

Synthesis, physicochemical characterisation and cytotoxic screening of new complexes of cerium, lanthanum and neodymium with Warfarin and Coumachlor sodium salts

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(Received 20 November 1997; accepted 17 June 1998)

Abstract – The complexes of the types $\text{Ln}(\text{HW})_3 \cdot n\text{H}_2\text{O}$ and $\text{Ln}(\text{HC})_3 \cdot n\text{H}_2\text{O}$ [where $\text{Ln} = \text{Ce}, \text{La}, \text{Nd}$; $\text{HW} = \text{Warfarin}$; $\text{HC} = \text{Coumachlor}$] have been synthesized by reaction of Warfarin Sodium, resp. Coumachlor Sodium and the appropriate lanthanide nitrates in various stoichiometric ratios. The formation of the complexes have been proved on the basis of elemental analysis, conductivities, IR spectroscopy and $^1\text{H-NMR}$ spectroscopy. It is concluded that the lacton- and the keto-carbonyl groups of the ligands are bonded to the metal ion as bidentate ligand. The conductivity measurements show non-electrolytic nature of the complexes. Cytotoxicity determination by MTT-assay shows that the inorganic salts and the complexes with Warfarin did not show any significant activity. The complexes with Coumachlor were found to be more cytotoxic. The most active compound was the complex of Cerium with Coumachlor. All tested complexes showed similar in vitro cytotoxic profiles. This fact is in agreement with the formation of the complexes. © Elsevier, Paris

metal complexes of Warfarin / Coumachlor / lanthanides / cytotoxic screening

1. Introduction

The complexes of rare earth ions have aroused much interest [1–3]. Lanthanide ion is a subject of increasing interest in bioinorganic and coordination chemistry [4–6]. Although data on studies in solution are available on transition metal complexes of coumarin derivatives, particularly with 3-acetyl-7-methoxycoumarin [7], 3-benzoyl-4-hydroxycoumarin [8], 3-phenyl-4,5,7-trihydroxycoumarin [9], 3-sulfo-4-hydroxycoumarin [10], 6-chloro-4-hydroxy-3-acetylcoumarin [11], 4-hydroxy-3-mercaptocoumarin [12], no studies in the solid state have been made on the metal complexes of Warfarin Sodium and Coumachlor Sodium with Ce(III) , La(III) and Nd(III) metal ions.

The present paper describes the preparation, isolation and characterization of Warfarin Sodium and Coumachlor

Sodium complexes. 4-Hydroxycoumarin derivatives are known for their antibacterial, antifungal activities [13] and significant anticoagulant action, especially the anticoagulant Warfarin and its derivatives [14].

The present work is in continuation of the authors' earlier work [15] on complexes of cadmium, copper and zinc with Warfarin Sodium. Coumachlor Sodium is not used as a ligand up to now. Our synthesis of complexes with lanthanides (III) is taken into consideration with cytotoxic screening and further pharmacological study.

Lanthanum chloride [16, 17] manifests an antitumor activity. Furthermore, literature data show that the coumarins have also these properties [18]. Coumarin and its 4-hydroxy and 7-hydroxy derivatives, as well as o-, m- and p-coumaric acid were tested against P-815 and P-388 tumor cells in vitro. All compounds were more or less cytotoxic against tumor cells [19]. The effect of Warfarin on tumor cell growth was studied [20]. Warfarin inhibits metastasis of Mtn3 rat mammary carcinoma without

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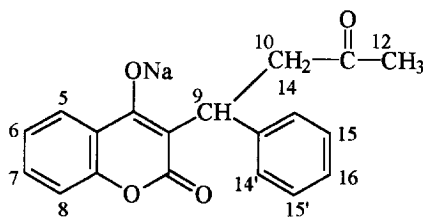
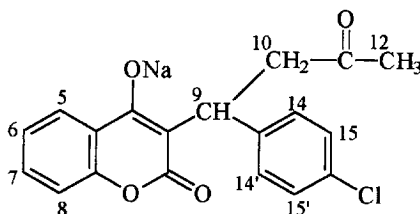
^aW - Warfarin Sodium^bC - Coumachlor Sodium

Figure 1. Structures of (a) Warfarin Sodium and (b) Coumachlor Sodium.

affecting primary tumor growth. 3,7-Diamino-4-hydroxy-coumarin is useful as an antitubercular agent [21]. Akman et al. had investigated synergistic cytotoxicity between menadione and the related anticoagulant Dicumarol, inhibited growth of murine leukemia L1210 in liquid suspension culture [22].

2. Chemistry

The compounds used for preparing the solutions were Merck products, p.a. grade: $\text{Ce}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$, $\text{La}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ and $\text{Nd}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$. The sodium salt of 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one (Warfarin Sodium) and the sodium salt of 4-hydroxy-3-[1-(4-chlorophenyl)-3-oxobutyl]-2H-1-benzopyran-2-one (Coumachlor Sodium) (figure 1) were prepared by reported procedure [14] and their purities were checked by TLC and melting point determination. They were used for the preparation of metal complexes as ligands.

All the complexes were obtained with the following general procedure: we synthesized the complexes by

Table I. Melting points of the complexes.

Complex	M.p. (°C)
$\text{Ce}(\text{HW})_3 \cdot 4\text{H}_2\text{O}$ ^a	235
$\text{La}(\text{HW})_3 \cdot 4\text{H}_2\text{O}$	> 300
$\text{Nd}(\text{HW})_3 \cdot 4\text{H}_2\text{O}$	> 300
$\text{Ce}(\text{HC})_3 \cdot 5\text{H}_2\text{O}$ ^b	260
$\text{La}(\text{HC})_3 \cdot 5\text{H}_2\text{O}$	> 300
$\text{Nd}(\text{HC})_3 \cdot 4\text{H}_2\text{O}$	> 300

^a HW = $\text{C}_{19}\text{H}_{16}\text{O}_4$; ^b HC = $\text{C}_{19}\text{H}_{15}\text{ClO}_4$.

mixing water solutions of lanthanide nitrates and the ligands in amounts equal to metal–ligand ratio of 1:2. At the moment of mixing of the solutions precipitates were obtained. The reaction mixture was stirred at 25 °C for one hour. The products thus obtained were separated from the solutions at pH 4–5, filtered off, washed three times with water and dried in a desiccator to constant weight.

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

The complexes were characterized by elemental analysis and were analysed for their metal contents employing standard procedures after destroying the organic matter. The presence of the sodium ions was checked up by means of flame photometry. The water content in the complexes was determined by Karl Fisher analysis. The structure of the compounds was confirmed by IR and ¹H-NMR spectroscopy. The compounds were investigated by thin layer chromatography and the results showed that the complexes were pure.

Table I shows the data of the elemental analysis of the compounds obtained, serving as a basis for the determination of their empirical formulas and the results of the Karl Fisher analysis and of the flame photometry analysis. Besides analytical data, the melting points of the compounds are reported in table I. The complexes are high melting solids and they decompose before their melting temperatures. All complexes behave as non-electrolytes ($\lambda_m < 14 \text{ Ohm}^{-1} \text{ cm}^2 \text{ mol}^{-1}$).

3. Pharmacology

3.1. Colorimetric MTT (tetrazolium) assay

In principle, this assay is based on the cellular reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] by the mitochondrial dehydrogenase of viable cells to a blue formazan product. The production of formazan can be measured spectrophotometrically following solubilization. We use the method described by Mossman [23] with some modifications.

Table II. Characteristic IR spectral bands of the compounds.

(a) Some bands of the spectra of Warfarin Sodium and its complexes.

Substance	ν_{OH}	$\nu_{\text{C=O}}$ (lacton)	$\nu_{\text{C=O}}$ (keto)	$\nu_{\text{C=C}}$
$\text{C}_{19}\text{H}_{15}\text{NaO}_4$	3562	1721	1668	1634–1597
$\text{Ce}(\text{HW})_3 \cdot 4\text{H}_2\text{O}$	3280	1685	1620	1580–1500
$\text{La}(\text{HW})_3 \cdot 4\text{H}_2\text{O}$	3273	1682	1618	1572–1495
$\text{Nd}(\text{HW})_3 \cdot 4\text{H}_2\text{O}$	3394	1695	1651	1580–1505

(b) Some bands of the spectra of Coumachlor Sodium and its complexes.

Substance	ν_{OH}	$\nu_{\text{C=O}}$ (lacton)	ν_{arom}		
$\text{C}_{19}\text{H}_{14}\text{NaClO}_4$	3400	1722	1522–1460	–	546
$\text{Ce}(\text{HC})_3 \cdot 5\text{H}_2\text{O}$	3300	1700	1510–1456	578	534
$\text{La}(\text{HC})_3 \cdot 5\text{H}_2\text{O}$	3204	1695	1510–1456	576	535
$\text{Nd}(\text{HC})_3 \cdot 4\text{H}_2\text{O}$	3378	1693	1514–1455	578	534

P3HR1 Burkitt lymphoma cells were seeded in 96-well plates (100 mL/well at a density of 1×10^5 cells/mL) and exposed to various concentrations of the tested compounds and cis-DDP(II) as reference compound. After incubation for 72 h, the 2 mg/mL MTT solution in PBS was added to each well and further incubated for 4 h at 37 °C. The formazan crystals formed were dissolved by adding 100 mL/well of 5% formic acid in 2-propanol. After a few minutes at room temperature to ensure that all crystals were dissolved absorption was measured by an ELISA reader, using a test wavelength of 580 nm. For each concentration at least 8 wells were used. Cell growth inhibition (T/C) was calculated according to $[\text{OD of drug treatment}/\text{OD of control}] \times 100$. Data processing was executed with Microsoft Excel, Microsoft Word and Sigma Plot Windows.

4. Results and discussion

The analytical data of the complexes (table I) are in agreement with the composition $\text{Ln}(\text{HW})_3 \cdot n\text{H}_2\text{O}$ and $\text{Ln}(\text{HC})_3 \cdot n\text{H}_2\text{O}$.

The mode of bonding of the ligands to Ce(III), La(III) and Nd(III) ions was elucidated by recording the IR spectra of the complexes as compared with those of the free ligands.

IR spectra of the compounds were recorded on solid state in Nujol in the range from 3600 to 400 cm^{-1} (table II).

4.1. IR spectra of the complexes of Warfarin Sodium

The bands appearing in the IR spectrum of the ligand at 3562; 1721; 1668; 1634, 1597; 1518, 1461; 1265,

1223; 1163; 1093 are shifted in the complexes. A band at 1721 cm^{-1} can be attributed to the stretching vibrations of the carbonyl group of the lacton ring. The band at 1668 cm^{-1} corresponds to keto-group. Two bands at 1634 and 1597 cm^{-1} can be related to the stretching vibrations of the conjugated olefinic system. The vibrations in the region 1518–1461 cm^{-1} correspond to the aromatic compounds system.

A broad band, characteristic of ν_{OH} of coordinated or crystalline water was observed in the region 3400–3270 cm^{-1} in the spectra of all the complexes. The weak band observed at 3562 cm^{-1} in the spectra of the free ligand shifted to lower wavenumber in the complexes. This assignment is corroborated by the occurrence of the corresponding rocking mode in the region 840–830 cm^{-1} . This is attributed to the presence of crystalline water.

The band at 1163 cm^{-1} assigned as $\nu_{\text{C-OH}}$ is observed at more or less the same position in the complexes and this may be due to the existence of nonionised OH group in all cases.

The $\nu_{\text{C=O}}$ band of the lacton ring at 1721 cm^{-1} exhibits 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 group in coordination.

The most notable change observed upon complex formation is a shift of the C=O band of the keto-group stretched to lower frequency. The same changes were observed with the vibrations of the conjugated olefinic system. The C–C and C–O stretch and the C–O–C band are all shifted to following frequency (1279–1244; 1165 and 1078 cm^{-1}) in the complexes.

Table III. ^1H -NMR spectral data (100 MHz, DMSO- d_6).(a) ^1H -NMR spectra of the Warfarin Sodium and its complexes.

Substance	δ (ppm)				
	$\text{H}_5\text{--H}_8$	H_9	2 H_{10}	3 H_{12}	$\text{H}_{14,15,16}$
W	7.00–7.50 m	4.90 t	3.25 d	2.05 s	7.50–7.80 m
CeW	7.00–7.50 m	5.60 bs	3.20 bs	1.70 bs	7.50–7.90 m
LaW	7.00–7.50 m	4.65 bs	3.25 bs	1.95 bs	7.50–7.90 m
NdW	6.95–7.20 m	6.85 bs	3.45 bs	1.85 bs	7.40–7.70 m

(b) ^1H -NMR spectra of the Coumachlor Sodium and its complexes.

Substance	δ (ppm)				
	$\text{H}_5\text{--H}_8$	H_9	2 H_{10}	3 H_{12}	$\text{H}_{14,15}$
C	6.95–7.30 m	4.90 t	3.27 d	2.05 s	7.55–7.80 d
CeC	6.90–7.30 m	5.50 bs	3.30 bs	1.90 s	7.20–7.50 d
LaC	6.95–7.30 m	4.90 bt	3.27 bd	2.05 s	7.55–7.85 d
NdC	7.35–7.65 m	6.85 bs	3.45 bs	1.90 s	7.15–7.60 d

4.2. IR spectra of the complexes of Coumachlor Sodium

The bands appearing in the IR spectrum of the free ligand at 3400; 1722; 1651; 1624, 1597; 1522, 1460; 1265, 1223; 1163; 1088 are shifted in the complexes.

A broad band characteristic of ν_{OH} of crystalline water was observed in the region 3400–3200 cm^{-1} in the spectra of all the complexes and had higher intensity than in the free ligand. This is attributed to the presence of crystalline water.

A band at 1722 cm^{-1} can be attributed to the stretching vibrations of the carbonyl group of the lacton ring and the band at 1651 cm^{-1} corresponds to keto-group of the side chain. Two bands at 1624 and 1597 cm^{-1} can be related to the stretching vibrations of the conjugated olefinic system. The vibrations of the region from 1522 to 1460 cm^{-1} correspond to the aromatic system. The C–C, C–O and C–O–C bands of the ligand are at 1265–1223; 1163 and 1088 cm^{-1} .

The $\nu_{\text{C=O}}$ band at 1722 cm^{-1} exhibits a shift of 20–30 cm^{-1} to lower wavenumber values on complexation, which may be taken as evidence for the participation of the C=O group of the lacton ring in coordination.

There were considerable changes upon complex formation of the C=O band of the keto-group and of the C=C band of the conjugated olefinic system. The vibrations of the aromatic system shifted to lower frequency in the complexes (1510–1455 cm^{-1}). The C–C, C–O and C–O–C bands were all shifted to higher frequency (1280–1228; 1180 and 1091 cm^{-1}) in the complexes. Similar frequency shifts were observed for other com-

plexes and were attributed to complexation of the positive ion with the carbonyl oxygen of coumarin [24].

The data of the IR analysis are presented in *table II* and they are in agreement with the compositions in *table I*.

4.3. ^1H -NMR analysis

Metal ion coordination with ligand by means of oxygen atoms of C=O groups was shown owing to data of ^1H -NMR spectra. Proton spectra of the compounds, recorded at 100 MHz in DMSO- d_6 as a solvent, confirmed the structures. The chemical shifts are given in δ -scale.

The typical chemical shifts of the ^1H -NMR spectra in DMSO- d_6 solvent are shown in *table III*.

The typical values of coupling constants in Hz are: H_9 : 7.0; H_{10} : 7.0; H_{14} : 8.1 and H_{15} : 8.1.

The chemical shifts of the H_9 and H_{10} protons vary in the lanthanide complexes, because of the shift properties of these metals. It is an evidence that in the La (III) complexes there is observable weak negative shift effect on the H_9 and H_{10} . The effect in the Nd(III) complexes is more complicated. It was observed a strong positive shift effect for H_9 and more weak for H_{10} . In the spectra of the Ce(III) complexes the shift effect is also positive for H_9 and for H_{10} .

These data confirm formation of the complexes and the coordination of metal ions with the oxygen atoms of the lacton carbonyl and of the side chain.

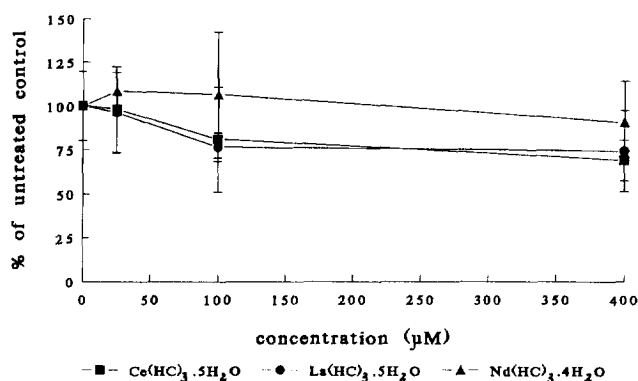


Figure 2. Cytotoxic effect of complexes of Coumachlor on P3HR1 cells (MTT-assay).

5. Conclusion

Pharmacological data from MTT-assay (*figure 2*) show that the complexes of Lanthanum and Cerium with Coumachlor induce approximately 25% reduction of the survival cells at concentration 100 and 400 μM . The complex of Neodymium with Coumachlor have not shown cytotoxic effect. Also, the inorganic salts and the complexes with Warfarin are inactive on P3HR1 cell line. Reference compound *cis*-DDP(II) has a strong growth

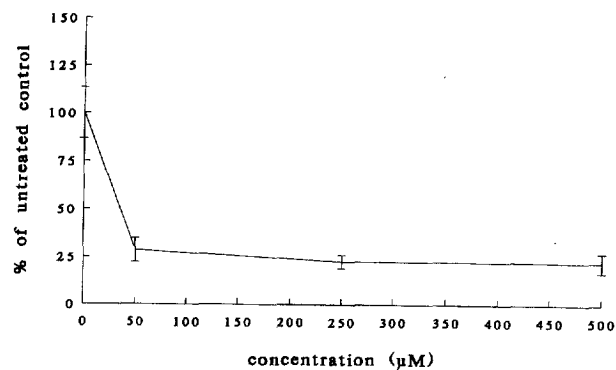


Figure 3. Cytotoxic effect of *cis*-DDP on P3HR1 cells (MTT-assay).

inhibitory effect: only about 20–30% of the cells are surviving (*figure 3*). The IC_{50} value is determined as 37 μM . These screening data confirm our previous observations that lanthanide complexes have some potential cytotoxicity [25]. Also for a first time we observe that cerium complexes can be cytotoxic. 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 tumours in order to find out the differences in their spectrum of activity (*table IV*).

Table IV. Spectrophotometrical data from MTT assay of the lanthanide complexes in comparison with the inorganic salts.

Compound	MTT-formazan absorbtion at 580 nm			
	Control	25 μM	100 μM	400 μM
Ce(NO ₃) ₃ •6H ₂ O	0.3936 ±0.0672	0.4835 ±0.0419	0.4216 ±0.0511	0.3649 ±0.0823
La(NO ₃) ₃ •6H ₂ O	0.3936 ±0.0672	0.4429 ±0.0579	0.4258 ±0.0801	0.4124 ±0.0775
Nd(NO ₃) ₃ •6H ₂ O	0.3936 ±0.0672	0.4874 ±0.0885	0.4143 ±0.0245	0.3613 ±0.0955
Ce(HW) ₃ •4H ₂ O	0.3332 ±0.0393	0.3649 ±0.0704	0.3466 ±0.0489	0.2795 ±0.0476
La(HW) ₃ •4H ₂ O	0.3332 ±0.0393	0.3681 ±0.0472	0.3138 ±0.0400	0.3503 ±0.0540
Nd(HW) ₃ •4H ₂ O	0.3332 ±0.0393	0.3939 ±0.0698	0.4313 ±0.0545	0.4218 ±0.0548
Ce(HC) ₃ •5H ₂ O	0.4099 ±0.0808	0.4019 ±0.1001	0.3313 ±0.1220	0.2819 ±0.0470
La(HC) ₃ •5H ₂ O	0.4099 ±0.0808	0.3941 ±0.0942	0.3129 ±0.0334	0.3030 ±0.0945
Nd(HC) ₃ •4H ₂ O	0.4099 ±0.0808	0.4441 ±0.0612	0.4354 ±0.1478	0.3700 ±0.0962

6. Experimental protocols

6.1. Chemistry

The carbon, hydrogen and chlorine content of the compounds were determined by elemental analysis.

The water content was determined by Metrohn Herizall E55 Karl Fisher Titrator and the presence of sodium ions was checked up by means of flame photometry.

Melting points were determined by using a Boetius melting point apparatus and are uncorrected.

Conductometric measurement was carried out at 25 °C on 10^{-3} M solutions, in DMSO, by using a Metrohm 660 AG CH-9101 Herisau conductometer with platinum electrode and a cell having cell constant of 0.79 cm^{-1} .

IR spectra (Nujol) were recorded on a IR spectrometer FTIR-8101M Shimadzu.

^1H -NMR spectra were recorded at room temperature on Bruker WP 100 (100 MHz) spectrometer in DMSO- d_6 . Chemical shifts are given in ppm.

General method of synthesis: Warfarin Sodium and Coumachlor Sodium were used for the preparation of metal complexes as ligands (*figure 1*). The complexes were synthesized by mixing a water solution of the ligand with a water solution of the corresponding metal (III) salts in amounts equal to metal/ligand molar ratio of 1:2. The reaction mixture was stirred with an electromagnetic stirrer at 25 °C for one hour. The products thus obtained were separated from the solutions at pH 4–5, filtered, washed with water and dried.

6.2. Biological evaluation

For cell culture was used Laminar Flow cabinet Haereus HV 2436 and CO_2 gassed incubator Haereus BB16. For MTT-assay measurement was used ELISA-reader Labsystems Uniskan I.

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