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Original article

# Cytotoxic activity of new lanthanum (III) complexes of bis-coumarins

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**Abstract**

Complexes of lanthanum (III) with bis-coumarins: bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane; bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane were synthesized by reaction of lanthanum (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 lanthanum (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 lanthanum (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 lanthanum (III) complexes were interpreted on the basis of comparison with the spectra of the free ligands. This analysis showed that in the La (III) complexes the ligands coordinated to the metal ion through both deprotonated hydroxyl groups. On the basis of the $\nu$(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 lanthanum complexes against the acute myeloid leukemia derived HL-60 and the chronic myeloid leukemia (CML)-derived BV-173. In addition the cytotoxic effects of La (III) complex with bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-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 for programmed cell death.


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

## 1. Introduction

Coumarin is used widely as a therapeutic agent and is administered clinically in the treatment of certain lymphedemas and malignancies. 7-Hydroxy- and 4-hydroxycoumarins are naturally occurring substances with a variety of biological activities, e.g. antitumoral action.

The antitumor activities of coumarin and its known metabolite 7-hydroxy-coumarin were tested in several human tumor cell lines by Steffen et al. [1]. Both compounds inhibited cell proliferation of a gastric carcinoma cell line, a colon-carcinoma cell line (Caco-2), a hepatoma-derived cell line (Hep-G2) and a lymphoblastic cell line (CCRF cem). Egan et al. [2] have synthesized, characterized and determined cytostatic and cytotoxic nature of 8-nitro-7-hydroxycoumarin using both human (including K-562 and HL-60) and animal cell lines grown in vitro. 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 [3]. The effect of Warfarin on tumor cell growth was studied [4]. Warfarin inhibits metastasis of Mtln3 rat mammary carcinoma without affecting primary tumor growth. Seven known coumarins, showing significant cytotoxic activities on P388 cell lines, were isolated from the roots of Angelica gigas (Umbelliferae) [5]. Akman et al. [6] had investigated synergistic cytotoxicity between menadione and the related anticoagulant Dicumarol, inhibited growth of murine leukemia L1210 in liquid suspension culture. The cytotoxicity of

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22 natural and semisynthetic simple coumarins was evaluated in GLC4, a human small cell lung carcinoma cell line, and in COLO 320, a human colorectal cancer cell line, using the microculture tetrazolium (MTT) assay [7].

A number of 4-hydroxycoumarin derivatives has been studied as to their HIV integrase inhibitory potency [8]. The main purpose was to simplify the large structure of the compounds while maintaining their potency. It was found that the minimum active pharmacophore consist of a coumarin dimer containing an aryl substituent on the central linker, methylene. The addition of 4- and 7-hydroxy substituents in the coumarin rings improved the potency of the compounds. Among the systems studied, the 3,3′-benzylidene-bis(4-hydroxycoumarin) has been tested as a HIV integrase inhibitor and has shown significant activity [8]. The complexation ability of the 3,3′-benzylidene-bis(4-hydroxycoumarin) with lanthanides was not reported so far. It is expected that the complexes with this ligand and with similar ligands will retain or improve its biological activity as in the case of other lanthanide complexes with hydroxycoumarin derivatives.

The complexes of rare earth ions have aroused much interest. Lanthanides are a subject of increasing interest in bioinorganic and coordination chemistry [9,10].

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 [11] and bis-(4-hydroxy-3-coumarinyl)-acetic acid [12] 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 lanthanum 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 [13–18]. 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.

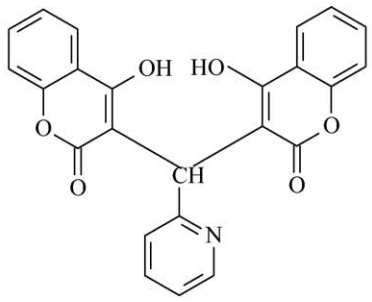
Unfortunately, little is known about the complexing ability of lanthanum (III) with coumarins. A survey of the literature reveals that no work has been done on the reactions of lanthanum (III) with 3,3′-benzylidene-bis(4-hydroxy-2H-1-benzopyran-2-one) and its pyridyl derivatives. It was, therefore, considered 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 bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane, bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane in complexation reaction with lanthanum (III). The obtained La (III) complexes with these coumarin ligands were characterized by elemental analysis, physicochemical methods, mass-, NMR- and IR-spectroscopy. The complicated vibrational spectra of lanthanum (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.

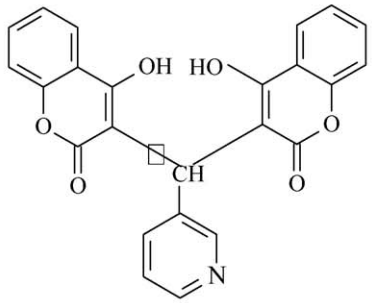
We observed that La (III) possess a cytotoxic activity and literature data show that the coumarins have also these properties. That is why our synthesis of complexes of La (III) is taken into consideration with cytotoxic screening and further pharmacological study.

## 2. Chemistry

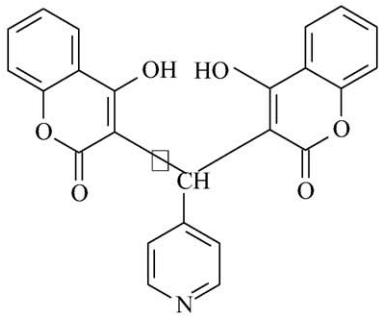
The compounds used for preparing the solutions were Merck products, p.a. grade: $La(NO_3)_3 \cdot 6H_2O$. Bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane, bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane were used for the preparation of metal complexes as ligands (Scheme 1). These ligands were obtained by condensation of 4-hydroxycoumarin and heterocyclic aldehyde (Scheme 2) in ethanol medium at reflux and stirring until crystals appeared. The ligands were prepared by following procedure [19]. The respective heterocyclic aldehyde was added to 4-hydroxycoumarin in ethanol in amounts equal to aldehyde/4-hydroxycoumarin molar ratio of 1:2. The mixture was stirred vigorously at boil temperature until the precipitate was obtained. The mixture was filtered washed several times with ethanol and dried in a desiccator to constant weight. The residue was recrystallized from dioxan. Yields: bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane ($H_2L1$): (40%); bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-me-




$H_2L1$= bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane ($H_2L1$)

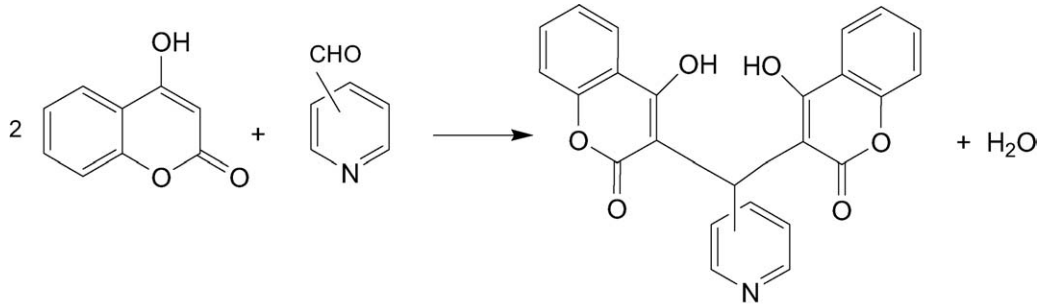



$H_2L2$= bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane ($H_2L2$)




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

Scheme 1. Structures of the ligands.




Scheme 2. Synthesis of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane ($H_2L1$); bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane ($H_2L2$); bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane ($H_2L3$).

thane ($H_2L2$): (84%); and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane ($H_2L3$): (48%).

The complexes of lanthanum (III) with bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane ($H_2L1$), bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane ($H_2L2$) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane ($H_2L3$) were synthesized by reaction of lanthanum (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 lanthanum (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.

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

## 3. Pharmacology

In the present study we performed comparative evaluation of the cytotoxic effects of the three newly synthesized lanthanum complexes against two leukemic cell lines of human origin, namely the acute promyelocyte leukemia HL-60 and the pre-B-cell lymphoma BV-173, using the standard MTT-dye reduction assay for cell viability. In addition the cytotoxic effects of the lanthanum complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane, were evaluated on the chronic lymphoid leukemia cells SKW-3.

In order to assess the ability of the lanthanum complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane to trigger programmed cell death, we isolated DNA from the cytosolic fraction of treated and untreated SKW-3 cells and analyze it via agarose gel electrophoresis, ethidium bromide staining and visualization.

## 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 La (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 La (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.

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

| | Found/calculated | | | | |
|---|---|---|---|---|---|
| Complex | % C | % H | % N | % $H_2O$ | % La |
| La(L1) (OH)·$H_2O$ | 49.61 | 3.14 | 2.78 | 3.42 | 23.48 |
| | 49.23 | 2.73 | 2.39 | 3.08 | 23.76 |
| La(L2) (OH)·2$H_2O$ | 48.19 | 3.41 | 2.58 | 6.28 | 23.43 |
| | 47.76 | 2.98 | 2.32 | 5.97 | 23.05 |
| La(L3) (OH)·$H_2O$ | 48.91 | 3.13 | 2.56 | 3.48 | 23.49 |
| | 49.23 | 2.73 | 2.39 | 3.08 | 23.76 |

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

### 4.2. IR spectra of the complexes

The mode of bonding of the ligands to La (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 bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane ($H_2L1$), bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane ($H_2L2$) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane ($H_2L3$) and of the lanthanum complexes with these ligands are presented in Table 3.

#### 4.2.1. IR-spectrum of the complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane ($H_2L1$)

The bands appear in the IR spectrum of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane ($H_2L1$) at 3122,

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

| Ligand | *m/z* | (%) | Complex | *m/z* | (%) |
|---|---|---|---|---|---|
| $H_2L1 = C_{24}H_{15}NO_6$ | 413 | 7 | La($L_1$) | 589 | 1 |
| | 395 | 2 | (OH)·$H_2O$ | 490 | 4 |
| | 252 | 7 | | 460 | 6 |
| | 162 | 30 | | 410 | 1 |
| | 120 | 28 | | 307 | 70 |
| | 92 | 38 | | 176 | 100 |
| $H_2L2 = C_{24}H_{15}NO_6$ | 413 | 7 | La($L_2$) | 603 | 1 |
| | 395 | 2 | (OH)·2$H_2O$ | 490 | 4 |
| | 252 | 30 | | 460 | 6 |
| | 162 | 62 | | 410 | 1 |
| | 120 | 74 | | 307 | 75 |
| | 92 | 86 | | 176 | 100 |
| $H_2L3 = C_{24}H_{15}NO_6$ | 413 | 0 | La($L_3$) | 589 | 1 |
| | 252 | 18 | (OH)·$H_2O$ | 490 | 4 |
| | 250 | 50 | | 460 | 7 |
| | 162 | 62 | | 410 | 1 |
| | 120 | 74 | | 307 | 80 |
| | 92 | 86 | | 176 | 100 |

Table 3
Selected experimental IR frequencies of the ligands and their La (III) complexes ($cm^{-1}$)[a]

| Compound | $\nu$OH/$H_2O$ | $\nu$(C=O) | $\nu$(C=C) | $\nu$(Py) | $\nu$(Ar) | $\delta$(COH) | $\nu$(C–O) | |
|---|---|---|---|---|---|---|---|---|
| $H_2L1 = C_{24}H_{15}NO_6$ | | | | 1620 | | | 1181m | |
| | 3122m | 1696s | 1608s | 1559 | 1489m | 1350m | 1164m | 770 |
| | 3060m | 1635s | 1539s | 1505 | | 1332m | 1111s | 751 |
| | | | | 1410 | | | 1039m | |
| La($L_1$) (OH)·$H_2O$ | | | | 1622 | | | 1211w | |
| | 3400br | 1652sh | 1520s | 1558 | 1436m | – | 1151w | 759 |
| | | 1599s | | 1506 | | | 1108m | |
| | | | | 1419 | | | 1076w | |
| $H_2L2 = C_{24}H_{15}NO_6$ | | | | 1620 | | | 1176m | |
| | 3070m | 1687s | 1610s | 1560 | 1491m | 1350m | 1120m | 807 |
| | 3051m | 1614s | 1536s | 1506 | | 1329m | 1104s | 753 |
| | | | | 1409 | | | 1050m | |
| La($L_2$) (OH)·2$H_2O$ | | | | 1620 | | | 1184w | |
| | 3368br | 1650sh | 1520s | 1560 | 1451m | – | 1120w | 760 |
| | | 1599s | | 1506 | | | 1107m | |
| | | | | 1418 | | | 1069w | |
| $H_2L3 = C_{24}H_{15}NO_6$ | | | | 1620 | | | 1181m | |
| | 3180m | 1699s | 1610s | 1558 | 1498m | 1340m | 1155m | 770 |
| | 3120m | 1635s | 1538s | 1520 | | 1315m | 1107s | 750 |
| | | | | 1405 | | | 1037m | |
| La($L_3$) (OH)·$H_2O$ | | | | 1622 | | | 1186w | |
| | 3390br | 1652sh | 1520s | 1559 | 1440m | – | 1150w | 760 |
| | | 1599s | | 1516 | | | 1109m | |
| | | | | 1419 | | | 1079w | |

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

3060; 1696, 1635; 1608, 1539; 1489, 1181, 1164, 1111, 1039 $cm^{-1}$. The bands at 1696 and 1635 $cm^{-1}$ can be attributed to the stretching vibrations of the carbonyl groups of the lacton rings. Bands at 1608 and 1539 $cm^{-1}$ can be related to the stretching vibrations of the conjugated olefinic system. The vibrations at 1489 $cm^{-1}$ correspond to the aromatic systems. Bands at 1620, 1559, 1505, 1410 $cm^{-1}$ can be attributed to the stretching vibrations of pyridine and they remain almost the same in the complex.

A broad band, characteristic of $\nu_{OH}$ of coordinated water was observed in the range 3300–3400 $cm^{-1}$ in the spectrum of the complex. The weak bands observed at 3122 and 3060 $cm^{-1}$ in the spectrum of the free ligand is missing in the spectrum of the complex. A comparison of the infrared spectra of the ligand and of the complex reveals the disappearance of absorption bands observed in the free ligand at 3122, 3060 $cm^{-1}$ and 1350, 1332 $cm^{-1}$ 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 1696 and 1635 $cm^{-1}$ 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 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 [20].

*4.2.2. IR-spectrum of the complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane ($H_2L2$)*

The bands appear in the IR spectrum of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane ($H_2L2$) at 3070, 3051; 1687, 1614; 1610, 1536; 1491, 1176, 1120, 1104, 1050 $cm^{-1}$. The bands at 1687 and 1614 $cm^{-1}$ can be attributed to the stretching vibrations of the carbonyl groups of the lacton rings. Bands at 1610 and 1536 $cm^{-1}$ can be related to the stretching vibrations of the conjugated olefinic system. The vibrations at 1491 $cm^{-1}$ correspond to the aromatic systems. Bands at 1620, 1560, 1506, 1409 $cm^{-1}$ can be attributed to the stretching vibrations of pyridine and they remain almost the same in the complex.

A broad band, characteristic of $\nu_{OH}$ of coordinated water was observed in the range 3300–3400 $cm^{-1}$ in the spectrum of the complex. The weak bands observed at 3070 and 3051 $cm^{-1}$ in the spectrum of the free ligand is missing in the spectrum of the complex. A comparison of the infrared spectra of the ligand and of the complex reveals the disappearance of absorption bands observed in the free ligand at 3070 and 3051 $cm^{-1}$ and 1350, 1329 $cm^{-1}$ 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 1687 and 1614 $cm^{-1}$ 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 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 [20].

*4.2.3. IR-spectrum of the complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane ($H_2L3$)*

The bands appear in the IR spectrum of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane ($H_2L3$) at 3180, 3120; 1699, 1635; 1610, 1538; 1498, 1181, 1155, 1107, 1037 $cm^{-1}$. The bands at 1699 and 1635 $cm^{-1}$ can be attributed to the stretching vibrations of the carbonyl groups of the lacton rings. Bands at 1610 and 1538 $cm^{-1}$ can be related to the stretching vibrations of the conjugated olefinic system. The vibrations at 1498 $cm^{-1}$ correspond to the aromatic systems. Bands at 1620, 1558, 1520, 1405 $cm^{-1}$ can be attributed to the stretching vibrations of pyridine and they remain almost the same in the complex.

A broad band, characteristic of $\nu_{OH}$ of coordinated water was observed in the range 3300–3400 $cm^{-1}$ in the spectrum of the complex. The weak bands observed at 3180 and 3120 $cm^{-1}$ in the spectrum of the free ligand is missing in the spectrum of the complex. A comparison of the infrared spectra of the ligand and of the complex reveals the disappearance of absorption bands observed in the free ligand at 3180, 3120 $cm^{-1}$ and 1340, 1315 $cm^{-1}$ 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 1699 and 1635 $cm^{-1}$ 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 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 [20].

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.

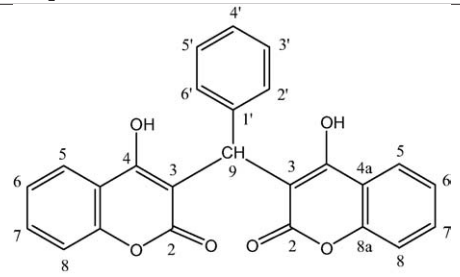
*4.3. $^1H$- and $^{13}C$-NMR spectra of the ligands and their La (III) complexes*

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 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 La (III).

Table 4
$^1H$-NMR spectral shifts, $\delta$ (ppm) of the ligands and their La (III) complexes (250 MHz, DMSO-$d_6$)

| Compound | $\delta$ (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 |
| La($L_1$) (OH)·$H_2O$ | 7.15–7.81 | 6.30 | 8.03–8.33 |
| $H_2L2 = C_{24}H_{15}NO_6$ | 7.22–7.57 | 6.42 | 7.79–8.70 |
| La($L_2$) (OH)·$2H_2O$ | 7.06–7.52 | 6.26 | 7.80–8.34 |
| $H_2L3 = C_{24}H_{15}NO_6$ | 7.22–7.58 | 6.46 | 7.80–8.68 |
| La($L_3$) (OH)·$H_2O$ | 7.15–7.85 | 6.33 | 8.15–8.38 |




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

$^{13}C$-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 $\delta$ (ppm) are presented in Table 5.

Due to electron transfer from the hydroxyl and carbonyl oxygen atoms to La (III), a difference in chemical shifts was observed for the neighboring C-4, 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. On the basis of the results thus obtained, it was suggested that the ligands act as tetradentate ones in the La (III) complex formation.

*4.4. Pharmacology*

*4.4.1. In vitro cytotoxicity*

The cytotoxic effects of the three newly synthesized lanthanum complexes of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane (La-1), bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (La-2) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (La-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 is sumarrized in Tables 6 and 7.

As evident from the obtained results, La-1 was the most potent cytotoxic agent against HL-60 cells among the tested La complexes (Figs. 1–3 and Table 6). It actually lacked any cytotoxic effects at concentrations below 50 μM and even induced some rise in cell viability at this concentration range. When applied at higher concentrations however, La-1 caused prominent reduction of the cell survival fraction by ca. 53% at 100 μM and by almost 90% at 200 μM. The two other com-

Table 5
$^{13}$C-NMR spectral shifts, $\delta$ (ppm) of the ligands and their La (III) complexes (62.9 MHz, DMSO-$d_6$)

| Atom | $\delta$ (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | $H_2L1$ | La($L_1$) (OH)·$H_2O$ | $H_2L2$ | La($L_2$) (OH)·2$H_2O$ | $H_2L3$ | La($L_3$) (OH)·$H_2O$ |
| C-2 | 168.6 | 164.2 | 168.1 | 170.4 | 168.2 | 170.7 |
| C-4 | 164.0 | 161.4 | 164.1 | 165.6 | 164.9 | 168.1 |
| C-8a | 157.6 | 152.2 | 152.8 | 155.0 | 164.2 | 165.8 |
| C-1′ | 152.9 | 152.1 | 142.9 | 152.6 | 152.8 | 153.5 |
| C-7 | 146.5 | 148.3 | 144.9 | 148.7 | 141.0 | 148.5 |
| C-3′ | 141.9 | 136.4 | – | – | 131.7 | 137.9 |
| C-5′ | 141.9 | 135.4 | 140.4 | 131.4 | 131.7 | 135.0 |
| C-4′ | 131.9 | 130.5 | 139.2 | 130.0 | – | – |
| C-6′ | 125.9 | 123.7 | 131.6 | 126.2 | 125.3 | 131.4 |
| C-2′ | – | – | 126.8 | 124.3 | 125.3 | 130.0 |
| C-5 | 124.4 | 122.2 | 124.3 | 122.4 | 124.3 | 126.3 |
| C-6 | 123.4 | 120.5 | 123.3 | 121.8 | 123.3 | 121.7 |
| C-4a | 119.3 | 119.9 | 119.6 | 119.8 | 119.5 | 120.0 |
| C-8 | 115.9 | 114.5 | 115.8 | 115.1 | 115.9 | 115.1 |
| C-3 | 100.5 | 103.0 | 101.7 | 102.3 | 101.5 | 102.8 |
| C-9 | 36.7 | 38.0 | 34.8 | 36.1 | 37.9 | 34.5 |

Table 6
Spectrophotometrical data from the MTT assay concerning the cytotoxic effects of the newly synthesized lanthanum complexes of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane (La-1), bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (La-2) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (La-3) on HL-60 and BV-173 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 | La-1 | 1.372 ± 0.133 | 1.828 ± 0.057 | 1.740 ± 0.058 | 1.590 ± 0.049 | 0.643 ± 0.073 | 0.145 ± 0.033 |
| | La-2 | 1.372 ± 0.133 | 1.745 ± 0.059 | 1.660 ± 0.052 | 1.655 ± 0.052 | 1.521 ± 0.062 | 0.302 ± 0.040 |
| | La-3 | 1.372 ± 0.133 | 1.760 ± 0.034 | 1.688 ± 0.021 | 1.715 ± 0.076 | 1.580 ± 0.086 | 0.338 ± 0.017 |
| BV-173 | La-1 | 1.049 ± 0.059 | 1.143 ± 0.051 | 1.122 ± 0.067 | 1.031 ± 0.037 | 0.123 ± 0.021 | 0.022 ± 0.011 |
| | La-2 | 1.049 ± 0.059 | 1.332 ± 0.059 | 1.309 ± 0.029 | 1.344 ± 0.037 | 1.249 ± 0.031 | 0.054 ± 0.018 |
| | La-3 | 1.049 ± 0.059 | 1.256 ± 0.051 | 1.222 ± 0.082 | 1.228 ± 0.049 | 1.092 ± 0.096 | 0.715 ± 0.085 |

Table 7
$IC_{50}$ values of the tested lanthanum (III) complexes, extrapolated from the corresponding concentration–response curves

| Cell line | $IC_{50}$ value (μM) | | |
|---|---|---|---|
| | La-1 | La-2 | La-3 |
| HL-60 | 97.78 | 168.44 | 172.01 |
| BV-173 | 76.8 | 160.72 | >200 |

plex compounds La-2 and La-3 failed to induce any decrease of cell viability when applied at concentrations up to 100 μM. When applied at concentration of 200 μM La-2 and La-3 exhibited practically equal cell growth inhibition with ca. 22% and 25% of viable cells, respectively.

The evaluation of the effects of the tested compounds on the lymphoid BV-173 cells revealed that La-1 exhibited superior activity as compared to the other two lanthanum agents as evident from the concentration response curves on Figs. 4–6 and the corresponding $IC_{50}$ values. Although some stimulation of cell viability and lack of cytotoxicity were found within the concentration range 12.5–50 μM, at the higher concentration of 100 μM La-1 caused a strong decrease of the cell survival fraction by approximately 88%. At the highest concentration tested (200 μM) La-1 almost totally eradicated the viable BV-173 cells (survival fraction = ca. 2%). Both La-2 and La-3 lacked cytotoxic effects at concentrations up to 100 μM and caused significant decrease of cell viability only at the higher concentration evaluated (survival fractions as follows: ca. 5% for La-2 and ca. 68% for La-3).

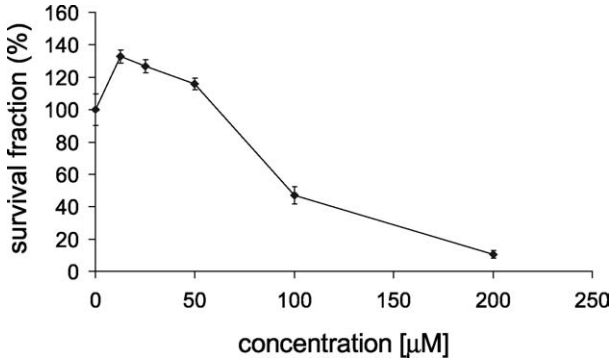



Fig. 1. Cytotoxic effect of of the newly synthesized lanthanum complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane (La-1) as assessed by the MTT-dye reduction assay following 72 h treatment of HL-60. Each data point represents the arithmetic mean ± S.D. of at least six independent experiments.

*4.4.2. DNA-fragmentation analysis*

Following 24 h treatment of SKW-3 cells with either 100 or 200 μM La-1, led to an apparent DNA-laddering phenomenon, that is considered as a major hallmark of programmed cell death (Fig. 7).

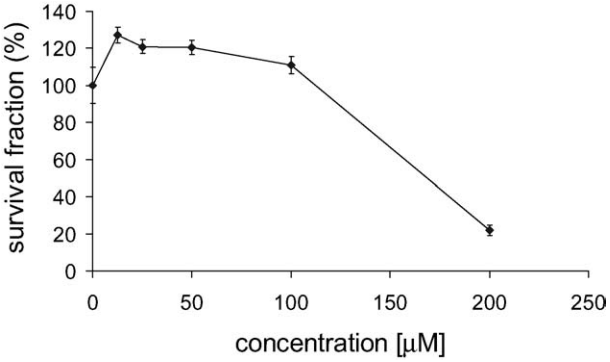



Fig. 2. Cytotoxic effect of the newly synthesized lanthanum complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane (La-2) as assessed by the MTT-dye reduction assay following 72 h treatment of HL-60. Each data point represents the arithmetic mean ± S.D. of at least six independent experiments.

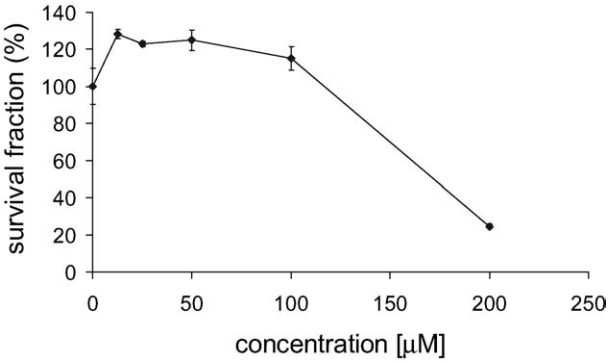



Fig. 3. Cytotoxic effect of the newly synthesized lanthanum complex of bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane (La-3) as assessed by the MTT-dye reduction assay following 72 h treatment of HL-60. Each data point represents the arithmetic mean ± S.D. of at least six independent experiments.

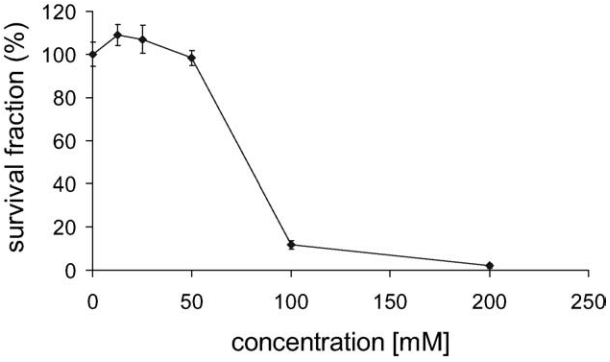



Fig. 4. Cytotoxic effect of La-1 as assessed by the MTT-dye reduction assay following 72 h treatment of BV-173. Each data point represents the arithmetic mean ± S.D. of at least six independent experiments.

These data show that the induction of apoptosis is implicated in the observed cytotoxicity of La-1 against BV-173 cells.

## 5. Conclusions

The coordination ability of the ligands has been proved in complexation reaction with lanthanum (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 La (III) complexes confirmed the suggested coordination of the ligands through both the hydroxyl and carbonyl oxygen atoms.

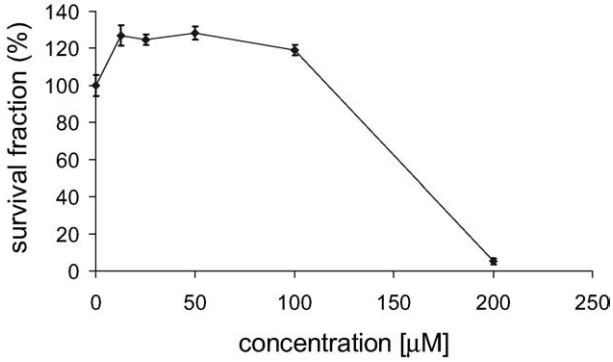



Fig. 5. Cytotoxic effect of La-2 as assessed by the MTT-dye reduction assay following 72 h treatment of BV-173. Each data point represents the arithmetic mean ± S.D. of at least six independent experiments.

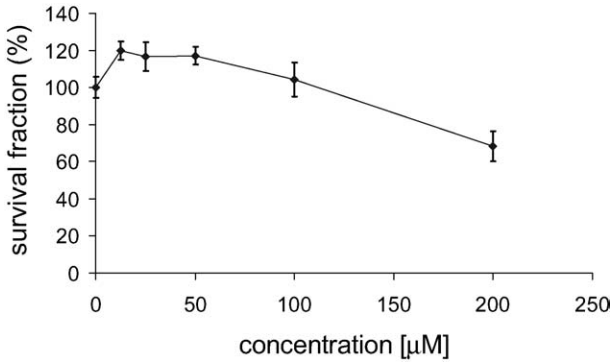



Fig. 6. Cytotoxic effect of La-3 as assessed by the MTT-dye reduction assay following 72 h treatment of BV-173. Each data point represents the arithmetic mean ± S.D. of at least six independent experiments.

All of the newly synthesized La (III) complexes under investigation exhibited cytotoxic activity in micromolar concentrations. Taken together, our experimental data give us reason to conclude that among the newly synthesized La (III) complexes La-1 proved to be superior in respect to relative potency and thus should be subset to further thorough pharmacological evaluation.

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

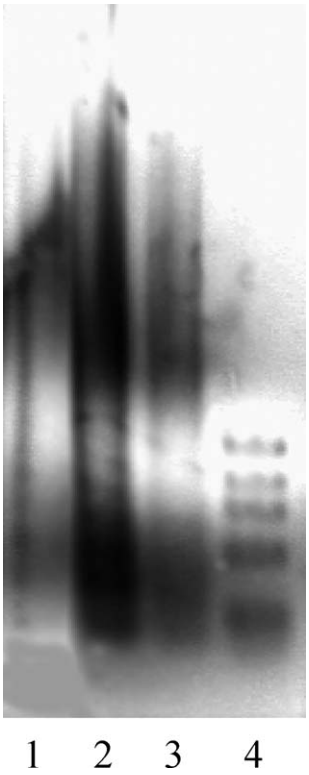



Fig. 7. Agarose gel electrophoresis of DNA, isolated from the cytosolic fraction of untreated SKW-3 cells (lane 1) or following treatment with La-1 at 100 μM (lane 2) and 200 μM (lane 3); lane 4—size marker.

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 lanthanum (III) with bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-2-yl-methane ($H_2L1$), bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-3-yl-methane ($H_2L2$) and bis(4-hydroxy-2-oxo-2H-chromen-3-yl)-piridin-4-yl-methane ($H_2L3$) were synthesized by reaction of lanthanum (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 lanthanum (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. Yields: $La(L_1)(OH)\cdot H_2O$: 73%; $La(L_2)(OH)\cdot 2H_2O$: 82%; $La(L_3)(OH)\cdot H_2O$: 76%.

*6.2. Pharmacology*

*6.2.1. Cell lines and culture maintenance*

The human tumor cell lines exploited in the present study were supplied from the Department of Human and Animal Cell Cultures at the German Collection of Microorganisms and Cell Cultures. They were maintained as suspension-type 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% fetal calf serum and 2 mM L-glutamine. Cells were kept in logarithmic phase via supplementation with fresh medium two or three times per week.

*6.2.2. Cytotoxicity determination*

The stock solutions of the investigational lanthanum complexes were freshly prepared in DMSO. The stock test samples were diluted with RPMI-1640 to a suitable concentration before being added to the cell suspension in the 96-well microplate. At the final dilutions obtained in the wells of the microplate the concentrations of DMSO never exceeded 1%. The cell viability was assessed by the standard MTT-dye reduction assay as previously described with some minor alterations [21,22]. Briefly, exponentially growing cells were seeded in 96-well microplates (100 μl aliquots per well) at a density of $1 \times 10^5$ cells per ml. Following 24 h incubation at 37 °C the cells were exposed to the novel lanthanum complexes (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). The microplates were further incubated for 4 h at 37 °C and the formazan crystals formed were dissolved through addition of 100 μl per well 5% solution of formic acid in 2-propanol (Merck). The absorption of the samples was then measured using an ELISA reader (Uniscan Titertec) at 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. The results were expressed as $IC_{50}$ values, extrapolated from the corresponding concentration response curves.

*6.2.3. DNA isolation and gel electrophoresis*

The DNA isolation and horizontal gel electrophoresis procedures were performed out as described elsewhere [21]. About $5 \times 10^6$ SKW-3 cells-treated with La-1 or untreated controls, were washed in PBS. Cell pellets were re-dissolved 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 on ice for 5 min and thereafter spun at 13,000 rpm for 20 min. The superna-

tants 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 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 water-soluble 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 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.

## Acknowledgements

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