Cerium(III) and Neodymium(III) Complexes as Scavengers of X/XO-Derived Superoxide Radical

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Abstract: The cerium (III) and neodymium (III) complexes with 3,3′-benzylidenebis[4-hydroxycoumarin] were synthesized and characterized by different analytical and spectral methods. The synthesis of these complexes is taken into consideration with cytotoxic screening and study of their antioxidant effect. Their cytotoxicity toward cancerous cell cultures correlated with the weakness of the coordinative bond between the cation and organic ligand and with the capability to scavenge superoxide radicals as well. On the basis of the data reported by us earlier and our new results, it was proposed that cerium (III) complex with 3,3′-benzylidenebis[4-hydroxycoumarin] might induce intracellular acidification along with control over the extracellular oxidative stress.

Key Words: 4-hydroxycoumarins; lanthanide (III) complexes; cytotoxicity; antioxidant activity, oxidative stress.

INTRODUCTION

Coumarins, an old class of compounds, are naturally occurring benzopyranone derivatives. The pharmacological and biochemical properties and therapeutic applications of simple coumarins depend upon the pattern of substitution. Coumarins have attracted intense interest in recent years because of their diverse pharmacological properties. Among these properties, their cytotoxic effects were most extensively examined. Yet, the results from different coumarins with various tumor lines are contradictory in part. There is still a long way to go until we know which cytotoxic agent will clinically be suitable for what tumor entity for treatment. Their ability to bind metal ions represents an additional means of modulating their pharmacological responses.

The coumarins have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities. The hydroxycoumarins are typical phenolic compounds and, therefore, act as potent metal chelators and free radical scavengers [1-5]. They are powerful chain-breaking antioxidants. The coumarins display a remarkable array of bio-chemical and pharmacological actions, some of which suggest that certain members of this group of compounds may significantly affect the function of various mammalian cellular systems.

Plant phenolics are widely consumed and have received considerable attention as anticarcinogens. Tumor-modulating effects of coumarin antioxidants also have been studied using carcinogens [1-5]. Protective mechanisms include inhibition of prooxidant carcinogenesis by peroxisome proliferators, antipromotion effects, and induction of detoxifying enzymes. The effects of antioxidants in animal studies are complex, however, and also include tumor promotion, carcinogenic, and co-carcinogenic activities. The most attractive candidate anticarcinogens, however, are those that suppress the rate at which initiated cells progress through the promotion-progression-metastasis pathway without appreciable toxicity, since their application does not require knowledge of the initiating carcinogen and, by definition, will not have tumor promotional properties.

The ability to control the amount and the rate of production of hydroxyl radicals may prove useful for examining the cytotoxic effects of hydroxyl radicals generated in biological systems [1]. Nishiyama T. *et al*. [2] compared the antioxidative activities of some hydrocoumarins with those of alpha-tocopherol for the oxidation of tetralin and linoleic acid in a homogeneous solution. Protective effects of coumarins against cytotoxicity induced by linoleic acid hydroperoxide were examined in cultured human umbilical vein endothelial cells [3]. An antioxidant auraptene (7-geranyloxycoumarin) has been reported to have chemopreventive effects on chemically induced carcinogenesis [4, 5]. The results in the study clearly indicate that oral administration of auraptene effectively enhances both macrophage and lymphocyte functions in mice. The study suggested involvement of the immune response in chemically induced carcinogenesis. Coumarin, in combination with cimetidine, has been subjected to separate clinical trials for the treatment of advanced renal cell carcinoma, malignant melanoma, and non-small cell lung cancer [6-8].

Nowadays, a lot of studies report complexes of coumarin derivatives with metals, which possess biological activity. Rare earth metals possess an antitumor activity [9-14]. Furthermore, literature data show that the coumarins have also these properties. These previous data are in accordance with our investigations [9-14]. They give our reason to suppose that complexes of coumarins with lanthanides could present interesting metalorganic compounds with antitumor activity.

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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. The studying of the *in vitro* effects of metal coordination complexes is interesting in connection with different cell lines and tumors in order to find out the differences in their spectrum of activity. Following the above encouraging results we decided to continue our recent investigations regarding anti-oxidant activity of antineoplastic/ cytotoxic synthetic coumarin derivatives, and especially their cytotoxic lanthanide complexes.

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*, as well as antioxidant properties. For that reason the synthesis of lanthanide complexes was taken into consideration with cytotoxicity and antioxidant activity screening, as well as with further pharmacological study. Recently we have reported the synthesis of series of Ce(III), La(III) and Nd(III) complexes of biologically active 4-hydroxycoumarins with very strong cytotoxic effects [15-20]. The most active of these coordination compounds have been chosen by us for further investigations, especially antioxidant ones, in order to find out the reasons for their increased biological activity.

In the present study we perform the coordination ability of 3,3′-benzylidenebis[4-hydroxycoumarin] in complexation reaction with Ce(III) and Nd(III) ions. The obtained lanthanide(III) complexes with 3,3′-benzylidenebis[4-hydro-xycoumarin] were characterized by different analytical, physicochemical and spectral methods. The most sensitive points to coordination modes of the ligand have been assigned and discussed. Recently, we observed that cerium (III) and neodymium (III) complexes with 3,3′-benzyl-idenebis[4-hydroxycoumarin] possessed a strong cytotoxic activity [19-20]. It is well known that a molecule which kills cancer cells *via* apoptotic pathway and eliminates extra-cellular superoxide radicals would be a promising anticancer drug. That is why our investigations on these complexes are taken into consideration with studying of their antioxidant effects. Our purpose was to find out whether the higher cytotoxicity accompanied with a better antioxidant activity.

RESULTS AND DISCUSSION

Characterization of Ce(III) and Nd(III) Complexes

The lanthanide(III) complexes with 3,3′-benzylidenebis [4-hydroxycoumarin], Fig. (**1**) were synthesized by the reaction of lanthanide(III) nitrates and the ligand in aqueous solution, in amounts equal to metal : ligand molar ratio of 1 : 2 [19]. As described previously, the formation of the complexes may be represented by the following equations:

$$L + 2NaOH \rightarrow 2Na^{+} + L^{2-} + 2H_2O \quad (1)$$

$$[Ln(H_2O)_n]^{3+} + H_2O \leftrightarrow [Ln(OH)(H_2O)_{n-1}]^{2+} + H_3O^{+} \quad (2)$$

$$[Ln(OH)(H_2O)_{n-1}]^{2+} + L^{2-} \rightarrow Ln(L^{2-})(OH)(H_2O) + n\text{-}2(H_2O), \quad (3)$$

where Ln = Ce(III) and Nd(III), L = $C_{25}H_{16}O_6$ and L^{2-} = $C_{25}H_{14}O_6^{2-}$.

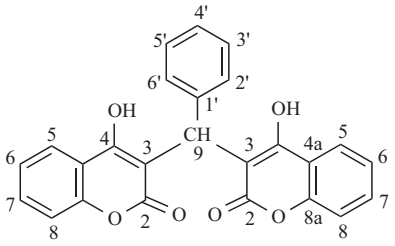

Fig. (1). Chemical structure of the ligand 3,3'-benzylidenebis[4-hydroxycoumarin].

The elemental analysis data of the Ln(III) complexes suggested the formula, $Ln(L^{2-})(OH).nH_2O$, where n= 2; 1 for Ce(III) and Nd(III) complex, respectively. The formula was further confirmed by mass spectral fragmentation analysis, NMR, UV-VIS and IR data. The results obtained were in agreement with metal: ligand ratio = 1: 1.

^{1}H and ^{13}C NMR Spectra

The Ln(III) complexes and 3,3′-benzylidenebis[4-hydroxycoumarin] were studied by their ^{1}H NMR spectra. The changes of chemical shifts of the ^{1}H NMR spectra were observed in the complexes and they were attributed to coordination of the ligand to Ln(III). The chemical shifts of the protons were different in the cerium and neodymium complexes, because of the shift properties of these metals. The ^{1}H NMR spectra of the complexes compared with the ligand spectrum reveal that the resonances due to protons are considerably broadened and also shifted indicating complexation. We considered these shifts as valuable and we used them to confirm the coordination. ^{1}H NMR shifts of this order are typical for coordination compounds of lanthanides and transition metals, as well. ^{13}C NMR spectra also showed valuable shifts and could be considered as a confirmation of the formation of the new compounds. Due to electron transfer from the hydroxyl and carbonyl oxygen atoms to Ln(III), chemical shifts moved to the higher field were observed for the neighboring C-4 and C-2 carbon atoms of the complexes 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. On the basis of the results thus obtained, it was suggested that the ligand acts as a tetradentate one in the Ln(III) complex formation.

UV-VIS Spectra

UV-spectra of DMSO versus PBS showed a single, very intensive band with maximum at 206 – 226 nm. This band shifted to the higher wavelength with increasing of DMSO concentration (from 206 to 218, and 226 for molar concentrations of DMSO of 0.0017, 0.0335 and 0.6710, respectively). Taking into account data for the UV spectrum of DMSO [21], this could be a $\pi \rightarrow \pi^*$ transition band at 188 nm being shifted due to an solvent effect of PBS on DMSO. UV spectra of the metal complexes in (DMSO+ PBS) solution with reference of (DMSO+PBS), the signal of DMSO at 206 – 226 nm should be not seen. The UV spectra of Ce(III) and Nd(III) complexes versus corresponding (DMSO+PBS) so-

lutions, consisted of complex bands, which shapes and intensities depended on the molar concentration of the metal complex, Fig. (**2**). The characteristic bands for the ligands interacting with the metal cations were detected between 410

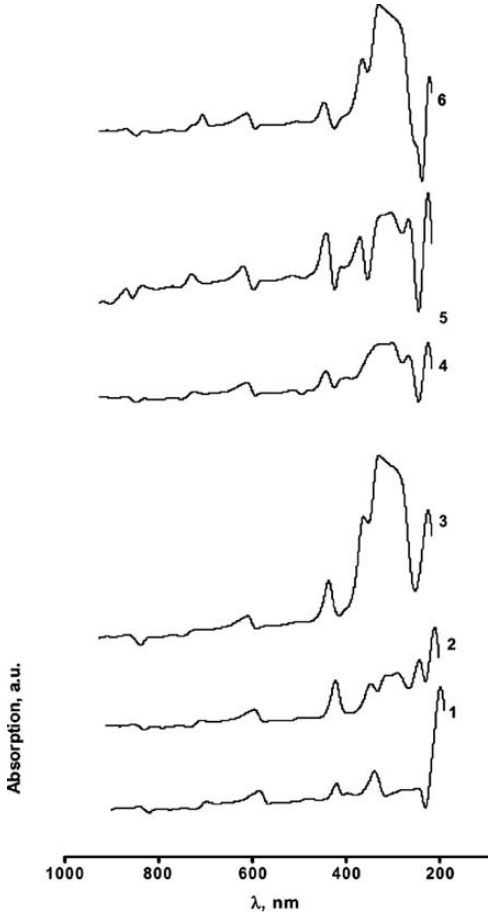

Fig. (2). UV-spectra of Ce(III) and Nd(III) complexes: **1-** 1μM Ce-1, **2-**10 μM Ce-1, **3-** 100 μM Ce-1, **4-** 1 μM Nd-1, **5-** 10 μM Nd-1, **6-** 100 μM Nd-1. Reference cuvettes contained DMSO in PBS, corresponding to DMSO in the sample cuvettes.

and 260 nm. The positions and intensities of the bands are presented in Table **2**. The bands at about 420-400, 334-340, 300 and 260-280 nm could be characteristic bands for the 3,3'-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one molecule, coordinatively bonded in the metal complexes. The band seen at 240-246 nm may be result of superposition of the characteristic band for the 3, 3'-benzylidene group of the ligand and this for *d*→*d* transition of type $^4T1_g(P)\leftarrow{}^4A_{2g}$ [22] of the metal complex. The bands at 303 and 277 nm corresponded to the characteristic rings' vibrations of the OH-influenced benzopyrane-2-one group. The bands at 400 - 420 nm, 330 - 340 nm and 240 - 220 nm, coincided by positions with the *d*→*d* transitions ($^4T_{2g}\leftarrow{}^4A_{2g}$, $^4T_{1g}\leftarrow{}^4A_{2g}$, and $^4T_{1g}(P)\leftarrow{}^4A_{2g}$, respectively), which may be calculated using theory [22]. It is known in the literature that the $^4T_{2g}\leftarrow{}^4A_{2g}$ transition energy (corresponding to the band at 420 – 400 nm) increases (λ shifts to the lower wavelengths) with increasing of the strength of the ligand's field [23] and decreases (λ shifts to the higher wavelengths) with increasing of the solvent's polarity [24]. As the bands 330-340 nm and 220-240 nm in the spectra of Nd(III) complex were very close to the characteristic UV bands of the ligand, their structure and exact positions were difficult for interpretation. As these bands appear in the UV spectra even at very low concentrations of Ce(III) and Nd(III) complexes (at which the characteristic bands for the ligand were very weak, as seen in Fig. (**2**), spectra 1 and 4), it could be proposed that *d*→*d* transitions occurred as components of the bands at 330-340 nm and 220-240 nm in the spectra of both Ce(III) and Nd(III) complexes.

The comparisons between UV spectra of solutions of different Ce(III) complex molar concentrations (Fig. (**2**), Table **2**) permitted to propose, that the disappearance of the band at 240 nm, accompanied by shift of the band at 276 nm to 256 nm seen in spectrum 3 of Fig. (**2**) may be related with submerging of both bands at 240 and 276 nm, due to the high concentration of the complex.

The UV spectra of the solutions of the complex Nd-1 (Fig. (**2**), spectra 4-6) were more complicated than those of the solutions of the Ce-1 (Fig. (**2**), spectra 1-3), especially in the areas where the *d*→*d* transitions lied (420–400, 330-340, and around 220-240 nm).

In the Nd-1 spectra, was observed a doublet at a (419±3), (404±5) nm (Fig. (**2**), spectra 4-6). In the UV spectrum of 10^{-4} M of Nd-1 (Fig. (**2**), spectrum 6) this was accompanied with a splitting of the band at about 200 nm to a doublet with maximums at 247 and 222 nm, and appearance of a low intensive sharp signal at above 600-680 nm. Latter could correspond to a spin-forbidden (such as $^2E_g\leftarrow{}^4A_{2g}$) transition due to a distorted symmetry of the Nd-1 complex in the (DMSO+PBS) solution. The position of the band at 222 nm very well correlated with the position of a $^4T_{1g}(P)\leftarrow{}^4A_{2g}$ type of *d*→*d* transition, calculated with taking into account the signal at about 400 nm. This might be result of an effect of the highly polar DMSO molecule on the Nd(III) complex. Similar effect on the Ce(III)-complex was not detected (Fig. (**2**), spectrum 3). Thus, higher effect of the polar molecule of DMSO on the Nd-1 than on the Ce-1 complex was proposed. If the Nd-1 complex was affected by DMSO, the strength and/or polarity of the bonds in the organic ligand surrounding the metal cation, would alter.

The binding mode of the ligand to Ln(III) ion was further elucidated by analysis of the IR spectra of 3,3′-benzylidenebis [4-hydroxycoumarin] and the complex formation. The very broad low intensive band with maximum at 3600 cm^{-1} indicated a strong intermolecular hydrogen bonding between the OH groups of the free ligand. The splitting of this band into two components in the spectra of Ce(III) and Nd(III) complexes proves that after being incorporated into the complex, the ligand looses its capability to create intermolecular H-bonds. The comparison between Ce(III) and Nd(III) complexes indicates that this is valid in less extent for the ligands around Nd^{3+} than for these surrounding Ce^{3+}. In other words, the shoulder 3680 –3600 cm^{-1} indicates that along with individual OH groups, some intermolecularly hydrogen bonded OH groups exist in the Nd(III) complex. The signals at 3406-3370 cm^{-1} may be assigned to stretching O-H vibrations of

phenyl groups being influenced by the metal cations. The fact that this signal in the spectrum of the Ce(III) complex was positioned at higher frequency than this in the spectrum of the Nd(III) complex, was related with higher vibrational energy of the O-H bonds in Ce(III), than in Nd(III) complex. This indicated that the OH groups responsible for the vibrations in the region 3400-3370 cm^{-1} might be more chemically reactive in the Ce(III) complex than these in the Nd(III) complex. A broad band, characteristic for ν(O-H) of coordinated water was observed in the spectra of the complexes in the range of 3500-3450 cm^{-1}.

The most notable change in the ligand spectral features when coordinated to Ln(III) is the observed C=O red shift. The ν(C=O) band at 1660 cm^{-1} in the ligand spectrum exhibited a red shift of 40 cm^{-1} in the spectra of the complexes. This finding may be taken as evidence for participation of the C=O group in coordination to the metal ion. Further, a comparison between the ligand and complex IR spectra revealed that the absorption bands associated with the stretching ν(O-H) of the phenolic groups (observed at 3074 cm^{-1} and 3032 cm^{-1} in the free ligand) disappeared in the Ln(III) complex spectra, indicating a loss of phenolic protons on complexation and thus forming a metal-oxygen bonds. The δ(COH)i.p. modes, which appeared at 1345 cm^{-1} and 1336 cm^{-1} in the spectrum of the ligand were not observed in the spectra of the complexes with Ln(III) and thus supported the suggestion that the ligand coordinates to the metal through its deprotonated form, L^{2-}. The formation of the Ce(III) and Nd(III) complexes lead to an increase of the characteristic symmetric and asymmetric C-O-C bond vibrations of the ligand in the coumarin structure.

The Ln(III) complex spectra showed new bands, in comparison with that of the free ligand, at 570 cm^{-1} and 410 cm^{-1}, and they were assigned to metal-oxygen stretching vibrations, in agrement with the literature data [17, 19]. The results of the IR spectra were presented in Table **1**.

On the base of the FT-IR spectra of the complexes a higher capability of the Nd(III) than the Ce(III) complex to interact with other molecules by hydrogen bonding might be expected. Also, it might be expected higher chemical reactivity of the OH groups in the Ce(III) than this of the Nd(III) complex. The higher capability of Nd(III) to interact with other molecules *via* hydrogen bonding might be the reason for splitting the $d \rightarrow d$ transition bands of the UV spectrum of this complex, in (DMSO+PBS) solution. The DMSO is a polar molecule which contains two potential electron donors, and is perfectly capable to accept a proton from OH groups of the Nd(III) complex.

Our NMR, UV-VIS and IR spectral data confirmed that the carbonylic and deprotonated hydroxylic oxygen atoms are included in the coordination to the metal ion in the complexes studied.

Pharmacology

The investigations carried out enabled the construction of dose response curves for the newly synthesized complexes and the corresponding inorganic salts. Furthermore the corresponding IC_{50} values were calculated [19].

The data for the cytotoxic efficacy of the tested compounds on HL-60 cells indicate that $Ce(L)(OH).2H_2O$ proved to be the most active cytotoxic compound with IC_{50} value of 21.37 μmol L^{-1}. Even at the lower concentrations of 25 and 50 μmol L^{-1} strong inhibitory effects were registered with ca. 33 and 16% vital cells respectively. At concentrations above 100 μmol L^{-1} the cerium complex caused almost total eradication of the vital cells by more than 97 % [19].

The neodymium complex under investigation also exerted concentration-dependent cytotoxic activity on HL-60 cells, though proved to be less potent than the corresponding cerium analogue on the basis of the IC_{50} value obtained (90.1 μmol L^{-1}) and maximal efficacy (about 25 % of viable cells at 500 μmol L^{-1}) [19].

In contrast to the observed effects of the cerium and neodymium complexes, the corresponding nitrate salts were practically devoid of cytotoxic effects at the same experimental conditions.

The evaluation of the effect of cerium complex with 3, 3'- benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) on

Table 1. Results of the IR Spectra

Compound	νOH/H_2O	ν(C=O)	ν(C=C)	ν (Ar)	δ(COH)	ν(C-O)	
$H_2L=C_{25}H_{16}O_6$	3074m 3032m	1660s 1617s	1605s 1568s	1496m	1345m 1336m	1182m 1160m 1092s 1074m	772 750
$Ce(L)(OH).2H_2O$	3400br	1620sh 1599s	1508s	1451m	-	1190w 1152w 1108m 1064w	758
$Nd(L)(OH).H_2O$	3391br	1625sh 1599s	1505s	1450m	-	1192w 1150w 1109m 1094w	757

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

Table 2. Characteristic Bands in the UV Spectra of Ce(III) and Nd(III) Complexes at Different Molar Concentrations (M)

Solution:	λ, nm(absorbance, a.u.) of samples with reference of (DMSO+PBS)
Ce1 (10^{-6} M)	411(0.11), 334(0.13), 303(0.09), 276(0.09), 246(0.08), 202(0.52)
Ce1 (10^{-5} M)	409(0.22), 334(0.19), 302(0.22), 276(0.22), 240(0.30), 204(0.49)
Ce1 (10^{-4} M)	410(0.25), 335(0.52), 302(0.78), 261(0.71), 199(0.54)
Nd1 (10^{-6} M)	417(0.13), 389(0.08), 341(0.08), 303(0.12), 272(0.23), 241(0.21), 199(0.25)
Nd1 (10^{-5} M)	419(0.16), 384(0.10), 340(0.16), 298(0.20), 277(0.18), 241(0.32), 204(0.25)
Nd1 (10^{-4} M)	422(0.25), 380(0.09), 340(0.35), 303(0.55), 247(0.44), 222(0.07), 198(0.34)

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 neodymium complex 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) [20].

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 [20].

The DNA isolated from the cytosolic fraction of BV-173 cells after 24h 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 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 [20].

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.

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-benzo-pyran-2-one) deserves further detailed pharmacological and toxicological evaluation.

Antioxidant Activity

The efficacy of an antineoplastic drug was related with the ability to kill tumor cells by inducing apoptosis, this way minimizing the negative impact of a tissue-damaging inflammatory response to the necrosis [26]. Although anticancer drugs induced apoptotic death *via* intra-cellular acidification due to mitochondrial-derived H_2O_2 [27], an extracellular oxidizing medium created by hydrogen peroxide was proved to switch the cell death from apoptotic to necrotic pathways [26]. Hydrogen peroxide is produced in mammalian tissues by transformation of superoxide radical or molecular oxygen [28,29]. A decreasing of extra-cellular superoxide radicals will diminish the formation of H_2O_2. This indirectly will increase the chance for an anticancer drug to kill the cancerous cells *via* apoptotic pathways. In agreement with [26-29] it might be proposed that a molecule which kills cancer cells *via* apoptotic pathway and eliminates extracellular superoxide radicals would be a promising anticancer drug.

The capability of the solutions of complexes of Ce(III) and Nd(III) to eliminate the Xanthine/Xanthine Oxidase derived superoxide radical was estimated using the MTT test reaction. The antioxidant activity of the coumarin containing complexes strongly depended on the nature of the cation forming the complex, Fig. (**3**). For the Nd(III) complex, in the range 0.01 – 0.1 μM, no statistically significant antioxidant effect was detected. The maximum effect (at about 10%, detected at 100 μM) indicated that the coumarin complex formed around Nd^{3+} exhibited very modest ability to interact with the superoxide radical. In contrast, the complex formed with participation of Ce^{3+} exhibited statistically significant antioxidant activity (at about 20%) even at concentration of 0.01μM. Within the range of 0.01 – 100 μM, the antioxidant effect of the Ce(III) complex solutions increased from 20 to more than 60%. The strength of the effect of both complexes depended on their concentrations in the solution. As the same molecule participated as ligand in both complexes, it was proposed that the antioxidant properties of the complexes differed because of the effect of the cation on the reactivity of the ligand toward the superoxide radical. The time-dependence of the formazan formation in presence of

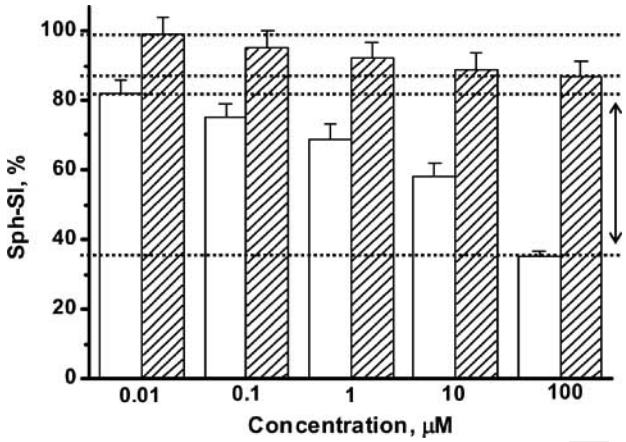

Fig. (3). Effect of the concentration of Ce1 (▭) and Nd1 (▨) complexes, on the SPh-SI measured in presence of X-XO-derived $^{\bullet}O_2^-$ radical. The effect of DMSO present in the solutions is being excluded.

solutions of Ce(III) and Nd(III) complexes was investigated at concentration of 100 μM, at which they both exhibited the strongest antioxidant effect, Fig. (**4**). The relative increasing with time of the absorbance at 560 nm, seen in Fig. (**2**), is due to the formation of formazan *via* MTT reduction with superoxide radicals, available at each moment in the solution. As much superoxide eliminated by the antioxidant

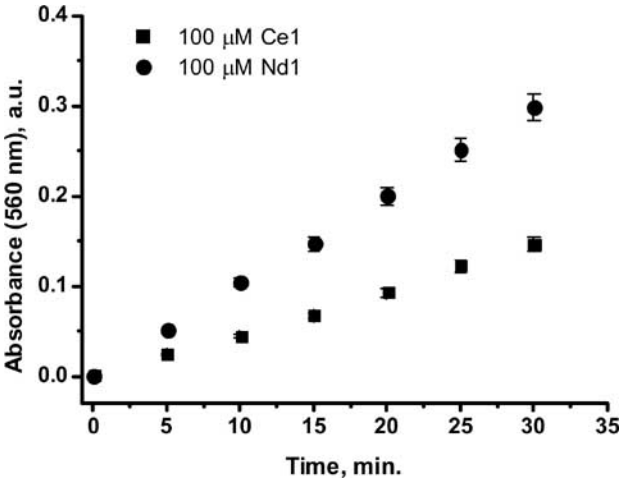

Fig. (4). Relative change of the absorbance at 560 nm with time, in presence of 100 μM solutions of Ce(III) and Nd(III) complexes.

molecule, as less would be available for reduction of MTT to formazan. At the first thirty minutes, a linear relationship between time and absorbance due to formazan, was observed. The absorbance at 560 nm was described by an equation of type *Abs*=b**t*, where b was a linear coefficient, ant *t* was the time in minutes. The coefficient b was found to be 0.00476 (R^2=0.9970) and 0.0098 (R2=0.9995) for the solutions of Ce(III) and Nd(III) complexes, respectively. As both curves in Fig. (**2**) represented the same reaction – the formation of formazan from MTT, the relative difference in their slopes might be explained with different effects of the complexes of Ce(III) and Nd(III) on the amount of superoxide available for MTT reduction. It was proposed that the elimination of $^{\bullet}O_2^-$ from the solution was faster in presence of Ce(III) than this in presence of Nd(III) complex.

Taking into account the UV and IR spectral characteristics of both coumarin-derived complexes, the remarkably higher antioxidant activity of Ce(III) complex in comparison with Nd(III) complex may be explained with the stronger bond between Nd^{3+} than this of Ce^{3+}, with the organic ligand. As strong the coordinative bond, as less the chance to participate in recombination of superoxide radical would be.

The high antioxidant activity of the Ce(III) complex might explain the elimination of cancerous cells *via* apoptotic pathway [20]. By eliminating superoxide radicals, the Ce(III) complex would locally diminish the extra-cellular H_2O_2. In agreement with [26-29], this would increase the opportunity for apoptotic death of the cancerous cells.

CONCLUSIONS

The coordination ability of 3,3′-benzylidenebis[4-hydroxycoumarin] have been proved in complexation reaction with lanthanide (III) ions. ^1H, ^{13}C NMR and IR spectral analysis of the ligand and its Ln(III) complexes confirmed the suggested coordination of 3,3′-benzylidenebis[4-hydroxycoumarin] through both the hydroxyl and carbonyl oxygen atoms.

Finally, the overall results from the preliminary screening program revealed that whereas the lanthanide nitrates lacked any cytotoxic effects the novel cerium and neodymium complexes with this ligand are potent cytotoxic agents. These results are in agreement with our previous investigations, concerning other lanthanide complexes with coumarins (13-15), whereby the complexes invariably exhibited superior activity in various tumors models as compared to the corresponding inorganic salts.

The analysis of the obtained IC_{50} revealed that the Ce complex exerted superior activity in comparison to the Nd compound. Taken together our data give us reason to conclude that the newly synthesized lanthanide complexes should be subset to further more detailed pharmacological and toxicological evaluation.

The higher cytotoxicity accompanied with better antioxidant activity of Ce(III) complex than these of Nd(III) complex might be related with the weaker coordinative bond between the organic ligand and the Ce^{3+} than this with Nd^{3+}. Thus, our investigation indicated that the Ce-complex would be better potential cytotoxic agent than this containing Nd.

EXPERIMENTAL

Synthesis of Ln(III) Complexes

The compounds used for the synthesis were Merck products, p.a. grade: $Ce(NO_3)_3.6H_2O$, $Nd(NO_3)_3.6H_2O$ and 3,3′-benzylidenebis[4-hydroxycoumarin] ($C_{25}H_{16}O_6$, H_2L). The complexes of lanthanides (III) with 3,3′-benzylidenebis [4-hydroxycoumarin] were synthesized by reaction of lanthanide(III) salts and the ligand for the first time by us and the method of synthesis was described previously [19].

Analytical and Spectroscopic Measurements

The elemental analyses for C, H, Ln and H_2O were performed according to standard microanalytical procedures. The IR spectra (Nujol and KBr) were recorded on IR-spectrometer FTIR-8101M Shimadzu (3800-400 cm^{-1}) and on IR-spectrometer Perkin-Elmer GX Auto image system (700-200 cm^{-1}). The ^1H NMR spectra were recorded at room temperature on Brucker WP 250 (250 MHz) spectrometer in DMSO-d_6. The ^{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.

All chemical compounds used in the analysis were of highest grade (SIGMA). The PBS (50 mM, pH 7.4) solutions were prepared using distilled water. The compounds to be analyzed were dissolved in DMSO prior preparation of the samples for UV-VIS spectral analysis. The spectra were recorded using a Perkin-Elmer 55a Spectrophotometer, and quartz cuvettes. The samples contained 1, 10 and 100 μM solutions of Ce(III) or Nd(III) complex in PBS (pH 7.4). For each solution, the reference cuvette contained the same amount of DMSO in PBS as the corresponding sample cuvette. The UV-spectra of DMSO in PBS were recorded using a reference cuvette with PBS.

The instrument was aligned by recording spectra of H_2O *vs* H_2O and PBS *vs* PBS, and finally, sample *vs* sample.

The experimental errors for determining the position of λ were (±2 nm) estimated by averaging of three spectra for each sample. The errors of determination of the intensities were within ±0.04 a.u. Relative differences of 5 nm and 0.08 a.u. in band position and band intensities, respectively, were considered statistically insignificant and were not discussed. The sample cuvettes contained metal complexes and DMSO as shown in the Table **1**. The interpretation of the spectra was made on the base of the UV data basis of NIST-Webbook spectra [25]. The calculations on the particular coumarin derivative were performed using the Woodwoard-Fieser rules [26] for calculating λ(max) for $\pi^* \rightarrow \pi$ transitions, taking into account coumarin structure [25].

Determination of the Antioxidant Activity

The antioxidant properties of the Coumarin complexes were estimated by monitoring their effects on the reduction of MTT in presence of the Xanthine/Xanthine Oxidase (X/XO) – *in vitro* derived superoxide radical $^{\bullet}O_2^-$. This method was focused on one of the many aspects of the antioxidant activity – the capability to scavenge the superoxide radical, which is generic for most of the Reactive Oxygen Species (ROS) and other free radicals. If a substance is able *in vitro* to scavenge $^{\bullet}O_2^-$, it might be promising antioxidant. The method was based on the ability of the superoxide radical to reduce the stain MTT (yellow) to Formazan (purple). An antioxidant molecule will diminish the superoxide radicals available for reduction of MTT, leading this way to a less intensive purple coloration of the solution in presence than in absence of the antioxidant. By measuring the absorbance at 560 nm, it is possible to estimate the relative change of the formazan concentration in the solution with time at fixed amount of the antioxidant molecule, for a fixed period of time at different concentrations of a same molecule, or for a fixed period of time and concentration and different antioxidant molecules. The effects on the absorbance at 560 nm of solvents were monitored, and considered during the examination of the antioxidant properties of all solutions in the experiment.

All chemicals and reagents were of finest grade (SIGMA and MERK, pa). The solutions were prepared using a gas-free deionized water. The K, Na- Phosphate buffer was of pH 7.45. Water solution of Xanthine (3 mM), and PBS solution of Xanthine oxidase (10 U/L) were used to generate superoxide radicals for the estimation of the antioxidant properties of the coumarin complexes of Ce(III) and Nd(III). MTT was dissolved in PBS to a concentration of 1 mg/ml. The complexes of Ce(III) and Nd(III) were dissolved in DMSO (0.671 M) to concentrations of 1000 μM, and then were diluted with PBS to concentrations of 100, 10, 1 0.1, and 0.01 μM.

The absorbance at 560 nm was detected using two-channels' Perkin-Elmer 552 spectrophotometer, connected to a PC, equipped with quartz cuvettes of volume 2 ml. The sample cuvette contained the complex examined (Ce(III) or Nd(III) complex), while in the reference cuvette the metal complex was omitted. Each sample contained 0.2 ml solution of XO (10 U/L), 0.1 ml Xanthine (3 mM), 0.05 ml MTT (1 mg/ml), and coumarin complex (Ce(III) or Nd(III) complex) of desired concentration (0.01, 0.1, 1, 10, and 100 μM), dissolved in PBS. The antioxidant activity of the solutions of Ce(III) and Nd(III) complexes was estimated by calculating the Spectrophotometric Scavenging Index (SPh-SI) after 30 minutes of MTT reduction in presence of different concentrations of a complex, and by monitoring the relative change of the coloration of each solution for 30 minutes.

Samples were kept for 30 minutes at 310K in a thermostat. The reaction was stopped by immediate introduction of 1 ml DMSO. After the vials reached 298 K, the absorbance at 560 nm was measured. The effect of DMSO present in the coumarin-containing samples on their AO activity was estimated, and considered in the data processing. Amounts of DMSO corresponding to its content in each coumarin-containing solution, were dissolved in PBS, and their antioxidant properties were measured. These data were subtracted from the effects of the corresponding coumarin containing solutions.

The Spectrophotometric Scavenging Index ,*SPh-SI*, of each sample was calculated using the formula:

$$SPh - SI = 100 * \frac{(Abs)_{Sample}}{(ABS)_{Control}},$$

where $(Abs)_{Sample}$ and $(Abs)_{Control}$ were the absorbances at 560 nm of the coumarin containing solution (after excluding the effect of DMSO in it), and this of a solution, containing X/XO only.

The time-dependence of the formazan formation in presence of solutions of Ce(III) and Nd(III) complexes was investigated at concentration of 100 μM, at which they both exhibited the strongest antioxidant effect.

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