\nu(CC)_{py} + \nu(CN)$
1457m	1458w	1452s	1460w	$\nu(CC) + \delta(CCH)_{ip}$
1431m	1421sh		1421s	$\delta(COH)_{ip}$
1412m	1405m			$\nu(CC)_{py} + \delta(CCH)_{ip(py)}$
1393m				$\nu(CC) + \delta(CCH)_{ip}$
1349m		1417m		$\nu(CO) + \delta(COH)_{ip}$
				$\nu(CO) + \delta(COH)$
1330m	1331s		1333s	$\delta(CCH)_{ip(py)}$
1288sh	1304vw		1280	$\nu(CN)_{py} + \delta(CCH)_{ip(py)}$
1275m	1277w	1280m		$\nu(CC)_{py} + \nu(CN)_{py}$
1254w	1248m		1249	$\delta(CCH)_{ip}$
1212m	1205s	1211m,d	1209s	$\nu(CO)_{lactone}$
1180m	1149m		1142m	$\delta(CCH)_{ip}$
1111s	1109w	1108	1105w	$\delta(CCH)_{ip(py)}$
1064m	1055vw	1062m	1043sh	$\nu(CO)_{lactone} + \nu(CC)_{met}$
1040m	1032s	1024sh	1023s	$\nu(CO)_{lactone} + \delta(CCH)_{ip}$
1017w		1012w		$\delta(CCC)_{ip(py)}$ (star of David)
995m	980w		992	$\delta(CCH)_{op(py)}$
945m	944w	950m	935	$\delta(CCH)_{op(py)}$
907m	899w	908m	893	$\delta(CCH)_{op}$
883m				$\delta(CCC)_{ip}$ (star of David)
869m	861vw			$\delta(CCH)_{op(py)}$
851m	846w	858w		$\delta(CCC)_{ip}$ (star of David)
791m		800w	789	$\delta(CCH)_{op}$
771m	–	–	–	$\delta(COH)_{op}$
761vs	763w			$\delta(COH)_{op}$
750s		759vs	747	$\delta(CCH)_{op}$
734m,sh				$\delta(CCH)_{op(py)}$
688sh	699w	687m	690s	$Ring_{op}$
672m	672s		662sh	$Ring_{ip}$
624m	630vw			$Ring_{op}$
602m	614m	613w	617w	$Ring_{ip(py)}$
546m	539m	547w	575m	$\delta(CC_{18}C) + ring_{ip}$
526w	523w	527w		$Ring_{op}$
482m	496w	479m		$\delta(CC_{18}C) + ring_{op(py)}$
447m	443m	440w	456m	$Ring_{ip}$
437sh	422m		402w	$Ring_{op}$

ip – In-plane; op – out-of-plane; py – pyridyl; s – strong; vs– very strong; w – weak; vw – very weak; sh – shoulder; m – medium.

4.3. ^1H and ^{13}C NMR spectra of the ligands and their Ce(III) complexes

Metal ion coordination with ligand by means of oxygen atoms of C=O groups and of the deprotonated hydroxyl groups was shown owing to data of ^1H and ^{13}C 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 ^1H NMR spectra in DMSO-d_6 are presented in Table 6. As it is seen from Table 6, different chemical shifts were observed in the complexes and these changes were attributed to coordination of the ligands to Ce(III). Comparison of the ^1H NMR spectra of the complexes with those of the ligands reveals that the resonances due to protons of the ligands are considerably broadened and also shifted indicating complexation. In agreement with literature data these shifts could be considered as valuable and we used them to confirm the coordination. A similar feature has often been observed in coordination compounds and metal complexes. As previously reported $\Delta\delta$ values are indicative of the coordinating mode of the coumarin-system [26–30].

^{13}C NMR spectra also showed valuable shifts and could be considered as a confirmation of the formation of the new Ce(III) compounds. Spectral integration is in agreement with the 1:1 metal-to-ligand stoichiometry, derived by elemental analysis. ^{13}C NMR spectra of the ligands and of the complexes were recorded at 62.9 MHz in DMSO-d_6. The results of ^{13}C NMR spectra of the compounds in δ (ppm) are presented in Table 7.

Due to electron transfer from the hydroxyl and carbonyl oxygen atoms to Ce(III), differences in chemical shifts were observed for the neighboring C-4, C-3 and C-2 carbon atoms of the complexes and they confirmed the expected coordination of the ligands through both deprotonated hydroxyl and carbonyl oxygen atoms. The other carbon atoms were only slightly affected from the coordination of the metal. On the basis of the results thus obtained, it was suggested that the ligands act as tetradentate ones in the Ce(III) complex formation. The data supporting this conclusion apparently agree with those reported recently by us in the literature regarding lanthanide complexes of coumarin ligands [12–15,23–30].

4.4. Pharmacology

4.4.1. In vitro cytotoxicity

The cytotoxic effects of the three newly synthesized cerium complexes of bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane (Ce-1), bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-yl-methane (Ce-2) and bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-4-yl-methane (Ce-3) against the human leukemic cell lines HL-60 (human promuelocytic leukemia) and BV-173 (pre-B cell lymphoma) were determined using the standard MTT–dye reduction assay for cell viability. The spectrophotometric data retrieved from these experiments are summarized in Tables 8 and 9.

All of the tested cerium complexes exhibited concentration-dependent cytotoxic effects after 72 h treatment of both HL-60 and BV-173 cells. The constructed dose response curves and the corresponding IC_{50} values obtained are shown in Figs. 1–6 and in Table 9, respectively.

As evident from the results obtained Ce-1 exerted the most pronounced cytotoxic effects against the myeloid HL-60 cells with IC_{50} value of ca. 70 μM (Fig. 1, Table 9). Whereas at

Table 4
Selected experimental (IR and Raman) wavenumbers of bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-yl-methane (H_2L2) and its Ce(III) complex

H_2L2		Ce complex		Approximate description
ν_{IR}	ν_{Ra}	ν_{IR}	ν_{Ra}	
3447br				$\nu(OH)_w$
3061w				$\nu(OH) + \nu(CH)_{py}$
2919w				$\nu(OH)$
2856w				$\nu(CH)_{met}$
1687vs		1653sh		$\nu(C{=}O)_{as}$
	1671m			$\nu(C{=}O)_s$
1636sh			1615sh	$\nu(CC)$
1615vs	–			$\nu(CC)$
–	1607vs	1599s	1604s	$\nu(CC)$
1565sh	1554m		1553	$\nu(CC)_{py}$
				$\nu(CC)_{py} + \nu(CN)$
1539vs	1532m	1506s	1500	$\nu(CC) + \delta(COH)_{ip}$
1534s	–			$\nu(CC) + \delta(COH)_{ip}$
1491w	1479vs		1480m	$\nu(CC)_{py} + \nu(CN) + \delta(CCH)_{ip(py)}$
1460m	1457w		1463w	$\nu(CC) + \delta(CCH)_{ip}$
1449sh	1444w	1455vs		$\nu(CC) + \delta(CCH)_{ip}$
	1431sh			$\delta(COH)_{ip}$
	1409s			$\nu(CC)_{py} + \delta(CCH)_{ip(py)}$
1407m				$\nu(CC)_c + \delta(CCH)$
1360w			1421m	$\nu(CO) + \delta(COH)_{ip}$
	1362vw	1417m	1384sh	$\nu(CO) + \delta(COH)$
1330w	1324s		1331m	$\delta(CCH)_{met}$
1277m	1296m	1280w	1308vw	$\nu(CC)_{py} + \nu(CN)_{py}$
1254m	1255s	1258w	1252w	$\delta(CCH)_{ip}$
1209sh	1207vs	1208m	1212m	$\nu(CO)_{lactone}$
1193sh	1195sh			$\nu(CO)_{lactone} + \nu(CC)_{met}$
1177m	1171w	1183m	1175sh	$\delta(CCH)_{ip(py)} + \nu(CO)$
1148w	1138s	1150vw	1144vw	$\delta(CCH)_{ip}$
1119m				$\delta(CCH)_{ip(py)}$
1109m	1120w	1108m	1108vw	$\delta(CCH)_{ip} + \delta(CCH)_{ip(py)}$
1080w		1069m	1063	$\delta(CCH)_{ip}$
1050m	1046s			$\nu(CO)_{lactone}$
1035m	1026vs		1023m	$\nu(CO)_{lactone} + \delta(CCH)_{ip}$
1020m				$\delta(CCH)_{ip}$
1003sh	1010m		992w	$\delta(CCC)_{ip(py)}$ (star of David)
	951m		–	$\delta(CCH)_{op}$
943m	936m	942w	–	$\delta(CCH)_{op(py)}$
904m	898m	908m	907	$\delta(CCH)_{op(py)}$
872m	873vw			$Ring_{ip}$ (star of David)
851m	847m	862vw		$\delta(CCH)_{op}$
	829s	821w	822vw	$\delta(CCH)_{op}$
809m	808vw	792vw	797vw	$Ring_{(ip)breathing}$
–	781s		789	$\delta(COH)_{op}$
760s	757sh			$\delta(COH)_{op}$
752sh	743m	761s	747w	$\delta(CCH)_{op} + \delta(CCH)_{op(py)}$
714m	713m			$\delta(OCO)_{op}$
695m	692m		693s	$Ring_{ip(py)}$
695m	–			$Ring_{ip}$
677m	674s	688m	659sh	$Ring_{op}$
630sh	623m			$Ring_{ip(py)}$
614m	605m	612w	608vw	$Ring_{ip(py)}$
565m	576vw		572m	$\delta(CC_{18}C)$
552m	557m	551w	527w	$Ring_{ip}$
530m	543m			$Ring_{op}$
492w	504s	482m		$Ring_{op(py)}$
448m	439s		456m	$Ring_{op}$

ip – In-plane; op – out-of-plane; py – pyridyl; s – strong; vs– very strong; w – weak; vw – very weak; sh – shoulder; m – medium.

Table 5
Selected experimental (IR and Raman) wavenumbers of bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-4-yl-methane (H_2L3) and its Ce(III) complex

H_2L3		Ce complex		Approximate description
ν_{IR}	ν_{Ra}	ν_{IR}	ν_{Ra}	
		3387br		$\nu(OH)_w$
3141w				$\nu(OH)$
2887w	2891w			$\nu(OH)$
2846w	–	2854w		$\nu(CH)_{met}$
1702,1694s	1695w			$\nu(C{=}O)_{as}$
1686,1683s	1682m	1653br	–	$\nu(C{=}O)_s$
1634m	1636w	1622s		$\nu(CC)_c$
1615s,sh	–	–	1605s	$\nu(CC)_c$
1610s	1607vs	1599vs	1577	$\nu(CC)_c$
1565m				$\nu(CC)_{py}$
1557m	1554m		1555	$\nu(CC)_{py} + \nu(C{=}N)$
1539m	1532w	1506vs	1500	$\nu(CC)_c + \delta(COH)_{ip}$
1531d,m				$\nu(CC)_c + \delta(COH)_{ip}$
1497s	1479s	1482sh	1478m	$\nu(CC)_{py} + \nu(CN)$
1467w				$\delta(COH)_{ip} + \nu(C{-}O)$
1460w	1463sh		1464w	$\nu(CC)_c + \delta(CCH)_{ip}$
1454w	1445m	1448m	–	$\nu(CC)_c + \delta(CCH)_{ip}$
	1435m			$\delta(COH)_{ip} + \nu(CO)$
1417w	1423m	1420m	1420	$\nu(CC)_{py}$
1407m	1399s		1396	$\nu(CC)c$
1400m				$\nu(CC)c$
1372w	1386sh	1416m	1349	$\nu(CO) + \delta(COH)_{ip}$
1359w	1353w		1330	$\nu(CO) + \delta(COH)_{ip}$
	1319m			
1277m	1250m	1281w	1253	$\nu(CN) + \nu(CC)_{py}$
1206m	1202vs	1211w	1209	$\nu(CO)_{lactone} + \delta(CCH)_{ip}$
Overlap.	1199m	1194sh	1190	$\nu(CO)_{lactone} + \delta(CCH)_{ip}$
1180m	1186w	1187m		$\delta(CCH)_{ip(py)} + \nu(CC)_{met}$
Overlap.	1174s	1149w	1146w	$\delta(CCH)_{ip}$
1148w	1143s			$\delta(CCH)_{ip}$
1106m	1100m	1109m		$\delta(CCH)_{ip}$
1066m	1061m	1062w		$\nu(CO)_{lactone} + \delta(CCH)_{ip(py)}$
1053m	1043m		1046w	$\delta(CCH)_{ip(py)}$
1036m	1031vs	1030w	1028m	$\nu(CO)_{lactone} + \delta(CCH)_{ip}$
1011w	1008m			$\delta(CCH)_{ip}$
989m	984m			$\delta(CCC)_{ip(py)}$ (star of David)
949m	969m	952w	962	$\delta(CCH)_{op(py)}$
940w	948m			$\delta(CCH)_{op(py)}$
904m	898m	908m	916	$\delta(CCH)_{op}$
853m	850m	839w		$\delta(CCH)_{op(py)}$
807m	814vw	811w		$\delta(CCH)_{op}$
795sh	788m	792w		$\delta(CCH)_{op}$
782sh	776s		778	$\delta(CCH)_{op(py)}$
763vs				$\delta(COH)_{op(sym)}$
760s	757w			$\delta(COH)_{op(as)} + \delta(CCH)_{op}$
752m	731w	762s	735m	$\delta(CCH)_{op} + \delta(CCH)_{op(py)}$
695w	692w	693w	685m	$\delta(OCO)_{op} + \delta(CCO)_{op}$
676w	674s		664w	$\delta(CCO)_{op(s)}$
481m	477m	479m	455s	$Ring_{py(op)}$
422w	445m		400m	$Ring_{op}$

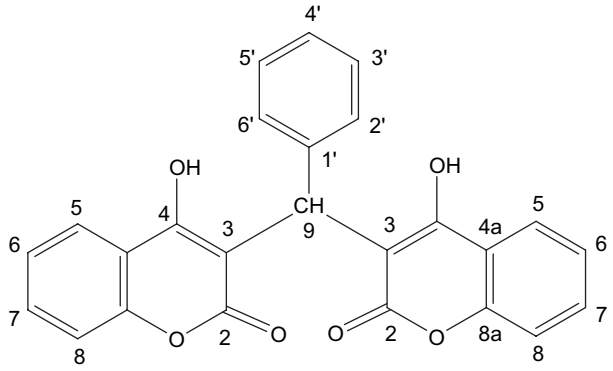
ip – In-plane; op – out-of-plane; py – pyridyl; s – strong; vs– very strong; w – weak; vw – very weak; sh – shoulder; m – medium.

concentrations up to 25 μM certain increase of the cell survival was encountered, at 50 μM Ce-1 produced a 30% reduction of the percentage of viable HL-60 cells. At 100 μM concentration Ce-1 decreased the cell survival fraction by ca. 81%. When applied at the highest concentration investigated (200 μM) it caused an almost absolute eradication of the

Table 6
^{1}H NMR spectral shifts, δ (ppm) of the ligands and their Ce(III) complexes (250 MHz, DMSO-d_6)

Compound	δ (ppm)		
	H_5–H_8[a]	H_9[a]	$H_{2'}$–$H_{6'}$[a]
$H_2L1 = C_{24}H_{15}NO_6$	7.24–7.58	6.54	7.80–8.64
$Ce(L_1)(OH)\cdot H_2O$	7.17–7.78	6.30	8.29–8.64
$H_2L2 = C_{24}H_{15}NO_6$	7.22–7.57	6.42	7.79–8.70
$Ce(L_2)(OH)\cdot H_2O$	7.06–7.53	6.24	7.90–8.94
$H_2L3 = C_{24}H_{15}NO_6$	7.22–7.58	6.46	7.80–8.68
$Ce(L_3)(OH)\cdot H_2O$	7.14–7.82	6.30	8.15–8.82

[a] The atom numbering is in agreement with the figure below.



malignant cells (survival fraction = ca. 3.12%). The other complexes under investigation were far less active against HL-60 with practical lack of cytotoxicity within the concentration range of 12.5–50 μM (Ce-2) and 12.5–100 μM (Ce-3) (Figs. 2 and 3). At the highest concentration of 200 μM applied both Ce-2 and Ce-3 showed profound maximal efficacy with less than 10% viable cells.

As evident from the IC_{50} values obtained Ce-1 and Ce-2 produced comparable cytotoxic effects on BV-173 cells, the former being slightly more active (Table 9). They both lacked cytotoxic effects at concentrations ranging between 12.5 and 50 μM, whereas at 100 μM they significantly reduced the percentage of viable cells by ca. 80% for Ce-1 and by ca. 54% for Ce-2 (Figs. 4 and 5). The treatment of BV-173 cells with either Ce-1 or Ce-2 at 200 μM resulted in almost total eradication of the viable cells (cell survival fractions less than 4%). The third complex compound evaluated Ce-3 was less active with respect to the IC_{50} value obtained, than Ce-1 and Ce-2, although at the highest concentration applied (200 μM) it produced comparable maximal efficacy with ca. 8.6% viable cells (Fig. 6).

Table 7
^{13}C NMR spectral shifts, δ (ppm) of the ligands and their Ce(III) complexes (62.9 MHz, DMSO-d_6)

Atom	δ (ppm)					
	H_2L1	$Ce(L_1)(OH)\cdot H_2O$	H_2L2	$Ce(L_2)(OH)\cdot H_2O$	H_2L3	$Ce(L_3)(OH)\cdot H_2O$
C-2	168.6	164.9	168.1	169.1	168.2	170.8
C-4	164.0	162.0	164.1	164.6	164.9	168.1
C-8a	157.6	156.6	152.8	156.2	164.2	164.7
C-1′	152.9	152.7	142.9	152.0	152.8	156.1
C-7	146.5	148.5	144.9	149.2	141.0	148.4
C-3′	141.9	136.0	–	–	131.7	137.8
C-5′	141.9	131.1	140.4	131.4	131.7	134.6
C-4′	131.9	126.3	139.2	130.7	–	–
C-6′	125.9	123.1	131.6	124.4	125.3	131.4
C-2′	–	–	126.8	123.2	125.3	130.5
C-5	124.4	121.1	124.3	122.4	124.3	126.5
C-6	123.4	120.2	123.3	116.8	123.3	122.2
C-4a	119.3	116.9	119.6	119.9	119.5	119.9
C-8	115.9	115.6	115.8	115.7	115.9	113.7
C-3	100.5	103.6	101.7	102.4	101.5	102.8
C-9	36.1	38.6	34.8	36.2	37.9	34.5

The additional investigation of the cytotoxic effects of the most active complex Ce-1 on the chronic lymphoid leukemia cells SKW-3 revealed considerable cytotoxic activity as evident from the concentration–response curve depicted in Fig. 7 and the retrieved IC_{50} value (Table 9). At the lowest concentration (12.5 μM) Ce-1 lowered the cell survival fraction by ca. 30%, at 100 μM the cell viability was decreased by ca. 56% and at the highest concentration evaluated (200 μM) the viable cells were only ca. 7%.

4.4.2. Apoptosis induction by Ce-1

The 24 h treatment of SKW-3 cells with Ce-1 (100 and 200 μM) led to DNA-laddering as depicted by the electrophoregram in Fig. 8. The DNA-laddering is indicative of mono- and oligonucleosomal fragmentation, recognized as

Table 8
Spectrophotometrical data from the MTT assay concerning the cytotoxic effects of the newly synthesized cerium complexes of bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane (Ce-1), bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-yl-methane (Ce-2) and bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-4-yl-methane (Ce-3) on HL-60, BV-173 and SKW-3 leukemic cells

Cell line	Complex	MTT-formazan absorption at 580 nm					
		Untreated control	12.5 μM	25 μM	50 μM	100 μM	200 μM
HL-60	Ce-1	0.801 ± 0.074	0.924 ± 0.048	0.955 ± 0.079	0.568 ± 0.031	0.148 ± 0.006	0.025 ± 0.011
	Ce-2	0.801 ± 0.074	0.908 ± 0.054	0.945 ± 0.063	0.873 ± 0.055	0.658 ± 0.037	0.058 ± 0.044
	Ce-3	0.737 ± 0.051	0.907 ± 0.026	0.880 ± 0.077	0.831 ± 0.054	0.824 ± 0.079	0.059 ± 0.028
BV-173	Ce-1	1.023 ± 0.086	1.028 ± 0.060	1.040 ± 0.075	0.976 ± 0.026	0.204 ± 0.030	0.037 ± 0.007
	Ce-2	1.023 ± 0.086	0.982 ± 0.066	1.022 ± 0.072	0.969 ± 0.047	0.476 ± 0.077	0.033 ± 0.020
SKW-3	Ce-3	0.986 ± 0.067	0.932 ± 0.049	0.987 ± 0.022	0.983 ± 0.039	0.840 ± 0.064	0.085 ± 0.033
	Ce-1	1.281 ± 0.091	0.917 ± 0.03	0.886 ± 0.081	0.749 ± 0.036	0.558 ± 0.059	0.089 ± 0.013

Table 9
Relative potency of the investigated compounds in the panel of human tumor cell lines, following 48 h treatment

Cell line	IC_{50} value (μM)		
	Ce-1	Ce-2	Ce-3
HL-60	69.9	142.8	159.5
BV-173	80.15	96.4	146.09
SKW-3	79.81	n.d.	n.d.

n.d. – Not detected.

a major hallmark of the programmed cell death. In order to characterize the cell death in SKW-3 we further observed morphological changes in the treated cells using phase-contrast light microscope. Apoptotic bodies' formation was encountered, through the observations carried out, as evident from the photomicrographs depicted in Fig. 9. These findings suggest that the 24 h treatment with Ce-1 is associated with induction of programmed cell death.

5. Conclusions

The coordination ability of the ligands has been proved in complexation reaction with cerium(III) ion. The elemental analysis and mass-spectral data confirmed the compositions of the compounds. ^{1}H NMR- and ^{13}C NMR-, IR- and Raman-spectral analysis of the ligands and their Ce(III) complexes confirmed the suggested coordination of the ligands through both the hydroxyl and carbonyl oxygen atoms.

All of the newly synthesized Ce(III) complexes under investigation exhibited cytotoxic activity in micromolar concentrations. Taking into consideration the superior relative potency of Ce-1, however, as well as its documented capability to induce programmed cell death we could conclude that this agent should undergo further detailed pharmacological evaluation.

According to our expectations the complexes of cerium(III) possess a cytotoxic activity and their in vitro effects are clearly expressed. These results confirmed our previous observations on the cytotoxicity of cerium(III) complexes.

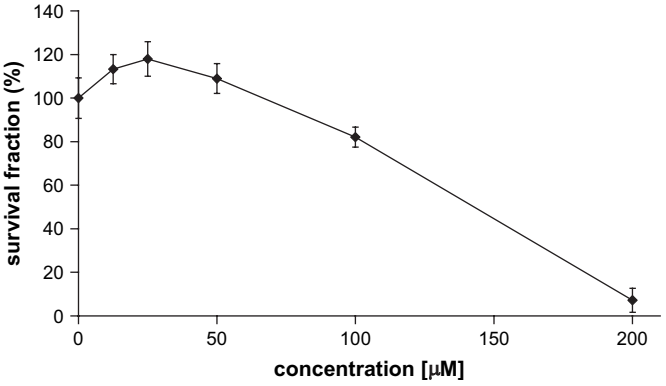


Fig. 2. Cytotoxic effects of Ce-2 on HL-60 cells, as assessed by the MTT–dye reduction assay following 72 h treatment. Each data point represents the arithmetic mean ± sd of at least six independent experiments.

6. Experimental protocols

6.1. Chemistry

6.1.1. Physical and spectroscopic measurements

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. 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 °C to 400 °C at a rate of 100 °C/min. The ionization current was 300 mA, the accelerating voltage was 3 kV and the chamber temperature was 150 °C.

The solid-state infrared spectra of the ligands and their complex were recorded in KBr in the 4000–400 cm^{-1} frequency range by FT-IR 113V Bruker spectrometer and by IR-spectrometer Perkin-Elmer GX Auto image system (700–200 cm^{-1}).

The Raman spectra of the compounds were recorded with a Dilor Labram microspectrometer (Horiba-Jobin-Yvon, model LabRam) equipped with 1800 grooves/mm holographic grating. The 514.5 nm line of an argon ion laser (Spectra Physics, model 2016) was used for the probes excitation. The spectra were

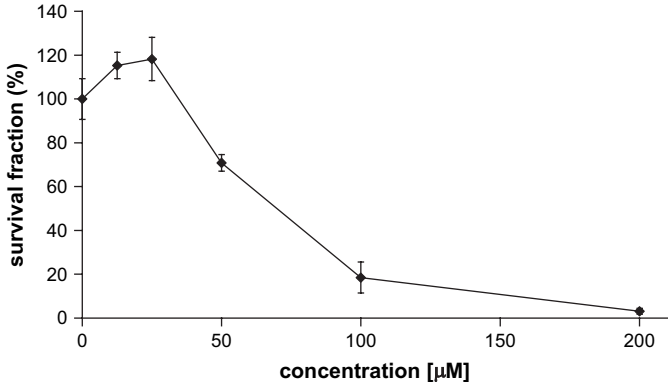


Fig. 1. Cytotoxic effects of Ce-1 on HL-60 cells, as assessed by the MTT–dye reduction assay following 72 h treatment. Each data point represents the arithmetic mean ± sd of at least six independent experiments.

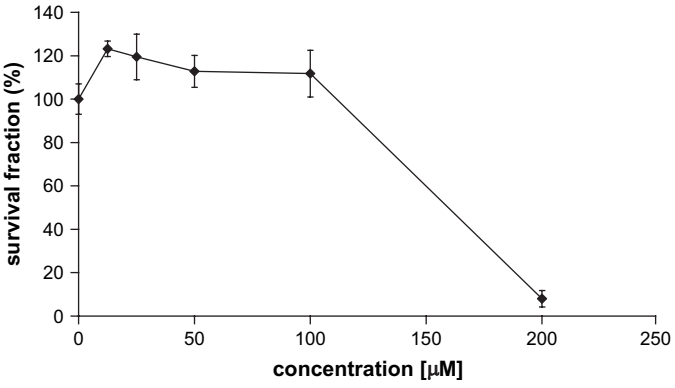


Fig. 3. Cytotoxic effects of Ce-3 on HL-60 cells, as assessed by the MTT–dye reduction assay following 72 h treatment. Each data point represents the arithmetic mean ± sd of at least six independent experiments.

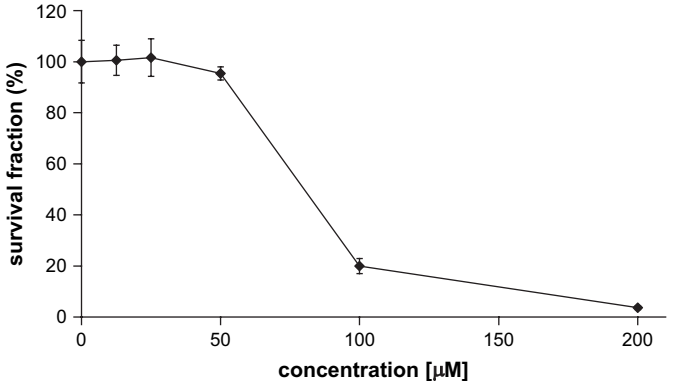


Fig. 4. Cytotoxic effects of Ce-1 on BV-173 cells, as assessed by the MTT–dye reduction assay following 72 h treatment. Each data point represents the arithmetic mean ± sd of at least six independent experiments.

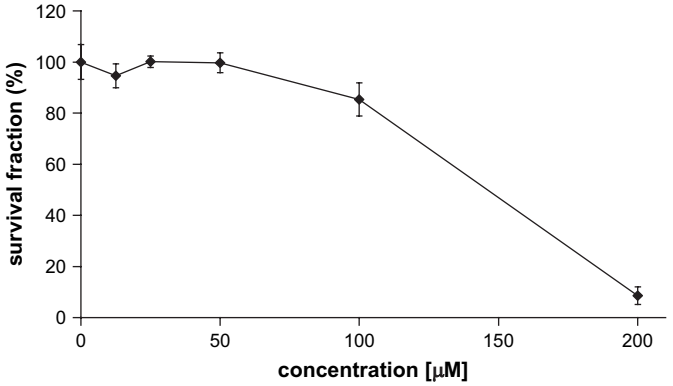


Fig. 6. Cytotoxic effects of Ce-3 on BV-173 cells, as assessed by the MTT–dye reduction assay following 72 h treatment. Each data point represents the arithmetic mean ± sd of at least six independent experiments.

collected in a backscattering geometry with a confocal Raman microscope equipped with an Olympus LMPlanFL 50× objective and with a resolution of 2 cm^{-1}. The detection of Raman signal was carried out with a Peltier-cooled CCD camera. The laser power of 100 mW was used in our measurements.

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

^{13}C NMR spectra were recorded at ambient temperature on Bruker 250 WM (62.9 MHz) spectrometer in DMSO-d_6. Chemical shifts are given in ppm, downfield from TMS.

6.1.2. General method of synthesis

The complexes of cerium(III) with bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-2-yl-methane (H_2L1), bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-3-yl-methane (H_2L2) and bis(4-hydroxy-2-oxo-2*H*-chromen-3-yl)-pyridin-4-yl-methane (H_2L3) were synthesized by reaction of cerium(III) salt and the ligand, in amounts equal to metal–ligand molar ratio of 1:2. The complexes were prepared by adding an aqueous solution of cerium(III) 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 h. 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 human leukemic cell lines HL-60, BV-173 and SKW-3 were provided by the German Collection of Microorganisms and Cell Cultures. They were grown as suspension-type cell cultures in a controlled environment (humidified atmosphere with 5% carbon dioxide, at 37 °C in a 'Heraeus' incubator) using RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine. The cell lines were re-fed with fresh medium twice or thrice weekly in order to maintain them in log phase.

The stock solutions of the novel cerium complexes were freshly prepared in DMSO and thereafter diluted with RPMI-1640 in order to obtain the desired final concentrations. At the final dilutions obtained in the microplate wells, the concentration of DMSO never exceeded 1%.

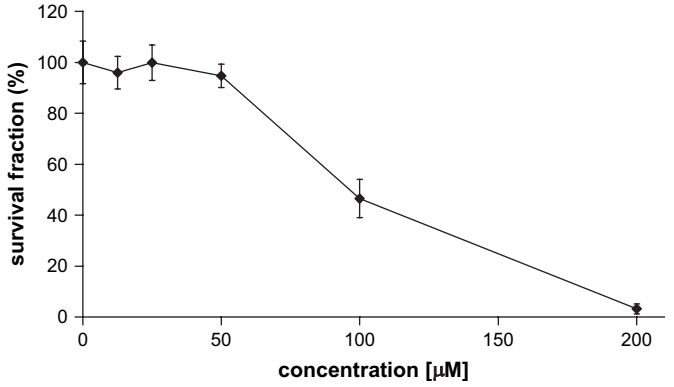


Fig. 5. Cytotoxic effects of Ce-2 on BV-173 cells, as assessed by the MTT–dye reduction assay following 72 h treatment. Each data point represents the arithmetic mean ± sd of at least six independent experiments.

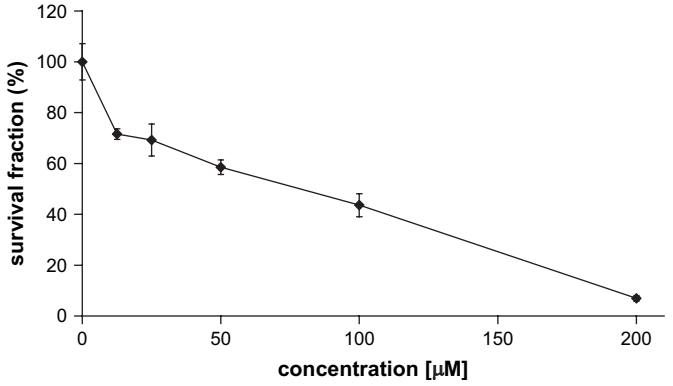


Fig. 7. Cytotoxic effect of Ce-1 on SKW-3 cells as assessed by the MTT–dye reduction assay following 72 h treatment. Each data point represents the arithmetic mean ± sd of at least eight independent experiments.

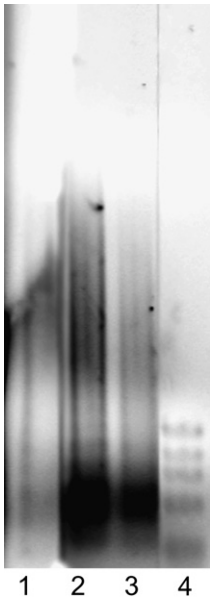


Fig. 8. Agarose gel electrophoresis of DNA, isolated from the cytosolic fraction of untreated SKW-3 cells (lane 1) or following treatment with Ce-1 at 100 μM (lane 2) and 200 μM (lane 3); line 4 – size marker.

The cell culture maintenance, the drug solution preparation as well as the treatment procedures were performed in a 'Heraeus' Laminar flow cabinet.

6.2.2. Cell viability determination (MTT assay)

The cytotoxic activity of the investigated complexes was determined using the standard MTT–dye reduction assay for cell viability. In brief, exponentially growing cells were seeded in 96-well microplates (100 μl aliquots/well) at a density of 1×10^5 cells/ml. Following 24 h incubation at 37 °C the cells were exposed to different concentrations of Ce-1, Ce-2 and Ce-3 for 72 h. After the incubation period, 10 μl MTT solution (10 mg/ml in PBS) aliquots were added to each well. The plates were then incubated for 4 h at 37 °C and the formazan crystals formed were dissolved through addition of 100 μl/well 5% formic acid in 2-propanol (Merck). The absorption of the samples was measured using an ELISA reader (Uniscan Titertec) at a wavelength of 580 nm. The blank solution was prepared with 100 μl RPMI-1640 medium (Sigma), 10 μl MTT stock and 100 μl 5% formic acid in 2-propanol. For each concentration survival fractions (% of untreated control) were calculated from the absorption values retrieved. The results were expressed as IC_{50} values, extrapolated from the corresponding concentration–response curves.

6.2.3. DNA isolation and gel electrophoresis

The isolation of cytosolic DNA and its horizontal gel electrophoretic analysis and visualization was performed. Briefly, about 5×10^6 SKW-3 cells (untreated controls, or treated with Ce-1 at 100 and 200 μM), were washed in PBS and pelleted. Thereafter the cell pellets were re-dispersed in 0.25 ml PBS and lysed through addition of 0.5 ml lysis-buffer (0.5% Triton X-100, 20 mM tris–HCl and 1 mM EDTA (pH = 7.4)). The samples were kept on ice for 5 min and thereafter spun at 13 000 rpm for 20 min. The supernatants were transferred into fresh 2 ml Eppendorf safe lock tubes and then 0.937 ml 2-propanol as well as 0.187 ml 6 M solutions of NaCl were added to each sample. The test tubes were gently agitated and incubated at −20 °C overnight in order to allow precipitation of the isolated DNA. The samples were centrifuged for 20 min at 13 000 rpm and the supernatants were decanted. Thereafter DNA was washed in 1 ml ice cold 70% ethanol and air dried. The isolated DNA was re-dissolved in 20 μl distilled water and analyzed by gel electrophoresis in 0.8% agarose gel and then stained with ethidium bromide. Finally DNA was visualized using an UV transilluminator and photographed with a fixed digital camera (Bio Doc IT™ system).

6.2.4. Statistics

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

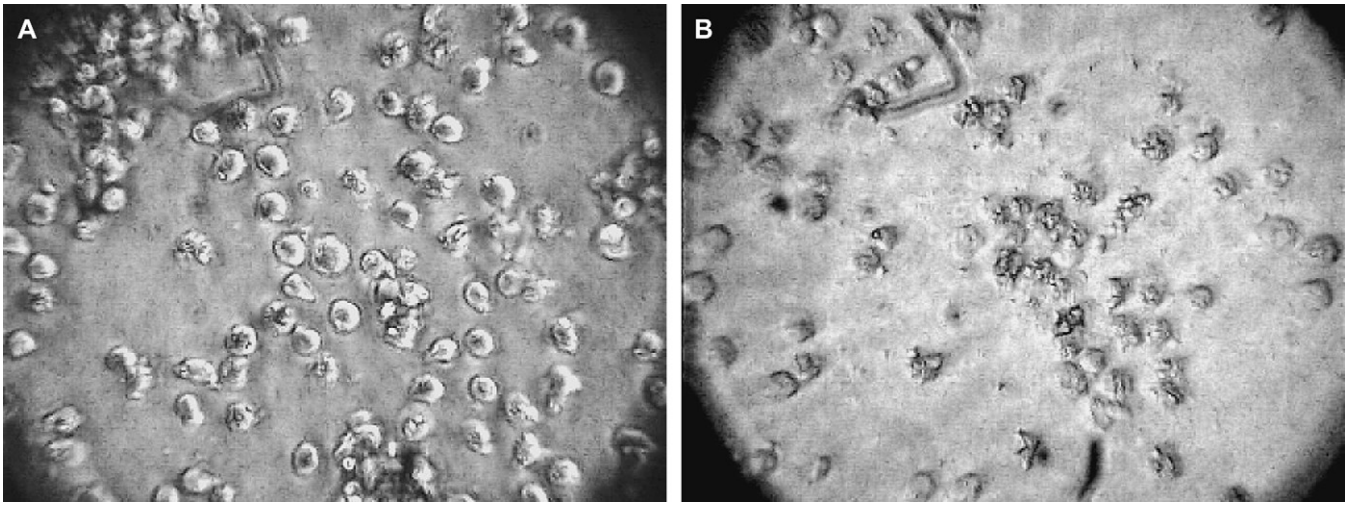


Fig. 9. Photomicrographs of SKW-3 cells: solvent treated (A) and following 24 h exposure to Ce-2 (200 μM) (B), showing typical apoptotic changes evoked by the exposure to the cerium coordination compound.

Acknowledgements

The ligands were synthesized by Doc. I. Manolov and their synthesis was published recently [27].

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