DOI: 10.1002/ejic.200900038

New Insights into the Chemistry of the Antineoplastic Lanthanum Complex Tris(1,10-phenanthroline)tris(thiocyanato-κ*N*)lanthanum(III) (KP772) and Its Interaction with Biomolecules

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Keywords: Antitumor agents / Lanthanum / N ligands / Heterocycles

The lanthanide complex tris(1,10-phenanthroline)tris(thiocyanato-κ*N*)lanthanum(III) [La(phen)$_3$(NCS)$_3$] (KP772) is a promising anticancer drug candidate, capable of overcoming resistance of tumors to established chemotherapeutics. The compound was characterized by elemental analysis, IR, ^{1}H NMR spectroscopy, TG/DTA measurements, mass spectrometry and X-ray diffraction analysis. The results indicate that KP772 is a neutral, nine-coordinate complex. In addition the behavior in water, important for the application as a chemotherapeutic drug, and the binding to biomolecules was investigated by capillary electrophoresis and ICP-MS.

Introduction

Lanthanide compounds, for example with cerium as metal center, have been used for the treatment of cancer (a Gd compound is in clinical development for the treatment of non-small-cell lung cancer), and as anti-emetics due to favorable pharmacological properties.[1,2] In addition, they found application in the treatment of burns and as phosphate binders against hyperphosphatemia.[2] The mode of action of anticancer-active lanthanides is related to their similarity to calcium: Ln^{3+} ions exhibit high affinity to Ca^{2+} binding sites in biomolecules because of their similar ionic radii, but are higher charged (HSAB principle).[2,3] Therefore, such compounds are able to inhibit calcium fluxes, required for cell cycle regulation, but they cannot only substitute for calcium but also for other metal ions such as Mg^{2+}, Fe^{3+} and Mn^{2+} in proteins, leading to the inhibition of their functions.[4,5] The tumor-inhibiting activity of lanthanum is considerably enhanced by complexation with various ligands such as phenanthroline derivatives.[6] Phenanthroline induces a cell cycle arrest in G_0/G_1, most likely based on its metal-chelating ability.[7,8]

The La complex tris(1,10-phenanthroline)tris(thiocyanato-κ*N*)lanthanum(III) (KP772; Figure 1) exerts potent activity against a wide range of tumor cell lines in vitro and a colon carcinoma xenograft model in vivo with properties comparable to cisplatin and methotrexate.[9] Notably, long-term treatment of KBC-1 cells with KP772 leads to a complete loss of drug resistance, and complementary studies showed that exposition of cells to subtoxic, stepwise increasing KP772 concentrations does not lead to acquired resistance.[10] KP772 is expected to be active against multidrug-resistant tumors, rendering it very interesting for further (pre)clinical development.[11]

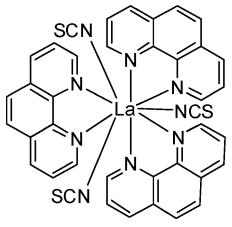

Figure 1. Structure of tris(1,10-phenanthroline)tris(thiocyanato-κ*N*)lanthanum(III) (KP772).

Herein, the characterization of KP772 with regard to chemical structure, stability in water and reactivity to biomolecules is described. Binding towards DNA is considered the most important step in the mode of action of successful metal-based anticancer compounds such as cisplatin.[12] Binding studies to nucleotides by capillary electrophoresis (CE) have been shown to be a suitable method to monitor the reaction of metal complexes to DNA model compounds.[13–15] Consequently, CE has emerged as a standard analytical procedure in anticancer research, especially due to its compatibility with physiological conditions. These features have made CE also interesting for the analysis of interactions of transition-metal complexes with proteins, especially when coupled to inductively coupled plasma mass

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/ejic.200900038.

spectrometry (ICP-MS) as the detector.[16] The binding towards serum proteins is considered essential for the transport of the drug to the target but can also be a reason for undesired side-effects and was therefore also of great interest in this study.[17–19]

Results and Discussion

Synthesis and Characterization

The synthesis of the complex was carried out by applying a procedure reported by Hart and Laming.[20] A single crystal was obtained by dissolving $[La(phen)_3(NCS)_3]$ in ethanol and subsequent evaporation of the solvent. The result of the X-ray diffraction study is shown in Figure 2. Crystal data, data-collection parameters, and structure-refinement details are given in Table 1. Selected bond lengths are quoted in the legend to Figure 2.

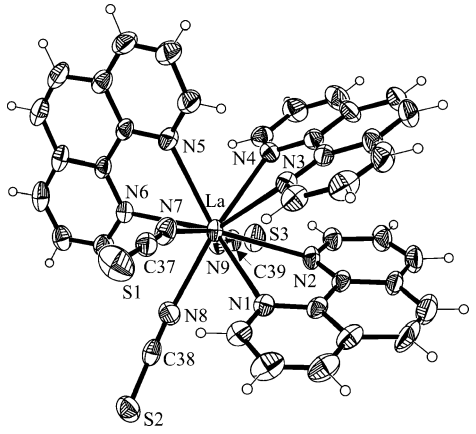

Figure 2. ORTEP view of $[La(phen)_3(NCS)_3]$ with thermal ellipsoids drawn at 30% probability level. Selected bond lengths [Å]: La–N1 2.750(4), La–N2 2.766(4), La–N3 2.758(4), La–N4 2.752(4), La–N5 2.742(5), La–N6 2.749(4), La–N7 2.541(5), La–N8 2.542(6), La–N9 2.543(5) Å.

The compound crystallizes in the triclinic centrosymmetric space group $P\bar{1}$, with no co-crystallized solvent molecules. Hart and Laming proposed the presence of the three phen ligands in the first coordination sphere for the La ion, the thiocyanates as counterions and an octahedral geometry.[20] However, the X-ray diffraction analysis of the single crystal obtained from ethanol revealed that the lanthanum(III) ion is nine-coordinate and binds to three bidentate 1,10-phenanthroline ligands and to the nitrogen atoms of the monodentate NCS^- ligands (in a *mer* fashion), as shown before for similar Pr and Nd compounds.[21] The average $La–N_{thiocyanate}$ bond [2.542(1) Å] is significantly shorter than that of $La–N_{phen}$ bonds [2.753(3) Å], but longer than in $[Pr(phen)_3(NCS)_3]\cdot C_2H_5OH$ [2.508(3) and 2.704(7) Å, respectively].[22] This is in accord with the anticipated "lanthanide contraction" in going from lanthanum to praseodymium. It should, however, be noted that the X-ray data collection for these two compounds was performed at different temperatures (300 K for La and 180 K for Pr). The thiocyanato ligands are linear with an average N–C–S angle of 178.5(2)°. A significant distortion from linearity at the nitrogen atoms occurs upon coordination to lanthanum(III) [La–N–C angles of 157.3(5)°, 158.3(5)°, and 163.2(5)°]. Two of the three phen ligands are involved in intermolecular π–π stacking interactions as shown in Figure 3. Of note are also two centrosymmetry-related interactions C8···S2 of 3.815 Å, which stabilize the central dimeric unit.

Table 1. Crystal data and details of data collection for $[La(phen)_3(NCS)_3]$.

	$[La(phen)_3(NCS)_3]$
Empirical formula	$C_{39}H_{24}LaN_9S_3$
Formula mass	853.76
Space group	$P\bar{1}$ (No. 2)
a [Å]	11.006(2)
b [Å]	12.465(2)
c [Å]	15.064(3)
α [°]	96.637(11)
β [°]	102.773(13)
γ [°]	103.317(12)
V [Å^3]	1930.9(6)
Z	2
λ [Å]	0.71073
$\rho_{calcd.}$ [g cm^{-3}]	1.468
Crystal size [mm]	$0.30 \times 0.20 \times 0.10$
T [K]	296
μ [mm^{-1}]	1.309
Reflections measured	70817
Independent reflections	7518 (R_{int} = 0.077)
$R1$[a]	0.0472
$wR2$[b]	0.1413
GOF[c]	1.087

[a] $R1 = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. [b] $wR2 = \{\Sigma[w(F_o^2 - F_c^2)^2]/\Sigma[w(F_o^2)^2]\}^{1/2}$. [c] GOF = $\{\Sigma[w(F_o^2 - F_c^2)^2]/(n-p)\}^{1/2}$, where n is the number of reflections and p is the total number of parameters refined.

NMR spectroscopy and ESI-MS data confirmed the structure obtained by X-ray diffraction. In ^{1}H NMR studies a remarkable downfield shift of the signals of the coordinated phen ligand was observed as compared with those of free phen,[23,24] and no signal for free, uncoordinated phen was found in the spectrum (Figure S1). The data of the spectra, the coupling constants, and the shifts are summarized in Table 2. ESI-MS data of KP772 were recorded in EtOH and H_2O. The major peaks in both spectra at m/z = 795.0 and 614.7 correspond to the pseudomolecular ions $[La(phen)_3(NCS)_2]^+$ and $[La(phen)_2(NCS)_2]^+$ (Supporting Information).

In the IR spectra the characteristic bands of ν(C=N) of the thiocyanate between 2050 and 2030 cm^{-1} and the ν(C–H) in the range 3100–3000 cm^{-1} were considered for characterization of the complex and free phen, which are easily distinguishable.[25] The position of the band corresponding to the C=N stretching vibration is used to distinguish between ionic, *N*-bonded and *S*-bonded thiocyanate ions.[26,27] The thiocyanate with band at 2052 cm^{-1} appears to be *N*-bonded in the case of KP772 as confirmed by X-ray diffraction analysis, whereas the C=N stretching frequency in *S*-bonded complexes is found at about 2100 cm^{-1}. No bands attributable to uncoordinated phen are present in the IR spectra of KP772.

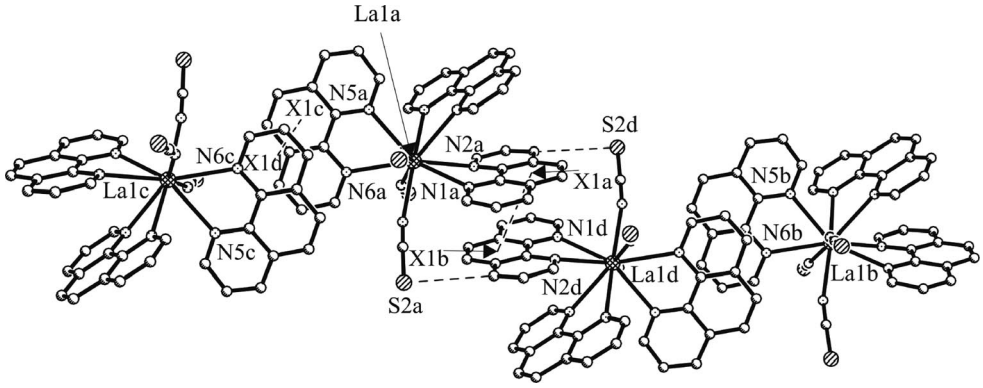

Figure 3. Portion of the crystal structure of $[La(phen)_3(NCS)_3]$ showing two types of π–π stacking interactions: (a) the interplanar distance between centrosymmetry-related phen rings coordinated via N1a, N2a and N1d, N2d is 3.48 Å, and the centroid···centroid distance (X1a···X1b) is 4.028 Å; (b) the interplanar separation between phen rings coordinated via N5a, N6a and N5c, N6c is 3.49 Å, and the centroid···centroid distance X1c···X1d is 3.705 Å.

Table 2. NMR spectroscopic data of the free phen and the complex measured in [D4]methanol. Chemical shifts are given in ppm.

Protons	δ (phen)	$^3J_{H,H}$ [Hz] (phen)	δ (KP772)	$^3J_{H,H}$ [Hz] (KP772)
2-H (9-H)	9.11	4.4	9.24 (0.13)[a]	3.5
3-H (8-H)	7.78	6.2	7.86 (0.08)[a]	6.3
4-H (7-H)	8.48	8.1	8.56 (0.08)[a]	8.1
5-H (6-H)	7.97	–	8.02 (0.05)[a]	–

[a] Values in parentheses show the shift of the signals of the complex in comparison to the free ligand.

Behavior in Aqueous Solution

Since the stability in aqueous solution over a prolonged period of time constitutes an essential requirement for pharmaceutical preparations,[28] the behavior of KP772 in water was studied by NMR, and analytical data of freshly prepared KP772 were compared with those obtained from the hydrolyzed complex, followed by lyophilization. The La complex is sufficiently soluble in water (S_{25} = 2.1 mM) and highly soluble in MeOH, DMSO, and THF.

^{1}H NMR spectra in D_2O revealed minor shifts to lower field of the phen signals when coordinated to La^{III}; however, they are distinguishable from each other (Supporting Information), and no free phen was observed after 7 d.

In order to study the influence of dissolution in water, solutions of KP772 were frozen with liquid nitrogen, assuming stability under these conditions (no substantial rearrangement in the frozen state and during the time of lyophilization of about 48 h is expected). Solutions of the complexes in triply distilled water (1–2 mM) were prepared, stirred at room temp. for 1, 3, and 6 h, lyophilized and characterized by elemental analysis. The data shows that $La(phen)_3(NCS)_3$ trapped a stoichiometric number of three crystal water molecules per complex (see the Supporting Information for the elemental analysis data), but was then stable over hours. This result was confirmed by differential thermogravimetric analysis (TG/DTA) and IR and NMR spectroscopy (see below). The shifts in the NMR spectra in D_2O remained constant for several days, a result that suggests stability of KP772 in aqueous solution. Furthermore, NMR spectra of KP772 and the lyophilisate were identical (in CD_3OD). In the IR spectra the lyophilized complex exhibits additional bands at 3337 and 3197 cm^{-1} as compared to the freshly prepared complex. These frequencies are attributable to coordinated or chemically combined water molecules, confirming the elemental analysis data.[29–31] The spectra of KP772 stirred in water for 1, 4, and 6 h before lyophilization were identical (Supporting Information) and no uncoordinated phen was present, as demonstrated by spiking experiments leading to an intense peak at 624 cm^{-1} (Figure 4).

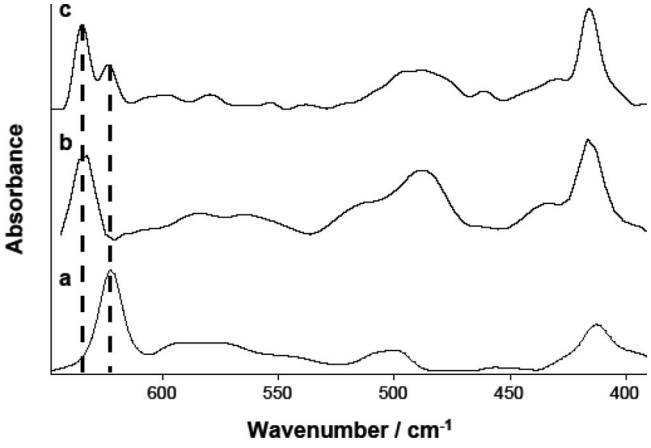

Figure 4. FIR spectra of (a) phen, (b) KP772, and (c) lyophilized KP772 with 1 equiv. phen in the range of 645–390 cm^{-1} recorded in CsI matrix.

The lyophilized samples were analyzed by thermogravimetry, and the resulting curves were virtually identical, independent of the stirring time in aqueous solution. When compared to freshly synthesized KP772, stepwise decomposition in the range 35–220 °C (three steps corresponding to single water molecules) was observed for the lyophilized product. All further steps were similar to that observed for the complex. To demonstrate that there is no free phen present in the lyophilisate, it was spiked with 1 equiv. of phen, which leads to a new decomposition step between 120 and 230 °C (Supporting Information).

Interactions with Biomolecules

Binding Towards Nucleotides

The complex was incubated with a mixture of all four nucleotides (5′-dAMP, 5′-dCMP, 5′-dGMP, 5′-dTMP) at a ratio of 1:2 under simulated physiological conditions (pH = 7.4, 37 °C) for up to 24 h. Measurements were carried out continuously with analysis times of less than 15 min. Because of their different charge states, the negatively charged nucleotides and the neutral complex exhibit different electrophoretic mobilities, making the different species easily distinguishable. No significant change in the peak areas corresponding to either the complex or each of the nucleotides was detected over time. This suggests that very little or no interaction between the nucleotides and the La compound takes place.

Binding Towards Human Plasma Proteins

CE has been shown to be a suitable technique to estimate the extent of binding towards human plasma proteins.[32,33] The La complex was first incubated with the abundant human serum proteins albumin and transferrin for up to 24 h under simulated physiological conditions, and the reaction mixtures were analyzed by CE. Because of their p*I* values of 5.92 and 6.81,[34] respectively, the proteins migrate as negatively charged species at pH = 7.4 and are therefore separated clearly from the lanthanide complex. However, as in the case of the nucleotides, no significant change in the peak area of the complex was detected up to a drug/protein ratio of 20:1. In another attempt to elucidate the binding ability of KP772 to proteins by ultrafiltration/ICP-MS, KP772 was incubated with human plasma for 2 h, ultrafiltered with a 5000 Da cut-off filter, and the La was surprisingly found to be to a high degree present as protein-bound species. This might be related to the high viscosity of human plasma and the small cut-off size causing incomplete filtration. However, additional studies are required to better understand the protein-binding profile of such compounds.

Conclusions

The antitumor drug candidate KP772 was characterized unambiguously by NMR and IR spectroscopy, TG/DTA, ESI-MS data, and X-ray diffraction analysis, which confirmed that three phen and three thiocyanato ligands are κ*N*-bound to the nine-coordinate lanthanum(III) ion. The stability in aqueous solution was found to be sufficient for potential application as intravenous infusion as demonstrated by NMR experiments. These results were confirmed by extensive studies with samples treated for different time periods in water followed by lyophilization. Elemental analyses, IR and NMR spectroscopy and TG/DTA revealed only minor changes in the structure of the complex, resulting from the three water molecules attached to the complex. Furthermore, the reactivity to biomolecules was studied, and no covalent binding to nucleotides used as DNA models was observed, whereas the protein binding studies were inconclusive and require further studies.

Experimental Section

Synthesis and Characterization

Materials and Methods: Chemicals for synthesis: All chemicals were obtained from commercial suppliers and were used without further purification. Ethanol was first dried with CaO and then with Mg immediately before use. Chemicals for CE: Sodium hydroxide solutions and sodium dihydrogenphosphate, the nucleotides (5′-dAMP, 5′-dCMP, 5′-dGMP, 5′-dTMP) and HEPES [*N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid)] were of analytical grade and supplied by Fluka (Buchs, Switzerland). Disodium hydrogenphosphate was purchased from Riedel-de Haën (Seelze, Germany), human serum albumin (HSA) and transferrin from Sigma–Aldrich (Vienna, Austria). High-purity water used throughout this work was obtained from a Millipore Synergy 185 UV Ultrapure Water system. Chemicals for ICP-MS: Human plasma (total protein concentration according to the supplied data sheet was 64 g/l) was a gift from Octapharma (Vienna, Austria), Centrisart I cut-off filters (5000 Da) were obtained from Sartorius (Göttingen, Germany). Instrumentation: The ^{1}H NMR spectra (Figure 5) were recorded with a Bruker Avance DPX 400 spectrometer (Ultrashield™ Magnet) at 400.13 MHz in [D$_4$]methanol or D$_2$O at 25 °C by using standard pulse programs. Chemical shifts were measured relative to the solvent. All IR spectra were measured with a Bruker Vertex 70 FT-IR spectrometer. IR spectra were recorded in the absorption mode by using KBr pellets (MIR, 4000–400 cm^{-1}) and CsI pellets (FIR, 650–200 cm^{-1}), respectively. TG/DTA was carried out simultaneously with a Mettler-Toledo TGA/SDTA851e. The thermograms were recorded in the temperature range 25–1100 °C with a heating rate of 3 °C/min and air as purge gas with a flow rate of 50 mL/min. Mass spectra (ESI-MS) were recorded with a Bruker Esquire$_{3000}$ ion-trap mass spectrometer in the positive-ion mode. The elemental analyses were measured with a Perkin–Elmer 2400 CHNS elemental analyzer by the microlaboratory of the Faculty of Chemistry, University of Vienna. Capillary electrophoresis experiments were performed with an HP^{3D} capillary electrophoresis system (Agilent, Waldbronn, Germany) equipped with an on-column diode-array detector. Fused silica capillaries (50 μm i.d., 50 cm total length, 42 cm to the detector) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Quantification of La was done by ICP-MS (Agilent 7500ce, Waldbronn, Germany).

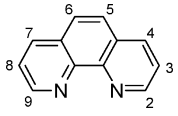

Figure 5. NMR numbering scheme of the phen ligand.

Synthesis of [La(phen)$_3$(NCS)$_3$] (KP772): The complex was prepared as published elsewhere.[20] A solution of $LaCl_3 \cdot 7H_2O$ in EtOH (0.51 g, 15 mL EtOH, 0.10 M) was slowly added to a solution of KSCN in EtOH (50 mL; 0.54 g, 0.11 M). The formed KCl was filtered off, and a solution of 1,10-phenanthroline monohydrate (1.10 g, 50 mL EtOH, 0.11 M) was slowly added whilst stirring. The solution was stirred at room temp. for 2 h, and a white precipitate was obtained, which was washed with ethanol, filtered off and dried in vacuo for 24 h. Yield: 1.44 g (94%). $C_{39}H_{24}LaN_9S_3$ (853.77): calcd. C 54.86, H 2.83, N 14.76, S 11.27; found C 54.56, H 2.83, N 14.51, S 11.04. ^{1}H NMR (400.13 MHz, [D$_4$]methanol): δ = 9.24 (d, $^3J_{H,H}$ = 3.5 Hz, 1 H, 2-H), 8.56 (dd, $^3J_{H,H}$ = 8.1 Hz, 1 H, 4-H), 8.02 (s, 1 H, 5-H), 7.86 (q, $^3J_{H,H}$ = 8.1 Hz, 1 H, 3-H) ppm. IR (KBr): $\tilde{\nu}$ = 2052, 2037 (ν_{C-N}) 1623, 1588, 1571, 1515, 1495, 1420, 1140, 1099, 1088, 861, 843, 729, 716 (δ, ν_{C-H}), 633,

487, 415, 271, 215 (ν_{La-N}) cm^{-1}. ESI-MS (EtOH): *m/z* (%) = 794.7 $[M - SCN]^+$, 614.6 $[M - phen - SCN]^+$.

Preparation of the Lyophilizate: KP772 (100 mg) was dissolved in triply distilled H_2O (50 mL) and then lyophilized for 48 h. The solid was dried in vacuo for 24 h. $C_{39}H_{30}LaN_9O_3S_3$ (907.07): calcd. C 51.59, H 3.33, N 13.89, S 10.57; found C 51.77, H 3.01, N 13.91, S 10.53. ^1H NMR (400.13 MHz, [D_4]methanol): δ = 9.25 (d, $^3J_{H,H}$ = 3.5 Hz, 1 H, 2-H), 8.57 (dd, $^3J_{H,H}$ = 8.1 Hz, 1 H, 4-H), 8.02 (s, 1 H, 5-H), 7.86 (q, $^3J_{H,H}$ = 8.1 Hz, 1 H, 3-H) ppm. IR: $\tilde{\nu}$ = 337, 3197 (ν_{O-H}) 2058, 2034 (ν_{C-N}) 1642, 1625, 1590, 1572, 1517, 1497, 1421, 1140, 1101, 1089, 862, 845, 729, 718 (δ, ν_{C-H}), 633, 489, 416, 268, 237, 217 (ν_{La-N}) cm^{-1}.

Crystallographic Structure Determination: X-ray diffraction measurements were performed with a Bruker X8APEX II CCD diffractometer. A single crystal was positioned at 40 mm from the detector, and 3024 frames were measured, each for 10 s over 1° scan width. The data were processed by using SAINT software.[35] Crystal data, data-collection parameters, and structure-refinement details for $[La(phen)_3(NCS)_3]$ are given in Table 1. The structure was solved by direct methods and refined by full-matrix least-squares techniques. Non-hydrogen atoms were refined with anisotropic displacement parameters. H atoms were placed at calculated positions and refined as riding atoms in the subsequent least-squares model refinements. The isotropic thermal parameters were estimated to be 1.2 times the values of the equivalent isotropic thermal parameters of the atoms to which the hydrogen atoms were bonded. The crystal structure contains voids of ca. 190 $Å^3$. The following computer programs were used: structure solution, SHELXS-97;[36] refinement, SHELXL-97;[37] molecular diagrams, ORTEP;[38] computer: Pentium IV; scattering factors.[39] CCDC-691376 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Capillary Electrophoresis

Sample Preparation: For the protein-binding analyses by CE, complex (1 mM) and protein (50 μM) were incubated at 37 °C. For the nucleotide-binding experiments, 0.5 mM complex and 1 mM of each of the DNA model compounds were used (37 °C). HEPES buffer (20 mM, pH = 7.4) was utilized as incubation medium and phosphate buffer (20 mM), pH = 7.4 as background electrolyte (BGE). The BGE and the samples were passed through a 0.45 μm disposable membrane filter (Sartorius, Göttingen, Germany) before being injected into the CZE (Capillary Zone Electrophoresis) system. The capillary and sample trays were thermostatted at 37 °C. Injections were performed at 50 mbar for 3 s; for all experiments a voltage of 20 kV was applied and kept constant throughout the analysis – the resulting current was about 35 μA. Detection was carried out at 200 and 254 nm. Prior to the first use, the capillary was flushed with HCl (0.1 M), water, NaOH (1 M), and again with water for 10 min each. After this procedure, the capillary was conditioned with the separation electrolyte for 10 min. Before each injection, the capillary was treated with NaOH (0.1 M) and water for 2 min each, and afterwards with the BGE for 3 min.

ICP-MS

Sample Preparation: A 100X solution of KP772 was prepared in water, diluted with human plasma (1.5 mL) to a final concentration of 3 μM, which equals typical IC_{50} concentrations in human tumor cell lines,[9] and incubated in Eppendorf tubes at 37 °C for 2 h. Cut-off filters were washed with water in order to remove glycerin from the membrane prior to quantitative transfer of the incubation mixture and consecutive centrifugation at 1800 rpm for 10 min followed by 3700 rpm for 30 min with a Hermle Megafuge 1R. As filtration was not complete due to the high viscosity of human plasma and the need for small cut-off filters, an aliquot (100 μL) was taken from the filtrate and acidified with sub-boiled nitric acid (0.5 mL) (quartz sub-boiling system from Milestone-MLS GmbH, Leutkirch, Germany). Furthermore, the amount of La in the Eppendorf tube (acidified with 1 mL of nitric acid, 50 μL aliquot thereof) and the amount of La adsorbed in the filter membrane (dissolution of the membrane by centrifugation of 1 mL of nitric acid with the Centrisart filter, 50 μL aliquot thereof for quantification) were determined by ICP-MS. Samples were acidified with nitric acid to a total volume of 0.5 mL of nitric acid, filled up to a total volume of 10 mL with milliQ water and internally standardized with In and Re (0.5 ppb). For the determination of protein-bound La, the La contents in the filtrate, in the Eppendorf tube and in the cut-off filter were subtracted from the total amount of La used for the binding study.

Supporting Information (see footnote on the first page of this article): NMR spectra, elemental analysis data, IR spectra, thermograms, ESI mass spectra, protein interaction studies, nucleotide interaction studies.

Acknowledgments

The authors would like to thank Dr. Vladimir Arion for X-ray crystal structure determination, Dr. Markus Galanski for assistance in compilation of this paper and Dr. Peter Unfried for assistance in the interpretation of the IR spectra and thermograms. M. G. thanks the Austrian Science Foundation (FWF) for financial support (Schroedinger Fellowship J2882-N19).

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Received: January 13, 2009
Published Online: August 26, 2009

