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Original article

Cytotoxic activity of new cerium (III) complexes of bis-coumarins

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Abstract

Complexes of cerium (III) with bis-coumarins: 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-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 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 cerium (III) complexes with bis-coumarins were characterized by different physicochemical methods—elemental analysis, IR-, ^{1}H- and ^{13}C-NMR-spectroscopies 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 two 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 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) were evaluated on the CML-derived K-562 and LAMA-84 cells, characterized by relative low responsiveness to chemotherapy. The DNA isolated from the cytosolic fraction of BV-173 cells after 24 h treatment with the same complex (at 100 and 200 μM) demonstrated a laddering phenomenon that is indicative for apoptotic cell death.

Keywords: Bis-coumarins; Cerium (III) complexes; IR- and NMR-spectra; Cytotoxic activity; DNA

1. Introduction

The coumarins constitute an important class of compounds, with several types of pharmacological agents possessing anticancer, anti-HIV, anticoagulant, spasmolytic and antibacterial activity among others. Of the many actions of coumarins, antioxidant and antiproliferative effects stand out. A large number of structurally novel coumarin derivatives have ultimately been reported to show substantial cytotoxic activity in vitro and in vivo.

Subsequent analysis of scientific literature revealed numerous reports on the antiproliferative and antitumor activities of a variety of coumarin compounds, e.g. both coumarin itself and 7-hydroxycoumarin have been reported to inhibit the proliferation of a number of human malignant cell lines in vitro [1–4] and have demonstrated activity against several types of animal tumors [5–9]. These compounds have also been reported in clinical trials to demonstrate activity against prostate cancer, malignant melanoma, and metastatic renal cell carcinoma [10–12].

For coumarins, generally the in vitro structure-activity relationship studies have shown that cytotoxicity is found with derivatives containing ortho-dihydroxy substituents (Kolodziej et al., 1997 [13]). Also, the chemical-structure/biological activity study of the coumarins showed that the addition of a cathecolic group to the basic structure induces increased cytotoxic activity in tumor cell lines (Kolodziej et al., 1997 [13]). The different cytotoxic values found for the coumarins could

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be related to presence and the positions of the hydroxyls in their structures.

The complexes of rare earth ions have aroused much interest. Lanthanides are a subject of increasing interest in bioinorganic and coordination chemistry [14,15].

Nowadays, a lot of studies report complexes of coumarin derivatives with rare earth metals, which possess biological activity. Thus, lanthanide complexes of 3-sulfo-4-hydroxycoumarin [16] and bis-(4-hydroxy-3-coumarinyl)-acetic acid [17] have been synthesized and characterized. The complexes have revealed good anticoagulant action.

Lanthanides manifest an antitumor activity. Furthermore, literature data show that the coumarins have also these properties. These previous data from literature are in accordance with our investigations. They give our reason to suppose that complexes of coumarins with cerium could present interesting metalorganic compounds with antitumor activity. As a result from our earlier work the cytotoxic profile of some complexes of Mendiaxon, Warfarin, Coumachlor and Niffcoumar with lanthanides against P3HR1, K-562 and THP-1 cell lines was proved [18–23]. The complexes of cerium, lanthanum and neodymium with these coumarin ligands induced approximately 30% reduction of the survival P3HR1 Burkitt lymphoma cells at concentration 100 and 400 μM. The cerium and lanthanum complexes of Mendiaxon and Niffcoumar induce similar low cytotoxic effect on AML derived THP-1 myeloleukemia cells. With the relatively resistant CML-derived erythroleukemic K-562 cell line we obtained very interesting in vitro results. It is noteworthy that the lanthanide complexes with Niffcoumar exert pronounced cytotoxic effects. They have a strong cell proliferation inhibiting effects (only about 30% of the cells were survival). This means that the resistant tumor cells may be very good inhibited with lanthanide complexes. This means also that the spectrum of cytotoxicity of these complexes is different from cis-DDP (II) and from Pt (II) complexes. These results are of some interest as a possibility to influence of resistant tumors. The corresponding lanthanide salts are found to be of very low or missing activity. So far we can conclude that the structure metal–ligand determines the antitumor spectrum of the newly complexes. Those in vitro effects are not so clearly expressed as it is in the case of cis-DDP (II). Nevertheless their studying is interesting in connection with other cell lines and tumors in order to find out the differences in their spectrum of activity.

Unfortunately, little is known about the complexing ability of cerium (III) with coumarins. A survey of the literature reveals that no work has been done on the reactions of cerium (III) with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) and its derivatives. 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 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-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- and IR-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 to coordination modes of the ligands have been assigned and discussed.

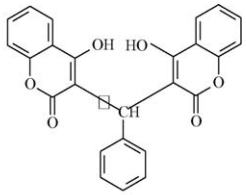
We observed that Ce (III) possess a cytotoxic activity and literature data show that the coumarins have also these properties. That is why our synthesis of complexes of Ce (III) is taken into consideration with cytotoxic screening and further pharmacological study.

2. Chemistry

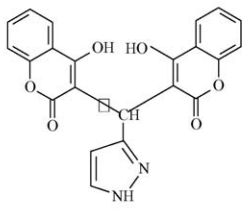
The compounds used for preparing the solutions were Merck products, p.a. grade: $Ce(NO_3)_3{\cdot}6H_2O$. 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane were used for the preparation of metal complexes as ligands (Scheme 1). These ligands were synthesized by us. They were obtained by condensation of 4-hydroxycoumarin and aromatic (Scheme 2) or heterocyclic (Scheme 3) aldehyde in ethanol medium at reflux and stirring until crystals appeared.

The complexes of cerium (III) with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) (H_2L1) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (H_2L2) were synthesized by reaction of cerium (III) salt and the ligands.

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

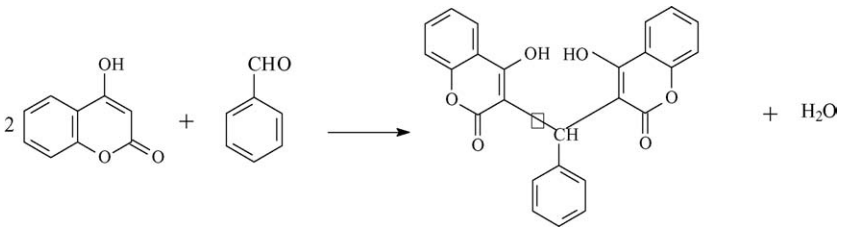


H_2L1= 3,3'-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one)

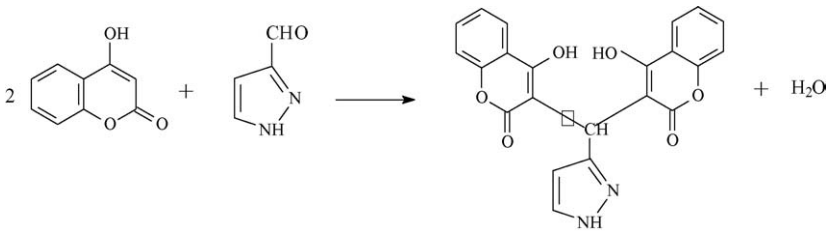


H_2L2= bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane

Scheme 1. Structures of the ligands.



Scheme 2. Synthesis of 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one.



Scheme 3. Synthesis of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane.

3. Pharmacology

In the present study we performed comparative evaluation of the cytotoxic effects of the two 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 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) were evaluated on the CML-derived K-562 and LAMA-84 cells, characterized by relative low responsiveness to chemotherapy.

In order to shed some light over the mechanistic particulars engaged in the cytotoxic mode of action the cerium complex of 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) we carried out DNA-isolation and gel electrophoresis to evaluate its ability to trigger apoptotic cell death.

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-, ^{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 formulae. 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. IR spectra of the complexes

The mode of bonding of the ligands to Ce (III) 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 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) (H_2L1) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (H_2L2) and of the cerium complexes with these ligands are presented in Table 3.

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

	Found/calculated				
Complex	% C	% H	% N	% H_2O	% Ce
$Ce(L1)(OH)\cdot 2H_2O$	50.13	3.41	–	6.08	23.05
	49.75	3.15	–	5.97	23.22
$Ce(L2)(OH)\cdot H_2O$	46.09	3.02	5.16	3.42	23.98
	45.91	2.61	4.87	3.13	24.35

$L_1 = C_{25}H_{14}O_6^{2-}$. $L_2 = C_{22}H_{12}N_2O_6^{2-}$.

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

Ligand	*m/z*	(%)	Complex	*m/z*	(%)
$H_2L1=C_{25}H_{16}O_6$	412	8	$Ce(L_1)(OH)\cdot 2H_2O$	586	8
	249	100		410	35
	221	17		305	98
	162	20		176	100
	120	37			
$H_2L2=C_{22}H_{14}N_2O_6$	402	0	$Ce(L_2)(OH)\cdot H_2O$	580	1
	241	16		490	3
	240	100		460	3
	162	72		410	1
	120	74		307	52
	92	98		176	100

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

Compound	νOH/H_2O	ν(C=O)	ν(C=C)	ν(Py)	ν(Ar)	δ(COH)	ν(C–O)	
$H_2L1=C_{25}H_{16}O_6$							1182m	
	3074m	1660s	1605s	–	1496m	1345m	1160m	772
	3032m	1617s	1568s			1336m	1092s	750
							1074m	
$Ce(L_1)(OH)\cdot 2H_2O$							1190w	
	3400br	1620sh	1508s	–	1451m	–	1152w	758
		1599s					1108m	
							1064w	
$H_2L2=C_{22}H_{14}N_2O_6$				1620			1187m	
	3139m	1669s	1610s	1559	1496m	1360m	1150m	770
	3070m	1635s	1539s	1507		1300m	1110s	748
				1417			1044m	
$Ce(L_2)(OH)\cdot H_2O$				1622			1194w	
	3390br	1652sh	1520s	1559	1460m	–	1145w	758
		1599s		1506			1109m	
				1420			1054w	

Broad bands, characteristic of ν_{OH} of coordinated water were observed in the range 3300–3400 cm^{-1} in the spectra of the complexes. The weak bands observed at 3074 and 3032 cm^{-1} in the spectrum of H_2L1 and at 3139 and 3070 cm^{-1} in the spectrum of H_2L2 are missing in the spectra of the complexes. A comparison of the infrared spectra of the ligands and of the complexes reveals the disappearance of absorption bands observed in the free ligands 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 at 1660 and 1617 cm^{-1} (H_2L1) and at 1669 and 1635 cm^{-1} (H_2L2) exhibit a shift of 30–40 cm^{-1} to lower wavenumber values on complexation which may be taken as evidence 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 complex. Similar frequency shifts are observed for the other complexes and are attributed to complexation of the positive ion with the carbonyl oxygen [24].

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, which have been assigned to the rocking, waggling and metal–oxygen stretching vibrations.

4.3. ^{1}H- 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 ^{1}H- and ^{13}C-NMR spectra.

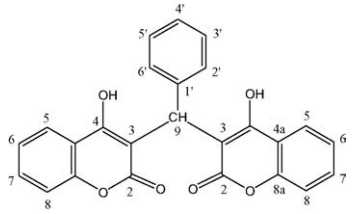
Proton spectra of the compounds recorded at 250 MHz in DMSO-d_6, confirmed the formation of the complex. The typical chemical shifts of the ^{1}H-NMR spectra in DMSO-d_6 are presented in Table 4. As it is seen from Table 4, different chemical shifts were observed in the complexes and these changes were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

^{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 5.

The ligand 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) (H_2L1) showed seven signals in the ^{13}C-NMR spectra resonating at δ 131.91, 128.08, 126.70, 125.58, 123.92, 123.76 and 115.95 ppm for 13 methine carbons (Table 5). In agreement with literature data, the peaks at δ 131.91, 123.92, 123.76 and 115.95 ppm were related to C-7, C-5, C-6 and C-8 (the atom numbering is in agreement with the scheme in Table 4 carbons, respectively, of the coumarin moieties. The signals at δ 128.08, 126.70 and 125.58 ppm were assigned to C-3′ (and C-5′), C-4′ and C-2′ (and C-6′) carbons of the phenyl ring. The chemical shifts at δ 165.36, 164.87, 152.23, 139.94, 117.96 and 104.13 ppm are due to the C-2, C-4, C-8a, C-1′, C-4a and C-3 quaternary carbons, respectively. Due to electron transfer from the hydroxyl and carbonyl oxygen atoms to Ce (III), a difference in chemical

Table 4
^{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]
$H_2L1=C_{25}H_{16}O_6$	7.11–7.39	6.37	7.56–7.92
$Ce(L_1)(OH)\cdot 2H_2O$	7.05–7.24	6.25	7.45–8.18
$H_2L2=C_{22}H_{14}N_2O_6$	7.23–7.55	6.36	7.83–8.15
$Ce(L_2)(OH)\cdot H_2O$	7.19–7.79	5.76	8.18–8.70



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

Table 5
^{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	$Ce(L_1)(OH)\cdot 2H_2O$	H_2L2	$Ce(L_2)(OH)\cdot H_2O$
C-2	165.3	167.8	167.9	170.0
C-4	164.9	164.7	163.9	157.4
C-8a	152.2	152.5	152.7	156.4
C-1′	139.9	142.3	150.5	152.6
C-7	131.9	130.9	134.3	131.0
C-3′	128.1	127.7	131.6	129.9
C-5′	128.1	127.7	–	–
C-4′	126.7	126.6	–	–
C-6′	125.6	124.8	–	–
C-2′	125.6	124.8	126.1	124.2
C-5	123.9	124.1	124.3	123.4
C-6	123.8	122.9	123.3	122.9
C-4a	117.9	119.9	119.5	120.8
C-8	115.9	115.4	115.8	116.7
C-3	104.1	103.4	101.4	103.0
C-9	35.9	36.5	30.1	35.5

shifts was observed for the neighboring C-3 and C-2 carbon atoms of the complex and they confirmed the expected coordination of the ligand through both deprotonated hydroxyl and carbonyl oxygen atoms. The other carbon atoms were only slightly affected from the coordination of the metal. Similar chemical shifts were observed for the other ligand and its complex (Table 5). On the basis of the results thus obtained, it was suggested that the ligands act as tetradentate ones in the Ce (III) complex formation.

4.4. Pharmacology

4.4.1. In vitro cytotoxicity

The spectrophotometric data regarding the MTT-dye reduction assay are summarized in Tables 6–7

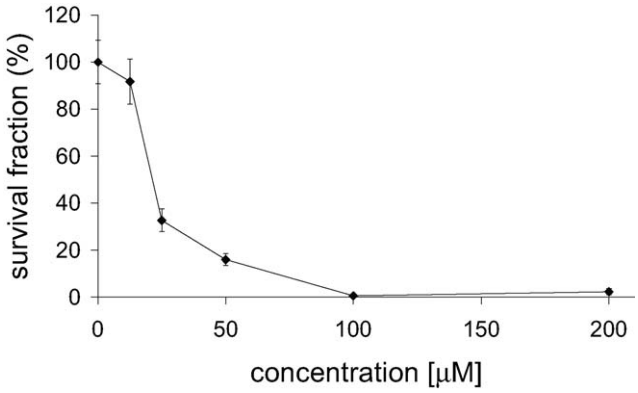


Fig. 1. Cytotoxic effects of cerium complex of 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one on the AML-derived HL-60 cells as assessed by the MTT-dye reduction assay following 72 h treatment of HL-60. Each data point represents the arithmetic mean least six independent experiments; the error bars represent the standard deviation.

The evaluation of the cell viability following 72 h treatment with cerium complexes revealed that the newly synthesized cerium complexes exerted cytotoxic effects in a concentration-dependent manner against HL-60 and BV-173 cells. The concentration–response curves drawn are presented on Figs. 1–4, whereas the corresponding IC_{50} are summarized in Table 8.

As evident from the concentration–response curve depicted on Fig. 1 cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) caused a drastic decrease of the viability of HL-60 at 25 μM by approximately 67%. At the higher concentration of 50 μM it decreased the cell survival fraction to ca. 16%, whereas at concentrations over 100 μM an almost total eradication of viable HL-60 cells was encountered. The other compound under investigation cerium complex with bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane failed to induce any cytotoxic effects

Table 6
Spectrophotometric data from the MTT assay concerning the cytotoxic effects of the newly synthesized cerium complexes with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) (Ce-1) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (Ce-2) on HL-60 and BV-173 leukemic cells

Cell line	Compl	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	0.735 ± 0.077	0.262 ± 0.039	0.128 ± 0.021	0.005 ± 0.002	0.019 ± 0.010
	Ce-2	0.737 ± 0.051	0.876 ± 0.065	0.920 ± 0.054	0.868 ± 0.044	0.730 ± 0.066	0.504 ± 0.035
BV-173	Ce-1	1.046 ± 0.078	1.116 ± 0.065	0.974 ± 0.068	0.249 ± 0.039	0.059 ± 0.014	0.016 ± 0.009
	Ce-2	0.986 ± 0.067	1.042 ± 0.029	0.947 ± 0.052	0.891 ± 0.035	0.766 ± 0.055	0.066 ± 0.025

Table 7
Spectrophotometric data from the MTT assay concerning the cytotoxic effects of the cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) (Ce-1) on the CML-derived LAMA-84 and K-562 cells

Cell line	MTT-formazan absorption at 580 nm					
	Untreated control	12.5 μM	25 μM	50 μM	100 μM	200 μM
LAMA-84	1.105 ± 0.044	1.093 ± 0.062	0.865 ± 0.053	0.135 ± 0.026	0.070 ± 0.018	0.066 ± 0.013
K-562	1.335 ± 0.058	1.531 ± 0.069	1.507 ± 0.055	1.039 ± 0.022	0.550 ± 0.048	0.382 ± 0.036

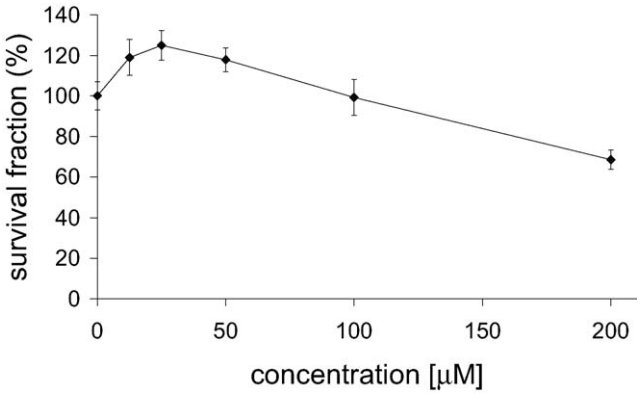
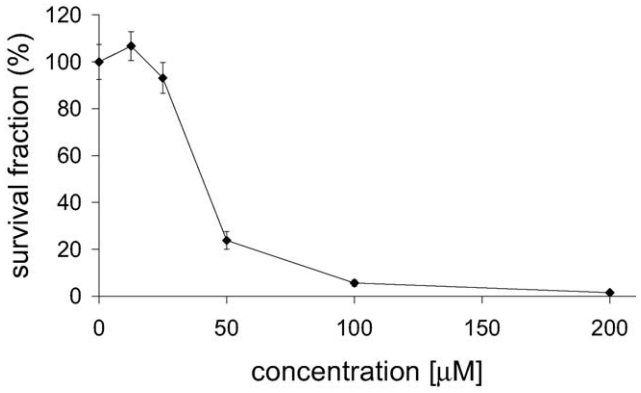


Fig. 2. Cytotoxic effects of cerium complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane on the AML-derived HL-60 cells as assessed by the MTT-dye reduction assay following 72 h treatment of HL-60. Each data point represents the arithmetic mean least six independent experiments; the error bars represent the standard deviation.



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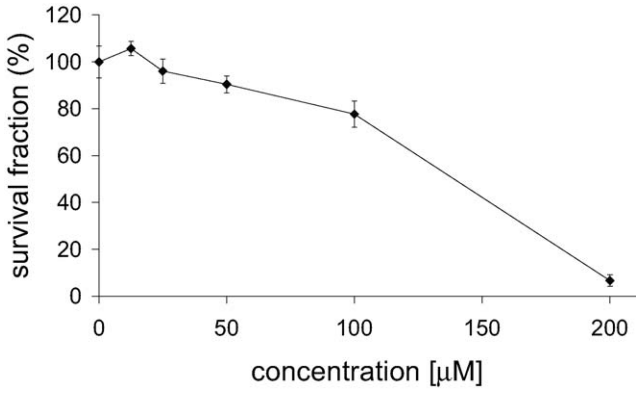

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Table 8
Relative potency of the investigated compounds in the panel of human tumor cell lines, following 48 h treatment

Cell line	IC_{50} value (μM)	
	Ce-1	Ce-2
HL-60	21.37	> 200
BV-173	40.52	138.92
K-562	87.74	n.d.
LAMA-84	35.42	n.d.

n.d.: not detected.

when applied in concentrations up to 100 μM and even a certain stimulation of the malignant cell proliferation was encountered within this concentration range. At the highest concentration exploited herein cerium complex with bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane decreased the viable cells to ca. 68%.

The evaluation of the effect of cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) on BV-173 showed a concentration-dependent cytotoxicity at concentrations exceeding 25 μM. At a concentration of 50 μM cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) reduced the cell survival fraction to ca. 24%, whereas at 100 μM the viable cells where ca. 6%. At the highest concentration evaluated of 200 μM the viable cells where almost completely abolished. The other cerium complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane also exhibited cytotoxic effect against BV-173 cells , although far less pronounced than that of cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one). At concentrations up to 100 μM only marginal decrease of the cell viability was encountered, whereas at the highest concentration applied (200 μM) the cell survival fraction was reduced by ca. 93%.

The profound cytotoxic activity of cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) against HL-60 and BV-173 cells encouraged us to probe its efficacy against the chronic myeloid leukemia derived LAMA-84 and K-562 cell lines. These cells express the characteristic for CML BCR-ABL protein, that represents a non-receptor tyrosinekynase, conditioning a relatively low responsiveness of K-562 and LAMA-84 cells to pro-apoptotic stimuli incl. treatment with chemotherapeutic agents. Interestingly, cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) caused concentration-dependent cytotoxic effects on both K-562 and LAMA-84 cells, although far more pronounced with the latter cell line (Figs. 5 and 6).

4.4.2. DNA-fragmentation analysis

The DNA isolated from the cytosolic fraction of BV-173 cells after 24 h treatment with cerium complex of 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) (at 100 and 200 μM) demonstrated a laddering phenomenon that is indicative for apoptotic cell death. The effects were more pronounced at the higher concentration of the complex under investigation. These finding suggest for that the capability of

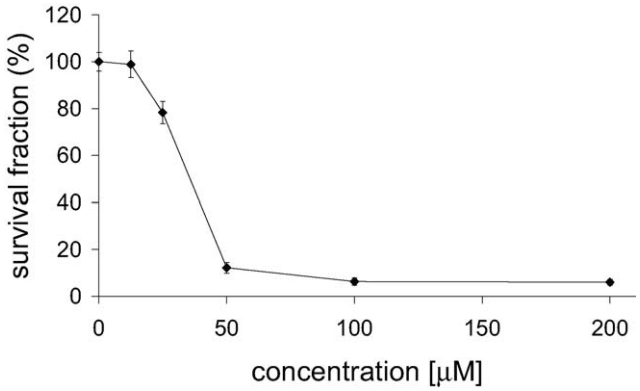

Fig. 5. Cytotoxic effects of cerium complex of 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one on the CML-derived cell line LAMA-84 as assessed by the MTT-dye reduction assay following 72 h treatment. Each data point represents the arithmetic mean least six independent experiments; the error bars represent the standard deviation.

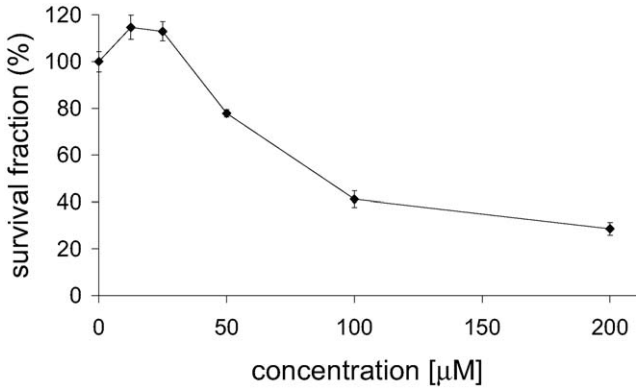

Fig. 6. Cytotoxic effects of cerium complex of 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one on the CML-derived cell line K-562 as assessed by the MTT-dye reduction assay following 72 h treatment. Each data point represents the arithmetic mean least six independent experiments; the error bars represent the standard deviation.

cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) to trigger activation of the apoptotic cellular machinery contributes to the observed cytotoxic effects on BV-173 (Fig. 7).

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-, ^{13}C-NMR- and IR- 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.

In our hands the two novel cerium complexes under investigation exhibited in vitro cytotoxic effects in micromolar concentrations. The cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) however demonstrated far more pronounced cytotoxic effects as compared to the cerium complex with bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane. On the basis of the observed considerable cytotoxic activity of cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) on K-562 and LAMA-84 cells, together with its documented ability to trigger programmed cell death it could be concluded that cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) deserves further detailed pharmacological and toxicological evaluation.

Fig. 7. Electrophoregram of cytosolic fraction—DNA, isolated from untreated BV-173 cells (lane1) and following 24 h treatment with cerium complex of 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one at 100 μM (lane 2) or 200 μM (lane 3).

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.

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 a 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.

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

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 cerium (III) with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) (H_2L1) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (H_2L2) 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 tumor cell lines HL-60, BV-173, LAMA-84 and K-562 exploited in the present study were supplied from 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 refed with fresh RPMI-1640 medium two or three times per week.

The stock solutions of the new cerium complexes were prepared in DMSO at 20 mM and consequently diluted in RPMI-1640; less than 1% of the DMSO was available at the final dilutions obtained.

All of the procedures concerning the cell culture maintenance, drug dissolution and treatment were carried out in a 'Heraeus' Laminar flow cabinet.

6.2.2. Cytotoxicity determination (MTT assay)

The MTT-dye reduction assay was carried out as previously described [25] with some minor modifications [26]. 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 to cerium complexes with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-(1H-pyrazol-3-yl)-methane (12.5–200 μM) 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 by means of 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. DNA-isolation and gel electrophoresis

The DNA extraction and horizontal submarine gel electrophoresis procedures were carried out as previously described [26]. About 5×10^6 BV-173 cells-treated with cerium complex with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) (at 100 or 200 μM) and untreated controls, were washed in PBS and spun at 2000 rpm for 5 min. The cell pellets were re-suspended in 0.25 ml PBS and lyzed through addition of 0.5 ml buffer containing 0.5% Triton X-100, 20 mM Tris–HCl and 1 mM EDTA (pH 7.4). Samples were incubated at 0 °C (on ice) for 5 min and thereafter spun at 13,000 rpm for 20 min. The supernatants were transferred into 2 ml 'safe lock' test tubes and then 0.937 ml 2-propanol as well as 0.187 ml 6 M solution of NaCl were added to each sample. The tubes were gently agitated and incubated at –20 °C for 12 h in order to allow precipitation of the hydrophillic DNA. The samples were centrifuged for 20 min at 13,000 rpm, the supernatants were decanted and DNA was washed in 1 ml ice cold 70% ethanol and then air dried. The isolated DNA was re-dissolved in 20 μl distilled water and analyzed by gel electrophoresis in 0.8% agarose gel. Finally DNA was stained with ethidium bromide and 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.

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