Evaluation of the Cytotoxic and Pro-Apoptotic Activities of Eu(III) Complexes with Appended DNA Intercalators in a Panel of Human Malignant Cell Lines

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Abstract: A series of 8 europium (III) tris-β-diketonates with common formula $Eu(L)_3Int$, where L is acetyl acetone, thenoyltrifluoroacetone, benzoylacetone, dibenzoylmethane and Int is 1,10-phenanthroline or 2,2`-bipyridine, together with an analog without intercalating moiety ($Eu(III)(acetyl\ acetone)_3(H_2O)_2$) were tested for cytotoxic activity in a panel of human tumor cell lines, using the MTT-dye reduction assay. The panel consisted of the leukemias HL-60, BV-173, SKW-3, K-562, LAMA-84 and the urinary bladder carcinoma 5637. The tested europium complexes with appended intercalator moieties exhibited profound cytotoxic effects with IC_{50} values lower or comparable to those of the referent drug cis-DDP, whereby the 1,10-phenanthroline bearing compounds were invariably more active than the corresponding 2,2'-bipyridine analogs. The established low cytotoxic potential of $Eu(III)(acetyl\ acetone)_3(H_2O)_2$ as compared to its highly potent analogs with either 1,10-phenanthroline or 2,2'-bipyridine ligand demonstrated that the abundance of intercalating motif is a mandatory structural prerequisite for optimal activity within this series of cytotoxic agents. Selected compound caused DNA-fragmentation when applied in cytotoxic concentration, which suggests that the induction of programmed cell death (apoptosis) at least partly mediates the cytotoxic effects of tested compounds. Taken together our data give us reason to conclude that the presented Eu(III) complexes represent a unique class of cytotoxic metal coordination compounds and necessitate further detailed evaluation in order to define the structure activity relationships as well as the predominant mode of action. To the best knowledge of the authors this is among the first reports of potent cytotoxic Eu(III) compounds.

Key Words: Europium(III), β-diketone, 1,10-phenanthroline, 2,2'-bipyridine, MTT-assay, apoptosis.

INTRODUCTION

The clinical success of the square planar complex cis-diamminedichloroplatinum(II) (cis-DDP) for the management of testicular teratoma and diverse solid tumors fueled intensive researches focused upon elaboration of better-tolerated and non-cross resistant analogs [1-4]. Apart from the thousands of platinum complexes synthesized the search for metal-based antineoplastic drugs was expanded upon other d^8 metal ions (e.g. Au(III), Pd(II)), isoelectronic to Pt(II) and capable of forming square planar complexes, analogs to cis-DDP [2,5,6]. Despite of the numerous complexes synthesized, however, neither the platinum drugs nor their isoelectronic/isostructural analogs proved to be superior to cis-DDP with respect to both low toxicity and wider spectrum of activity [2,5,6]. Currently, only 5 platinum agents other than cis-DDP (namely carboplatin, nedaplatin, oxaliplatin, lobaplatin and heptaplatin), are used clinically as drugs, and none of the non-platinum drugs has proved suitable for clinical utility yet [1,3].

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In an alternative fashion much attention has been paid to platinum-dissimilar transition metals, including the lanthanide series of rare earth elements, whose electronic configuration and chemical properties condition alternative mode of action and anticipated lack of cross-resistance to platinum drugs. The current preclinical status of lanthanide-based cytotoxic/anticancer complexes is extensively reviewed very recently [7].

Unlike the platinum (II) the lanthanides form complexes predominantly with O-containing ligands, rather than with S- and N-based ligands. Conversely, series of lanthanide complexes with diverse O-containing ligands such as coumarine, bis-coumarines, aminocycloalkancarboxylic acids etc. have been found to exert cytotoxic effects against different tumor cell lines [7-10].

As the lanthanide salts themselves are either non-cytotoxic or exert only marginal effects in the majority of tumor-inhibiting lanthanide agents the abundant ligands are characterized *via* intrinsic cytotoxic activity [7].

An intriguing approach towards the design of cytotoxic metal coordination compounds is based upon the appending

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of a DNA-intercalator moiety therein, thus combining the pharmacological properties of the metal center with those of the DNA-targeted pharmacophore in a single molecule. This strategy has met appreciable interest towards design of platinum, palladium, ruthenium, gold complexes and more recently extended to the design of rare-earth coordination compounds, with anticipated cytotoxic/antitumor activity [2,4,6]. Thus Wang *et al*., have designed cytotoxic lanthanum (III) complexes with 1,10-phenanthroline-2,9-bis-alpha amino acid conjugates, some of which were found to exert superior activity as compared to cis-DDP and to interact with DNA *via* complex mechanism, involving both covalent and intercalative binding modes [11,12].

The most stable and common lanthanide complexes are those formed with O-containing chelating ligands, such as EDTA-type anions, hydroxyacids as well as β-diketonates such as acetyl acetone. Owing to their photochemical properties lanthanide β-diketonates, including Eu(III) compounds have found wide application as light-converting optical devices, as emitters in liquid lasers and in electroluminescent devices, as chemiluminescence activators, as thermoluminescence dosimeters, for luminescent determination of Ln^{3+} etc. [14]. To our best knowledge however, Eu(III) β-diketonates have not been evaluated as cytotoxic/antineoplastic agents.

The aim of this study is to evaluate the cytotoxic potential of Eu(III) complexes bearing bulky DNA-intercalating motif (Int) in their structure. To meet this objective a series of 8 europium (III) tris-β-diketonates with common formula $Eu(L)_3Int$, where L is acetyl acetone, thenoyltrifluoroacetone, benzoylacetone, dibenzoylmethane and Int is 1,10-phenanthroline or 2,2'-bipyridine were synthesized according to a known procedure and their structures were confirmed by comparison of the literary data. In order to elucidate the structure activity relationships an analoge without intercalating moiety ($Eu(III)(acetyl\ acetone)_3(H_2O)_2$) was tested for biological activity as well.

RESULTS AND DISCUSSION

Chemistry

The synthesis of $Eu(L)_3.2H_2O$ was accomplished according to a previously described procedure [15] by adding a stoichiometric quantity of a ethanolic solution of the appropriate β-diketonate L anion (prepared by neutralization with an aqueous solution of NaOH) to an aqueous solution of $EuCl_3.6H_2O$. The compound precipitates and separated by filtration (Fig. (**1**)). So formed intermediates are used to the next stage without further purification.

The synthesis of the target 2,2'-bipyrdyl- and 1,10-phenantroline-tris[β-diketonato]europium(III) complexes **(9-16)** was carried out using previously described synthetic strategies [15,16,17].

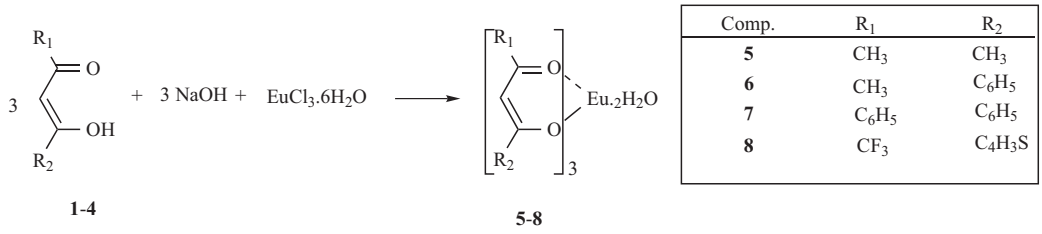
In this way, the preparation of Eu(III) β-diketonates **9-16** (Fig. (**2**)) occurs by mixing of methanol/acetone solutions of compounds **5**-**8** with methanol solution of equimolar amounts of 2,2'-bipyridine or 1,10-phenantroline. The formed products are recrystallized from ethanol/acetone and their structures are confirmed with melting points and ^{1}H-NMR spectra (experimental part).

According to the ^{1}H-NMR data and the melting points values, the purity of the target Eu^{3+} compounds is in the range of the analytical grade (≥ 99.7%). They were recrystallized several times before being used.

Pharmacology

In this study the cytotoxic potential of the target compounds, containing either 2,2'-bipyridine (**9**,**11**,**13** and **15**) or 1,10-phenanthroline (**10**,**12**,**14** and **16**) moieties was tested against a panel of human tumor cell lines characterized *via* diverse cell type and origin, using the MTT-dye reduction assay. The panel of tumor cell lines consisted of the acute promyelocyte leukemia HL-60, the pre-B cell leukemia BV-173, the chronic lymphoid leukemia SKW-3, the chronic myeloid leukemias K-562 and LAMA-84 and the urinary-bladder carcinoma 5637. In order to evaluate the influence of the intercalating motifs on the pharmacological activity the diaqua-analog **5** was tested as well. Throughout the cytotoxicity assessment cis-DDP was used as referent drug. The IC_{50} values obtained are summarized in Table **1**.

With the only exception of compounds **5** and **7** the europium complexes proved to be potent cytotoxic agents causing 50% inhibition of the cell viability at low micromolar concentrations and moreover most of them displayed lower IC_{50} values in comparison to the referent drug cis-DDP. Generally speaking the cell lines did not demonstrate significant differences in their responsiveness to the tested complexes with the exception of the CML-derived K-562 and LAMA-84. These are characterized *via* the strong expression of the BCR-ABL oncoprotein, a non-receptor tyrosine kinase which conditions the high proliferative activity of these cells together with their relatively low responsiveness to pro-



1-4 5-8

Comp.	R_1	R_2
5	CH_3	CH_3
6	CH_3	C_6H_5
7	C_6H_5	C_6H_5
8	CF_3	C_4H_3S

Fig. (1). Preparation of the intermediates 5-8 and chemical structures thereof.

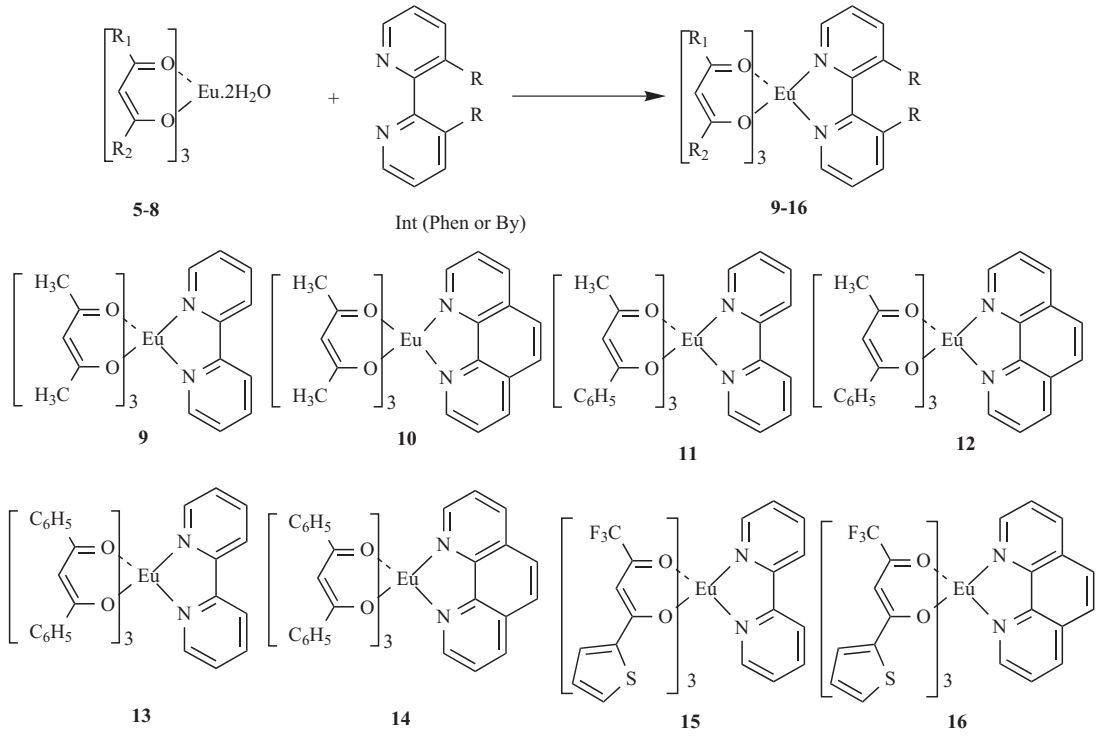


Fig. (2). Synthesis and chemical structures of the target compounds **9-16**.

apoptotic stimuli, including the antineoplastic drugs [18]. Interestingly, the BV-173 cells are also known to express BCR-ABL, but to lower extend than K-562 and LAMA-84 and conversely they were found to be more sensitive to the investigated compounds as compared to both CML-cell lines [18,19].

This study was aimed principally at determining the structure-activity relationships for the tested europium (III) tris-β-diketonates and more precisely to determine the influence of the intercalating motif and of the type of the diketonato ligand on the cytotoxic potential of the respective complexes.

Table 1. Cytotoxic Effects of the Tested Eu(III) tris-(β-diketonato) Complex Compounds and cis-DDP Against the Panel of Human Tumor Cell Lines as Assessed by the MTT-dye Reduction Assay After 72 h Exposure

complex	IC_{50} value (μM) [a]					
	HL-60	**BV-173**	**SKW-3**	**K-562**	**LAMA-84**	**5637**
5	> 100	> 100	> 100	> 100	> 100	> 100
9	69.21	45.49	> 100	> 100	> 100	> 100
10	7.29	5.97	6.46	10.60	8.49	6.25
11	18.14	7.53	8.18	34.82	16.72	12.36
12	5.37	6.17	7.02	9.04	7.82	12.44
13	3.81	2.52	7.71	7.49	6.49	5.31
14	4.22	3.56	2.18	12.13	8.02	3.53
15	27.41	18.64	16.14	26.30	22.01	12.35
16	4.94	2.75	2.20	5.89	5.02	4.79
cis-DDP	8.14	11.02	11.24	32.04	18.21	5.91

[a]means of three independent runs of MTT-assay

As clearly evident from the obtained IC_{50} values, the agents with appended intercalator moieties exhibited profound cytotoxic effects with IC_{50} values lower or comparable to those of the referent drug cis-DDP. Thus the diaqua-analog **5**, lacking Int motif in its structure exerted only marginal effects, failing to induce 50% inhibition of cell viability throughout the concentration range evaluated, while the introduction of a 2,2'-bipydine (**9**) or especially of a 1,10-phenanthroline (**10**) motif, while retaining the acetyl acetone ligand resulted in a significant increase of the cytotoxic activity (Table **1** and Fig (**3**)). Conversely, the juxtaposition of the activities of complexes bearing either of the intercalating motifs revealed that the 1,10-phenanthroline-analogs were generally more active than the corresponding 2,2'-bipyridine analogs. These data clearly indicate that the presence of a intercalator motif is a mandatory structural prerequisite for optimal activity within this series of cytotoxic agents. Conversely, the appending of such intercalating moieties have been widely used in order to modulate the kinetics of DNA-binding of diverse metal complexes including platinum(II), palladium(II), ruthenium(III), gold (III) etc [4,6]. This approach is not only aimed at increasing the rates of complexes binding to DNA, but to reduce their interactions with protein or peptide nucleophiles, implicated in both resistance mechanisms and organ toxicities of metal-based drugs. Furthermore the combination of the properties of a metal center and an intercalating pharmacophore in a single molecule is expected to result in augmentation of the cytotoxic potential of the resultant complexes [4].

Apart from the well-documented impact of the intercalating motif upon the bioactivity of the complexes we aimed at determining the influence of the β-diketonato ligand as well. As a general trend the acetylacetono-compounds **9** and **10** were the least active amongst the europium complexes tested. In the thenoyltrifluoroacetono-analogs (**15** and **16**) and especially in the benzoylacetono-complexes (**11** and **12**) the introduction of electron-withdrawing function and/or steric bulk was consistent with a significant increase of the cytotoxic activity - almost invariably these complexes were more potent than the referent drug cis-DDP. The symmetric, bulky dibenzoylmethane ligand afforded for the most prominent cytotoxic activity amongst the agents under investigation, rendering the analogs **13** and **14** two- to ca. five-fold more potent than cis-DDP (Table **1** and Fig. (**1**)). Since the high molecular weight of all of the complexes is generally detrimental for their transmembrane passage, the higher cytotoxic activity encountered with **13** and **14** could be attributable to the overall increase of the lipophilicity afforded by the abundant dibenzoylmethane ligands.

Considering the fact that the MTT-endpoint only detects the antiproliferative/cytotoxic potential giving no account on

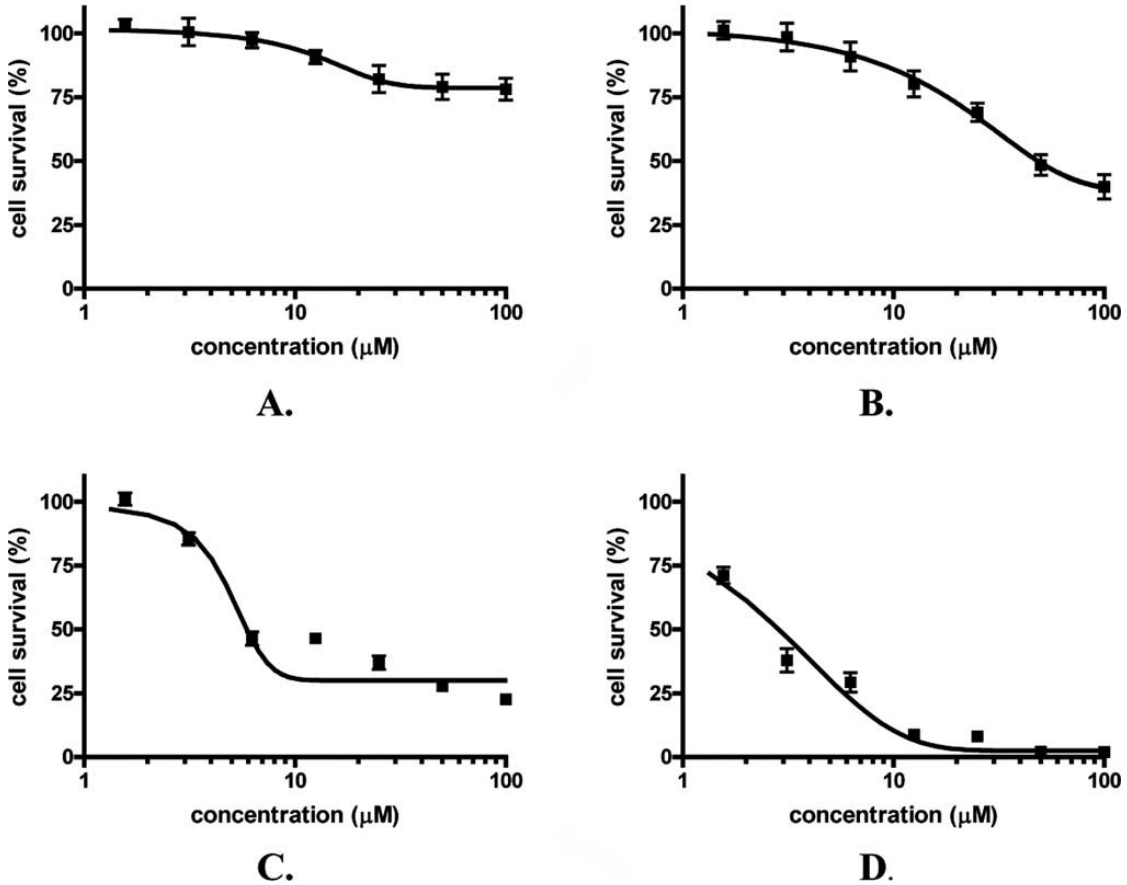


Fig (3). Representative concentration response curves as determined by the MTT-dye reduction assay, following 72 h exposure of the pre-B-cell leukemia BV-173 cells with compounds **5**, **9**, **10** and **13**.

the mechanistic aspects of the established effects we furthermore evaluated the ability of selected agents to induce oligonucleosomal DNA fragmentation, which is a well established cardinal feature of programmed cell death (apoptosis). The DNA isolated from the cytosolic fraction of SKW-3 cells, following 24 h exposure to compounds **15** and **16** at concentrations below their respective IC_{50} values demonstrated a typical laddering phenomenon, indicative for DNA-fragmentation and conversely for induction of apoptosis (Fig (**4**), left panel). When the cells were exposed to multifold-higher concentrations of the tested Eu(III) agents, however, a diffuse fluorescent smear was encountered, suggesting that in this concentration range the cytotoxic effect is conditioned *via* induction of necrotic cell death (Fig (**4**), right panel).

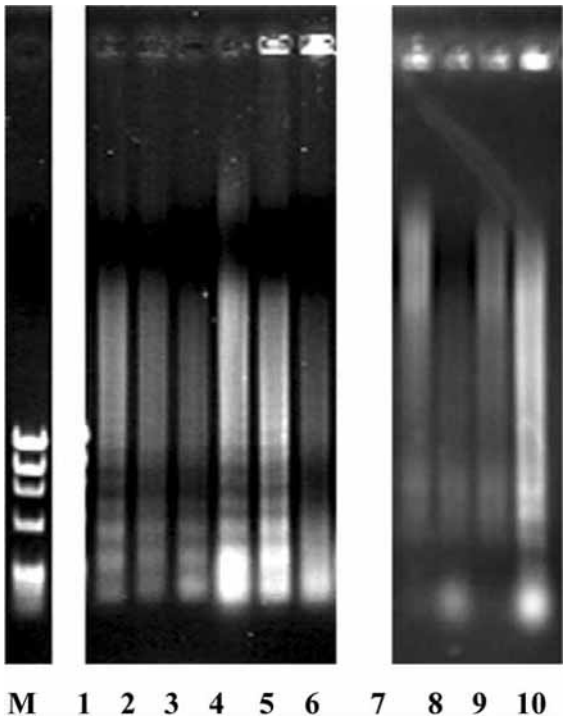


Fig. (4). Gel electrophoresis of DNA, isolated from the cytosolic fraction of SKW-3 cell following 24 h exposure to **15** (3.125 μM – lane 1; 6.25 μM – lane 2; 12.5 μM- lane 3; 25 μM lane 7 and 50 μM lane 8) and **16** (1 μM – lane 4; 2 μM – lane 5; 6.25 μM – lane 9 and 12.5μM- lane 10), versus untreated control – lane 6; M-DNA laddering marker. Typical DNA-laddering phenomena are evident on the left panel of the figure, corresponding to the lower concentrations of the compounds, whilst on the right panel, whereby the concentrations exceed the IC_{50} values of both agents a diffuse destruction of the chromatin is present, indicative for necrosis.

CONCLUSIONS

To our best knowledge this is the first report to demonstrate the potent cytotoxic potential of Eu(III) tris-β-diketonato coordination compounds against a wide spectrum of tumor test systems. The SAR analysis clearly indicates that the abundance of an intercalating moiety such as 2,2'-bipyridine or 1,10-phenanthroline is a dominant requirement for optimal activity. Furthermore, the symmetric hydrophobic dibenzoylmethane ligand proved to afford superior activity as compared to the remaining β-diketonato ligands employed. The observed propensity of the tested compounds to trigger DNA-laddering in SKW-3 cells suggest that the recruitment of the apoptotic machinery is involved in the cytotoxic mode of action of the presented compounds. Taken together our data give us reason to conclude that the presented Eu(III) complexes represent a unique class of cytotoxic metal coordination compounds and necessitate further more detailed evaluation in order to define more precisely the structure activity relationships as well as the predominant mode of action.

EXPERIMENTAL SECTION

A) Chemistry

Acetyl acetone (AA)(**1**), benzoylacetone (BA)(**2**), dibenzoylmethane (DBM)(**3**), thenoyltrifluoroacetone (TTA) (**4**), 2,2'-bipyridine, 1,10-phenantroline and $EuCl_3.6H_2O$ are commercially available reagents and are used without further purification. Melting points of the final products (**9-16**) are determined on a Kofler apparatus and are uncorrected. ^{1}H-NMR spectra were obtained on a Bruker 250 MHz instrument in DMF-d_7.

Synthesis of Eu(III) β-diketonates (5-8) [15]

To a solution of 0.031 mol NaOH in 5 ml H_2O 0.03 mol of the correspondent β-diketone (**1-4**) dissolved in 10 ml ethanol are added. The formed clear mixture is added to an aqueous solution of 0.01 mol $EuCl_3.6H_2O$ and the resulting white precipitate, is suction filtered and air dried. The isolated compounds **5-8** is recrystallized from a mixture ethanol: acetone (1:2) and dried in desiccator.

Synthesis of 2,2'-bipyrdyl- and 1,10-phenantroline-tris[β-diketonato]europium(III) Complexes

[15-17] 0.01 mol of Eu(III) β-diketonates **(5-8),** (prepared according to the aforementioned procedure), is dissolved in a mixture of 10 ml methanol and 5-6 ml acetone. To the solution of europium complex a methanol solution of 0.01 mol 2,2'-bipyridine or 1,10-phenantroline is added. The formed precipitate is suction filtered, washed with methanol and air dried. The final europium complexes (**9-16**) are recrystallized from ethanol/acetone mixture (1:2).

Tris(acetonylacetonato)-mono(2,2'-bipyridine)europium (III) 9

^{1}H-NMR (δ, ppm, DMF-d_7): 0.51 br s (18 H, CH_3), 1.3 br s (3H, CH), 8.22-9.00 m (8H, 2,2'-bipyridine). M.p. 198-200^{o}C (lit. 200-202^{o}C). Yield 98%.

Tris(acetonylacetonato)-mono(1,10-phenantroline)europium(III) 10

^{1}H-NMR (δ, ppm, DMF-d_7): 0.44 br s (18 H, CH_3), 1.5 br s (3H, CH), 9.2-11.7 m (8H, 1,10-phenatroline). M.p. 227-229^{o}C (lit.226-228^{o}C). Yield 97%.

Tris(benzoylacetonato)-mono(2,2'-bipyridine)europium (III) 11

^{1}H-NMR (δ, ppm, DMF-d_7): 2.31 s (9H, CH_3), 5.19 s (3H, CH), 5.22 s (2H, CH-2,2'-bipyridine), 6.40-8.50 m (21H, Ar). M.p. 160-161oC. Yield 95%.

Tris(benzoylacetonato)-mono(1,10-phenantroline)europium(III) 12

^{1}H-NMR (δ, ppm, DMF-d_7): 1.90 s (9H, CH_3), 5.09 s (3H, CH), 5.11 s (2H, CH-1,10-phenantroline), 6.51-6.66 m (15H, phenyl), 8.45-11.6 m (6H, 1,10-phenantroline). M.p. 193-194°C (191-192°C). Yield 97%.

Tris(dibenzoylmethane)-mono(2,2'-bipyridine)europium (III) 13

^{1}H-NMR (δ, ppm, DMF-d_7): 5.53 br s (3H, CH and 2H, CH-2,2'-bipyridine), 6.63-6.73 m (30H, Ar), 7.25-8.90 m (6H, 2,2'-bipyridine). M.p. 204-206°C, Yield 90%.

Tris(dibenzoylmethane)-mono(1,10-phenantroline)europium(III) 14

^{1}H-NMR (δ, ppm, DMF-d_7): 5.61 br s (3H, CH), 5.64 s (2H, CH-1,10-phenantroline), 6.54-9.21 m (36H, Ar). M.p. 175-177°C (lit.172-173°C). Yield 94%.

Tris[4,4,4-trifluoro-1-(2-thienyl)-1,3-butanediono]-mono (2,2'-bipyridine)europium(III) 15

^{1}H-NMR (δ, ppm, DMF-d_7): 6.36 s (3H, CH), 7.31-8.71 m (17H, Ar). M.p. 218-220°C Yield 91%.

Tris[4,4,4-trifluoro-1-(2-thienyl)-1,3-butanediono]-mono (1,10-phenantroline)europium(III) 16

^{1}H-NMR (δ, ppm, DMF-d_7): 6.27 s (3H, CH), 7.54-8.83 m (17H, Ar). M.p. 234-235°C Yield 94%.

B) Pharmacology

All of the procedures concerning the cell culture maintenance, solution preparation and treatment were carried out in a 'Heraeus' laminar flow cabinet after UV-sterilization of the environment. Stock solutions of the europium complexes **5**, **9-16** and of the referent drug cis-DDP were freshly prepared in DMSO and were consequently diluted to the desired extend with RPMI-1640 medium. At the final dilutions obtained the concentration of the solvent DMSO never exceeded 0.25 %.

Cell Culture Conditions

The human tumor cell lines involved in this study were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). For all cell lines RPMI-1640 liquid medium supplemented with 10 % FBS and 2 mM L-glutamine was used. Leukemic cells were grown as suspension type cultures in a controlled environment – cell culture flasks at 37°C in an incubator 'BB 16-Function Line' Heraeus (Kendro, Hanau, Germany) with humidified atmosphere and 5% CO_2. Cells were kept in log phase by supplementation with fresh medium after removal of cell suspension aliquots, two or three times a week. The urinary bladder carcinoma cell line 5637 was grown as a monolayer culture and was subcultured every 3 or 4 days after detaching the cells with 0.1% trypsin solution.

Cytotoxicity Assessment (MTT-Dye Reduction Assay)

Cell viability was used as bioactivity end-point and was assessed using the standard MTT-dye reduction assay as described by Mossman [20] with minor modifications [21], whereby at least 6 wells/concentration were used. All cell viability experiments were run in triplicate. Cell survival fractions were calculated as percentage of the untreated control (untreated control=100%). The results were fitted to sigmoidal dose response curves and the IC_{50} values were derived from the concentration-response curves using Origin and GraphPad Prizm software and for PC.

DNA-Fragmentation Analysis

Horizontal gel-electrophoresis of cytosolic DNA, isolated from treated SKW-3 cells was performed in order to test the ability of the compounds under investigation to trigger programmed cell death (apoptosis). The method was executed as previously described with some modifications [21]. In brief: cell suspension aliquots (10 ml) of SKW-3 (treatment groups and untreated control respectively) at a density of 0.5×10^6 cells/ml were transferred in sterile plastic petri-dishes. The cells were exposed to the tested compounds 24h. After the incubation period the cells were pelleted and washed in PBS. Cell pellets were re-dispersed in 0.25 ml PBS and lysed through addition of 0.5 ml buffer containing 0.5% Triton X-100, 20mM Tris-HCL and 1mM EDTA (pH = 7.4). Samples were incubated on ice for 5 min and thereafter spun at 13 000 rpm for 20 min. The supernatants were transferred into fresh 2ml Eppendorf safe lock tubes and then 937μl 2-propanol as well as 187 μl 6M 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. After that DNA was re-dissolved in 20μl 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).

Data Processing and Statistics

The statistical significance of the evaluated parameters was assessed using the Student's t-test with $p \leq 0.05$ set as significance level. The data processing was performed using MicrosoftEXCEL and Origin software for PC.

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ABBREVIATIONS

By	=	2,2'-bipyridine
cis-DDP	=	cis-diamminedichloroplatinum (II)
FBS	=	fetal bovine serum
IC_{50}	=	Concentration causing 50% reduction of cell viability
Int	=	DNA-intercalating motif
Phen	=	1,10-phenanthroline
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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