

## [DL-1,2-Bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II), a new compound for the therapy of ovarian cancer

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**Summary.** The synthesis of diastereoisomeric [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes, DL-3-PtCl<sub>2</sub> and *meso*-3-PtCl<sub>2</sub>, and their evaluation on the hormone-independent, human MDA-MB231 breast cancer cell line, on the cisplatin-sensitive and -resistant L1210 leukemia cell line, on the cisplatin-resistant human NIH:OVCAR 3 ovarian cancer cell line, on the P-388 leukemia of the mouse and on the cisplatin-sensitive and -resistant Ehrlich ascites tumor of the mouse are described. On all tumor models DL-3-PtCl<sub>2</sub> produces a marked inhibitory effect. The diastereoisomer *meso*-3-PtCl<sub>2</sub> is less active and more toxic. It is striking that DL-3-PtCl<sub>2</sub> leads to a pronounced inhibition of all cisplatin-resistant tumors. At non-toxic concentrations DL-3-PtCl<sub>2</sub> produces cytotoxic effects on the NIH:OVCAR 3 cell line. Therefore DL-3-PtCl<sub>2</sub> is of interest for further evaluation for the therapy of ovarian cancer.

**Key words:** [DL-1,2-Bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) – Ovarian cancer

### Introduction

The first communication on the capability of cisplatin to initiate regression in advanced ovarian cancer was made by Wiltshaw and Carr (1974; see also Barker 1983). Cisplatin produces a therapeutic response even in patients with refractory ovarian carcinoma (Wiltshaw and Kroner 1976). In high dosage cisplatin leads to response rates up to 63% and in some cases to complete remissions (18%) with a survival time greater than 2 years in several of these patients (Barker and Pring 1981; compare Ozols et al. 1985; Ozols and Young 1985; Eisenhauer et al. 1986).

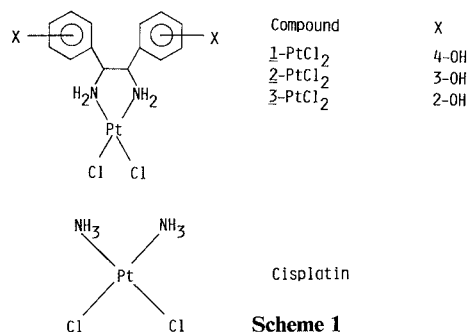
The reason for the short overall survival time is the rapid development of resistance in ovarian tumor cells (Ozols and Young 1984, compare also Bruntsch 1985;

Weiss 1986). Cisplatin-resistant tumor cells show multiple biochemical differences in comparison to the related sensitive cells, e.g. a reduced capability to accumulate cisplatin, an increased intracellular level of glutathione and an enhanced DNA repair, i.e. cisplatin-nucleoside-adduct removal (see Lai et al. 1988 for references).

The Sonderforschungsbereich 234 has been working for some time on the development of platinum complexes that show an activity on cisplatin-resistant tumor models. Such compounds are of interest: (a) in combination with cisplatin for first-line treatment of ovarian cancer to avoid or retard the development of resistance; (b) for the second-line therapy of ovarian cancer after development of resistance against cisplatin.

In recent publications (Wappes et al. 1984; Jennerwein et al. 1989 a, b) we have shown that stereoisomeric dichloro(1,2-diphenylethylenediamine)platinum(II) complexes with hydroxy groups in the 3 or 4 positions of both benzene rings (Scheme 1, compounds 1-PtCl<sub>2</sub> and 2-PtCl<sub>2</sub>) possess a marked activity on the Ehrlich ascites tumor of the mouse. On the cisplatin-resistant variant of this tumor, however, only the 3-hydroxy derivatives (2-PtCl<sub>2</sub>) lead to an inhibition of tumor growth (Jennerwein et al. 1989 b). Their effect was most outstanding in the case of the compound with *R,R/S,S* configuration<sup>1</sup>, which produced 40% cures at a dose of 3 × 2.5 mg/kg, i.p.

<sup>1</sup> *R,R/S,S* = racemate = DL; *R,S* = *meso*



**Scheme 1**

(days 1–3). The combination of compound 2-PtCl<sub>2</sub> (*S,S* configuration) with cisplatin results in a weakly synergistic effect in cell culture experiments using the hormone-independent human MDA-MB231 breast cancer cell line (Jennerwein et al. 1989 b).

To our surprise, the shift of both OH groups of the *R,R/S,S* compound 2-PtCl<sub>2</sub> from the 3 into the 2 position (compound DL-3-PtCl<sub>2</sub>) produces a distinct elevation of antitumor activity on the cisplatin-resistant Ehrlich ascites tumor (Scheme 1).

This publication will report on the synthesis of the two diastereoisomeric [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) compounds (DL-3-PtCl<sub>2</sub> and *meso*-3-PtCl<sub>2</sub>) and their antitumor activity on several tumor models.

## Materials and methods

**Chemistry.** *meso*-1,2-Bis(2-hydroxyphenyl)ethylenediamine (*meso*-3), originally described by Japp and Hooker (1884), was synthesized from salicylic aldehyde and ammonia using an improved method published by Vögtle and Goldschmitt (1976). *N,N'*-Disalicylidene-*meso*-1,2-bis(2-methoxyphenyl)ethylenediamine (*meso*-3c) was synthesized by the stereospecific [3,3] sigmatropic diaza-Cope rearrangement reaction according to the method of Vögtle and Goldschmitt (1976) from *meso*-1,2-bis(2-hydroxyphenyl)ethylenediamine (*meso*-3) and 2-methoxybenzaldehyde in boiling CH<sub>3</sub>CN (Scheme 2, method A). Upon hydrolysis with 3M H<sub>2</sub>SO<sub>4</sub> *meso*-1,2-bis(2-methoxyphenyl)ethylenediamine (*meso*-3a) was obtained (Scheme 2, method B). The reaction of *meso*-3a with 2-methoxybenzaldehyde led to the symmetrical diimine *meso*-3b (Scheme 2, method C). The compound *meso*-3b was melted. At temperatures higher than 120° C there is equilibrium between the diimines *meso*-3b and DL-3b. The diastereoisomeric products were separated by their different solubility in CH<sub>2</sub>Cl<sub>2</sub> (Scheme 2, method D). DL-1,2-Bis(2-methoxyphenyl)ethylenediamine (DL-3a) was prepared by hydrolysis of DL-3b in 3M H<sub>2</sub>SO<sub>4</sub> (Scheme 2, method E). DL-1,2-Bis(2-hydroxyphenyl)ethylenediamine (DL-3) was obtained by ether cleavage of DL-3a (Scheme 2, method F). It was difficult to find an appropriate method for this reaction. Various ether cleavages with BBr<sub>3</sub>/CH<sub>2</sub>Cl<sub>2</sub> abs., HBr, HI and BBr<sub>3</sub>/boiling CHCl<sub>3</sub> abs. were tested, and the best results seemed to be achieved by using BBr<sub>3</sub> in CH<sub>2</sub>Cl<sub>2</sub> at room temperature for 3 days. Trials to purify DL-3 further caused

its decomposition and were avoided. [DL-1,2-Bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) (DL-3-PtCl<sub>2</sub>) was synthesized by reaction of DL-1,2-bis(2-hydroxyphenyl)ethylenediamine (DL-3) with K<sub>2</sub>PtCl<sub>4</sub> (Scheme 3, method G). For steric reasons the complexation of *meso* ligands proceeds very slowly. Therefore faster complexation was achieved with the reaction of K<sub>2</sub>PtI<sub>4</sub> and *meso*-1,2-bis(2-hydroxyphenyl)ethylenediamine (*meso*-3) (Scheme 3, method H). To exchange iodine for chlorine, I<sup>-</sup> was precipitated with Ag<sub>2</sub>SO<sub>4</sub> and subsequently the intermediate di-aqua complex was converted to the dichloro complex (*meso*-3-PtCl<sub>2</sub>) by addition of KCl (Scheme 3, method I). The purity of the platinum compounds was confirmed by elemental analysis (Table 1) and HPLC (Table 4). The infrared and <sup>1</sup>H-NMR data (Table 2) are in good agreement with former results. In the phenolic ligands (*meso*-3 and DL-3) the hydroxy and the amine protons appear as one signal (Table 2). After complexation the peaks for OH and NH<sub>2</sub> are separated.

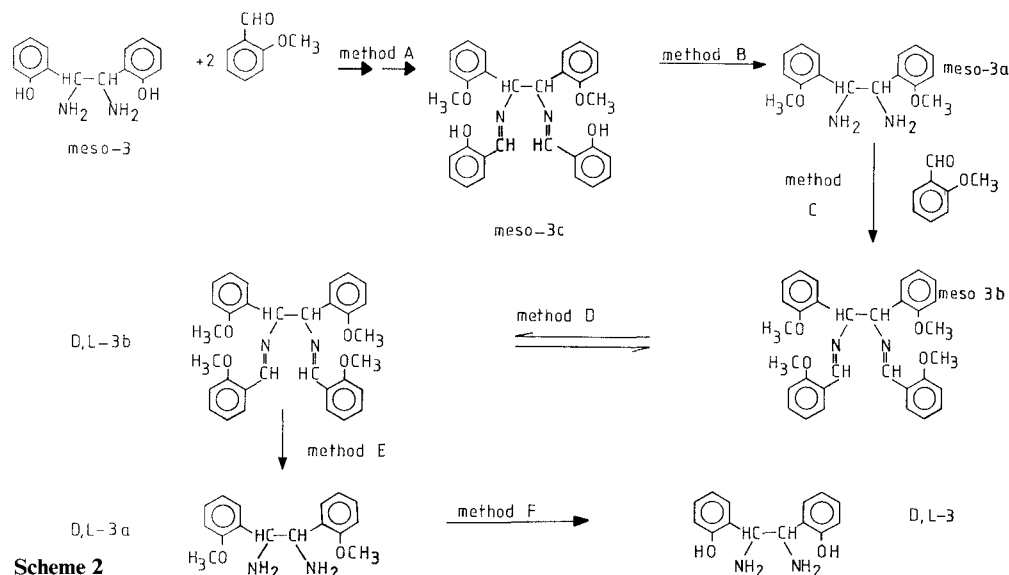
*meso*-*N,N'*-Disalicylidene-1,2-bis(2-methoxyphenyl)ethylenediamine (*meso*-3c) **method A.** *meso*-1,2-Bis(2-hydroxyphenyl)ethylenediamine (*meso*-3, 47.52 g = 0.195 mol) and 2-methoxybenzaldehyde (52.97 g = 0.389 mol) are refluxed in 300 ml MeCN for 3 h. The solution is concentrated to 150 ml. After standing in the refrigerator for 1 h the precipitate is collected in a Buchner funnel, washed with small amounts of ice-cold MeCN and dried over P<sub>2</sub>O<sub>5</sub>. Yield: 92%; yellow powder, mp: 211°–212° C.

*meso*-1,2-Bis(2-methoxyphenyl)ethylenediamine (*meso*-3a) **method B.** *Meso*-3c (77.52 g = 0.161 mol) is hydrolyzed with 700 ml 3M H<sub>2</sub>SO<sub>4</sub> and the salicylic aldehyde formed is removed by steam distillation. The hot solution is filtered and made alkaline with 20% NaOH under vigorous stirring and cooling by ice. The diamine is extracted with CH<sub>2</sub>Cl<sub>2</sub> and the organic phase is dried over MgSO<sub>4</sub>. The solvent is removed under reduced pressure and the residue is dried over P<sub>2</sub>O<sub>5</sub>. Yield: 89%; colorless solid, mp: 75°–76° C.

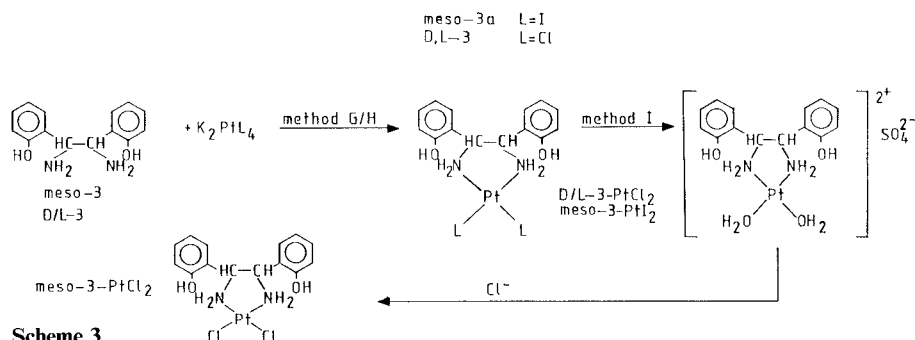
*meso*-*N,N'*-Bis(2-methoxybenzylidene)-1,2-bis(2-methoxyphenyl)ethylenediamine (*meso*-3b) **method C.** *Meso*-3a (37.36 g = 0.137 mol) is treated as described in method A. Yield: 81%; colorless powder, mp: 215°–217° C.

DL-*N,N'*-Bis(2-methoxybenzylidene)-1,2-bis(2-methoxyphenyl)ethylenediamine (DL-3b) **method D.** *Meso*-3b (56.38 g = 0.111 mol) is melted under vigorous stirring. After 10 min the liquid is allowed to cool. The glassy mass is stirred with 250 ml CH<sub>2</sub>Cl<sub>2</sub> for 16 h. The precipitate (= *meso*-3b) is collected in a Buchner funnel and the solvent is removed from the filtrate under vacuum. The residue (DL-3b) is dried over P<sub>2</sub>O<sub>5</sub>. Yield: 51% *meso*-3b, 44% DL-3b; yellow powder, mp: 165–168° C.

DL-1,2-Bis(2-methoxyphenyl)ethylenediamine (DL-3a) **method E.** DL-3b is hydrolyzed in the same manner as described in method B. Yield: 90%; brown oil.



Scheme 2



Scheme 3

**DL-1,2-Bis(2-hydroxyphenyl)ethylenediamine (DL-3) method F.** A solution of DL-3a (3.00 g = 11.02 mmol) in 150 ml  $\text{CH}_2\text{Cl}_2$  abs. is cooled under stirring to  $-60^\circ\text{C}$ .  $\text{BBr}_3$  (11.04 g = 44.07 mmol) is added slowly with a syringe. The reaction mixture is stirred for 30 min at  $-60^\circ\text{C}$  and for 3 further days at room temperature, then 50 ml  $\text{CH}_3\text{OH}$  are added slowly under cooling and the solvent is removed under reduced pressure at  $30^\circ\text{C}$ . This procedure is repeated twice. The residue is dissolved in 50 ml  $\text{H}_2\text{O}$ . The solution is filtered through a no. 3 fritted glass filter. The product is precipitated from the filtrate with 20%  $\text{NaOH}$  at pH 9–11 under cooling. The reaction mixture is extracted with ethyl acetate. The organic layer is dried over  $\text{MgSO}_4$ . After filtration the organic layer is removed under reduced pressure at  $30^\circ\text{C}$  and the residue is dried over  $\text{P}_2\text{O}_5$ . Yield: 75%; orange glassy mass.

**[DL-1,2-Bis(2-hydroxyphenyl)ethylenediamine] dichloroplatinum(II) (DL-3-PtCl<sub>2</sub>) method G.** An aqueous solution of  $\text{K}_2\text{PtCl}_4$  (322 mg = 0.78 mmol in 10 ml) is added slowly to a solution of DL-3 (190 mg = 0.78 mmol in 10 ml  $\text{H}_2\text{O}$  and 2 ml 2M  $\text{HCl}$ ). The mixture is stirred in the dark at room temperature. The pH is kept at 2.5–3.5 for 2 days and at 5.5–6.5 for 3 further days. The complex is collected in a no. 3 fritted glass filter and washed three times with 1 M  $\text{HCl}$ , and three times with water and dried under vacuum over silica gel/ $\text{CaCl}_2$  at  $80^\circ\text{C}$ . Yield: 78%; yellow powder.

**[meso-1,2-Bis(2-hydroxyphenyl)ethylenediamine] diiodoplatinum(II) (meso-3-PtI<sub>2</sub>) method H.** An aqueous solution of  $\text{K}_2\text{PtI}_4$  (1.7 g = 4.09 mmol in 40 ml water) and  $\text{KI}$  (13.61 g = 82 mmol) is stirred for 30 min at room temperature. meso-3 (1.00 g = 4.09 mmol) is dissolved in 40 ml water at  $40^\circ\text{C}$  by adding 2 M  $\text{HCl}$  dropwise. The dark  $\text{K}_2\text{PtI}_4$  solution is added slowly to the ligand solution. The reaction mixture is stirred for 24 h at room temperature in the dark. The pH is kept between 5.5 and 6.5. The complex is collected in a no. 3 fritted glass filter and washed three times with 2 M  $\text{HCl}$  and three times with water and dried under vacuum over silica gel/ $\text{CaCl}_2$  at  $80^\circ\text{C}$ . Yield: 67%; yellow powder.

**[meso-1,2-Bis(2-hydroxyphenyl)ethylenediamine] dichloroplatinum(II) (meso-3-PtCl<sub>2</sub>) method I.** meso-3-PtI<sub>2</sub> (504 mg = 0.73 mmol) and  $\text{Ag}_2\text{SO}_4$  (227 mg = 0.79 mmol) are stirred for 5 days at room temperature in the dark. The precipitated  $\text{AgI}$  is filtered off with a no. 4 fritted glass filter.  $\text{KCl}$  (1.09 g = 14.6 mmol) is added to the filtrate. After 1 day of stirring at room temperature the product is collected in a no. 3 fritted glass filter, washed three times with 1 M  $\text{HCl}$  and water and dried over silica gel/ $\text{CaCl}_2$  at  $80^\circ\text{C}$ . Yield: 35%; yellow powder.

**General procedures.** Melting points (uncorrected) were determined on a Büchi 510 melting point apparatus; for  $^1\text{H-NMR}$  spectra of the ligands a Varian EM 360-L 60-MHz spectrometer was used and the  $^1\text{H-NMR}$  spectra of the platinum complexes were received on a Bruker PFR-NMR spectrometer WM 250 at 250 MHz. Elemental analyses were performed by the microlaboratory of the University of Regensburg. For the HPLC (Altax model 420; pump: Altax model 110 A;  $\text{C}_{18}$ -reversed phase column, Merck, Lichrosorb), 1 mg platinum complex dissolved in 1 ml methanol is eluted with the solvent system: 60% methanol (Merck Lichrosolv)/40% bidistilled water, and assayed at 276 nm (Uvikon 720 LC spectrometer).

### Biological methods

**MDA-MB231 human breast cancer cell line.** The MDA-MB231 cell line (Lippman et al. 1977) was kindly provided by Dr. M. E. Lippman, NCI, Bethesda, USA. Cells were grown in a humidified incubator in 5%  $\text{CO}_2$ , at  $37^\circ\text{C}$ . McCoy 5a medium, supplemented with gentamicin (40  $\mu\text{g/ml}$ ), 10% newborn calf serum, and  $\text{NaHCO}_3$  (11 g/5 l) was used as culture medium. The cells were harvested with trypsin/EDTA, diluted with medium containing 5% newborn calf serum, and syringed gently to prevent clumping. Approximately  $2 \times 10^4$  cells in 2 ml were plated in duplicate in 6-well dishes (Costar). Then 2 days later the medium was changed and the Pt complexes were added as freshly prepared 1000-fold concentrated solutions in dimethylformamide, leading to a final solvent concentration of 0.1%. The cells of control wells contained an equal volume of dimethylformamide. After an incubation time of 2 days, which complied with a triple duplication time, the cells were labeled with 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine/well for 2 h. Cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested with PBS/EDTA buffer. After centrifugation, the cell pellet was resuspended in 1 ml PBS and divided into two 0.5-ml aliquots. One part was counted in a ZI Coulter counter, the other was sonicated. After addition of 4 ml 10% trichloroacetic acid, the acid-insoluble fraction was collected on a 0.45- $\mu\text{m}$  filter (Metricel, Gelman) and counted, after addition of 10 ml scintillation liquid (LS 8000 Beckman scintillation counter).

The [ $^3\text{H}$ ]thymidine was obtained from New England Nuclear, Dreieich, FRG, the newborn calf serum from Biochrom, Berlin, the McCoy 5a medium and the trypsin (0.05%)/EDTA (0.02%) solution from Boehringer, Mannheim, FRG, and the scintillation liquid Quickszint 212 from Zinsser, Frankfurt, FRG. The PBS contained  $\text{NaCl}$  (8 g),  $\text{KCl}$  (0.2 g),  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (1 g),  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (0.15 g), and  $\text{KH}_2\text{PO}_4$  (0.2 g) in 1 l  $\text{H}_2\text{O}$ . To prepare PBS/EDTA buffer 0.02% EDTA was added to the PBS.

**NIH:OVCAR-3 ovarian cancer cell line.** The NIH:OVCAR-3 (ATCC no. HTB 161), a human adenocarcinoma of the ovary (Hamilton et al. 1983), was obtained from the American Type Culture Collection in passage 17. Cell-line banking and quality control were performed according to the "seed stock concept" reviewed by Hay (1988). The cells were maintained in RPMI-1640 (Sigma) containing  $\text{NaHCO}_3$  (2 g/l), gentamicin (50 mg/l), 10% BMS (Seromed) and insulin (10  $\mu\text{g/ml}$ ) (Sigma) in 75- $\text{cm}^2$  flasks at  $37^\circ\text{C}$

**Table 1.** [1,2-Bis(2-hydroxyphenyl)ethylenediamine]dihaloplatinum(II) complexes: analytical data

Compound	C (%)		H (%)		(H%)	
	Calc.	Found	Calc.	Found	Calc.	Found
meso-3-PtI <sub>2</sub>	24.3	24.1	2.33	2.42	4.0	3.9
meso-3-PtCl <sub>2</sub>	33.0	32.9	3.16	3.31	5.5	5.4
DL-3-PtCl <sub>2</sub>	33.0	33.0	3.16	3.24	5.5	5.0

**Table 2.**  $^1\text{H-NMR}$  data of [1,2-bis-(2-hydroxyphenyl)ethylenediamine]dihaloplatinum(II) complexes (250 MHz, dimethylformamide- $d_7$ ) and of their ligands (60 MHz,  $\text{CDCl}_3$ ;  $\text{Me}_3\text{Si}_{\text{int}}$ )

Compound	$\delta$ (ppm)				
	Aromatic H	CH benzyl	NH	OH	$\text{OCH}_3$
<i>meso-3</i>	6.75–7.30 (m, 8H)	4.28 (s, 2H)		4.72 (s, br, 6H)	
<i>meso-3a</i>	6.78–7.56 (m, 8H)	4.54 (s, 2H)	1.88 (s, 4H)		3.73 (s, 6H)
<i>DL-3a</i>	6.74–7.60 (m, 8H)	4.52 (s, 2H)	1.85 (s, 4H)		3.84 (s, 6H)
<i>DL-3</i>	6.66–7.51 (m, 8H)	4.26 (s, 2H)		5.56 (s, br, 6H)	
<i>meso-3-PtI<sub>2</sub></i>	6.67–6.73 (m, 4H)	4.80 (br, 2H)	5.13 (br, 2H)	9.49 (s, 2H)	
	7.01–7.08 (m, 2H)		6.07 (br, 2H)		
	8.09–8.12 (m, 2H)				
<i>meso-3-PtCl<sub>2</sub></i>	6.68–6.73 (m, 4H)	4.83 (br, 2H)	5.25 (br, 2H)	9.50 (s, 2H)	
	7.01–7.07 (m, 2H)		6.12 (br, 2H)		
	8.16–8.19 (m, 2H)				
<i>DL-3-PtCl<sub>2</sub></i>	6.57–6.63 (m, 2H)	4.71 (br, 2H)	5.27 (br, 2H)	10.56 (s, 2H)	
	6.86–6.89 (m, 2H)		6.10 (br, 2H)		
	7.01–7.07 (m, 4H)				

**Table 3.**  $^1\text{H-NMR}$  of *N,N'*-dibenzylidene-1,2-diphenylethylenediamines (60 MHz,  $\text{CDCl}_3$ ;  $\text{Me}_3\text{Si}_{\text{int}}$ )

Compound	$\delta$ (ppm)			
	Aromatic H	CH benzyl	N=C–H	$\text{OCH}_3$
<i>meso-3c</i>	6.52–7.58 (m, 16H)	5.45 (s, 2H)	8.20 (s, 2H)	3.61 (s, 6H)
<i>meso-3b</i>	6.66–8.33 (m, 16H)	5.44 (s, 2H)	8.85 (s, 2H)	3.64 (s, 6H)
				3.80 (s, 6H)
<i>DL-3b</i>	6.54–8.29 (m, 16H)	5.54 (s, 2H)	8.84 (s, 2H)	3.53 (s, 6H)
				3.64 (s, 6H)

**Table 4.** HPLC analysis of the platinum complexes 3<sup>a</sup>

Compound	$t_R$ (min)
<i>DL-3-PtCl<sub>2</sub></i>	4.8
<i>meso-3-PtI<sub>2</sub></i>	3.8
<i>meso-3-PtCl<sub>2</sub></i>	3.0

<sup>a</sup> All compounds eluted as single peaks

in a  $\text{H}_2\text{O}$ -saturated atmosphere of 95% air and 5%  $\text{CO}_2$ . The cells were serially passaged weekly following trypsinization using trypsin/EDTA (Boehringer).

For chemosensitivity testing the cells (in passage 30) were plated in 96-well microplates (100  $\mu\text{l}$ /well) at a density of about 19 cells/microscopic field (Leitz Diavert, 320 $\times$ ) and were allowed to attach. After 73.5 h, the medium was removed by suction and replaced with fresh medium (200  $\mu\text{l}$ /well) containing drug (drugs were added as a 1000-fold stock solution in dimethylformamide) or pure solvent. On every plate the rows 5 and 6 ( $n = 16$ ) acted as controls, whereas two vertical rows ( $n = 16$ ) per drug concentration and time point were used. After varying times of incubation the cells were fixed with glutaraldehyde and stored under PBS at 4 $^\circ\text{C}$ . All plates were stained with crystal violet simultaneously. The processing procedure and data analysis were performed as described by Reile et al. (1989). Drug effects were calculated as corrected  $T/C$  values according to:  $T/C_{\text{corr}} = (T - C_0)/(C - C_0)$  where  $T$  is the absorbance of treated cells,  $C$  the absorbance of the controls and  $C_0$  the absorbance at the time ( $t=0$ ) when drug was added. The experimental errors for  $T/C_{\text{corr}}$  range from approximately  $\pm 20\%$  after short times of incubation (small values for  $\Delta T$  and  $\Delta C$  compared to  $C_0$ ) to  $\pm 5\%$  with prolonged incubation.

*L1210 leukemia cell line (a)* (experiments concerning Fig. 7). The cytotoxicity of platinum compounds was determined by cloning cells of the L1210 murine leukemia or of its subline L1210/Pt (25-fold resistant) in soft agar. Treatments were for 1 h at 37 $^\circ\text{C}$  in the presence

of 10% fetal calf serum (Seromed, Biochrom company). Cells were not washed before cloning in soft agar since the medium was diluted 1:400 during this procedure. Cells were then plated in 30-mm petri dishes (Falcon) and incubated for 7 days in 5%  $\text{O}_2$ , 5%  $\text{CO}_2$  and 90%  $\text{N}_2$ . The final concentration of fetal calf serum was 20% in soft agar (Bacto Agar, Difco). Colonies (> 50 cells) were scored by inspection of petri dishes on an inverted Leitz microscope and fractional survival was plotted semilogarithmically. Cisplatin (Platinex, Bristol Myers company) was available as a stock solution in physiological saline and diluted appropriately with RPMI-1640 medium supplemented with 10% fetal calf serum. Compounds *meso-3-PtCl<sub>2</sub>* and *DL-3-PtCl<sub>2</sub>* were provided as crystalline white powders and were made up as stock solutions of 5.1 mg/ml with dimethylsulfoxide. Drug resistance to cisplatin was induced by intermittent exposures of L1210 cells to increments of 0.2  $\mu\text{g}/\text{ml}$  of the drug in tissue culture if the cell doubling time during exposure and in the subsequent passage without drug was no more than 2–3 days. A total of 78 passages over 15 months were necessary to achieve the level of resistance found in the present experiment. During this time concentrations of cisplatin in the medium were increased from 0.1  $\mu\text{g}/\text{ml}$  to 10  $\mu\text{g}/\text{ml}$ .

*L1210 leukemia cell line (b)* (experiments concerning Table 7). Sensitive and resistant L1210 cells have been developed and described by Eastman and Illenye (1984) and by Richon et al. (1987). Suspension cultures are grown in McCoy's 5a (modified) medium supplemented with  $\text{NaHCO}_3$  (2.2 g/l), penicillin (250 U/ml), streptomycin (250 U/ml), fungizone (1  $\mu\text{g}/\text{ml}$ ) and 16% calf serum. For growth inhibition 4 ml cell suspension (approximately  $5 \times 10^4$  cells/ml) was incubated in triplicate with varying concentrations of drug over a 3-day period. The drug was added as a 1000-fold stock solution in dimethylformamide or dimethylsulfoxide. Stock solutions, especially dimethylsulfoxide solutions, were kept no longer than 5–10 min. After 3 days 1-ml aliquots were counted on a Coulter counter.

*P388 leukemia.* The P388 leukemia (Dawe and Potter 1957; Geran et al. 1972) was maintained by routine passage in female DBA/2 mice (Ivanovas, Kisslegg, FRG). For determination of antitumor activity, female CDF<sub>1</sub> mice (18–22 g, Zentralinstitut für Versuchstiere, Hannover, FRG) were inoculated i.p. with  $10^6$  leukemia cells in

0.1 ml PBS buffer (day 0). The animals were assigned randomly to groups of six (ten animals to the solvent control) and the complexes were administered i.p. on days 1, 5, and 9 or 1–9 as a solution in polyethylene glycol 400/1.8% NaCl in H<sub>2</sub>O (1:1). The antitumor activity was evaluated as the median survival time (days) compared to the untreated control.

**Ehrlich ascites tumor.** A hyperdiploid Ehrlich ascites tumor (ET<sub>WT</sub>) was maintained by weekly transplantation of  $2.5 \times 10^7$  tumor cells into female random-bred NMRI mice, as has been described earlier (Seeber et al. 1982). Tumor lines with documented *in vivo* resistance towards daunorubicin, doxorubicin, etoposide, cisplatin, 4-hydroxycyclophosphamide and also towards a combination of doxorubicin and cisplatin were developed by biweekly i.p. injections of increasing doses of the drugs as described (Seeber et al. 1982; Seeber 1982). In order to maintain a >32-fold anthracycline resistance, a >8-fold etoposide resistance and a >4-fold cisplatin resistance the tumor lines were intermittently exposed to the drugs *in vivo* using 5 mg/kg daunorubicin, 12 mg/kg etoposide and 8 mg/kg cisplatin at 2- to 4-week intervals. For comparative analyses of the *in vivo* activities of cisplatin and the platinum complexes groups of ten animals were formed and  $2.5 \times 10^7$  tumor cells were inoculated i.p. The drugs to be tested were injected i.p. 24 h later on days 1, 2, 3 as a solution in polyethylene glycol 400/1.8% NaCl (1:1). All animals were followed for survival for at least 30 days.

## Results and discussion

The diastereoisomeric [1,2-bis(2-hydroxyphenyl)ethylenediamine]-dichloroplatinum(II) complexes DL-3-PtCl<sub>2</sub> and *meso*-3-PtCl<sub>2</sub> were tested on the hormone-independent, human MDA-MB231 breast cancer cell line (Table 5). DL-3-PtCl<sub>2</sub> shows a significantly stronger inhibitory effect on [<sup>3</sup>H]thymidine incorporation and cell growth than its diastereoisomer *meso*-3-PtCl<sub>2</sub>. The antitumor activity of DL-3-PtCl<sub>2</sub> is one-third that of cisplatin.

For testing the activity of DL-3-PtCl<sub>2</sub>, *meso*-3-PtCl<sub>2</sub> and cisplatin against ovarian cancer the NIH:OVCAR-3 cell line was used. Hamilton et al. (1983) have described this cell line to be resistant to clinically relevant concentrations of Adriamycin, melphalan and cisplatin.

In order to provide the most information, the data are presented as growth curves (absorbance plotted versus time of drug exposure) as well as corrected  $T/C$  values versus time of drug exposure. A gradual decrease of  $T/C_{\text{corr}}$  values with time indicates an inhibition of cell growth (i.e. a slowing down or a stopping of cell proliferation = cytostatic drug action). No change or increase of  $T/C_{\text{corr}}$  values with time of drug exposure represents primary drug resistance or recovery of the cells from drug-induced damage, which may result in full reproductive integrity of

the cells (i.e. development of resistance).<sup>2</sup>  $T/C_{\text{corr}}$  values < 0 indicate cytotoxic drug action.

Figure 1 shows the effect of cisplatin on the NIH:OVCAR-3 cell line. At the lowest concentration (0.1 μM) the cells are weakly inhibited initially but fully recover after about 200 h drug exposure. This behavior can be explained by a resistance of the cell line against cisplatin, which is described by Hamilton et al. (1983). At higher (therapeutically non-relevant) concentrations clear-cut dose-dependent inhibition is observed. Compared to cisplatin for both DL-3-PtCl<sub>2</sub> (Fig. 2) and *meso*-3-PtCl<sub>2</sub> (Fig. 3) higher doses are required to achieve equiactive inhibition. Whereas DL-3-PtCl<sub>2</sub> is cytotoxic at 2.5 μM and 5.0 μM, *meso*-3-PtCl<sub>2</sub> exhibits a cytotoxic effect only in its highest concentration (5 μM). In contrast to DL-3-PtCl<sub>2</sub> the onset of action of *meso*-3-PtCl<sub>2</sub> is delayed. Although the equiactive *in vitro* concentrations of the most active diastereoisomer DL-3-PtCl<sub>2</sub> are higher than those of cisplatin, there may be an advantage with this compound in the therapy of ovarian cancer owing to its markedly lower toxicity (i.e. the realization of cytotoxic but non-toxic drug concentrations under *in vivo* conditions).<sup>3</sup> Therefore, DL-3-PtCl<sub>2</sub> could be useful for the first-line treatment of ovarian cancer in combination with cisplatin to avoid development of resistance, as well as for the second-line therapy of the cisplatin-resistant tumor.

In the P388 leukemia experiments *in vivo* DL-3-PtCl<sub>2</sub> proved to be very active (Table 6). In contrast, *meso*-3-PtCl<sub>2</sub> showed only marginal antitumor effects even at the highest tolerated dose. DL-3-PtCl<sub>2</sub> is also much better tolerated than *meso*-3-PtCl<sub>2</sub>. DL-3-PtCl<sub>2</sub> (30 μmol/kg) has a better effect if it is applied in nine doses (days 1–9;  $T/C = 205\%$ ) instead of three doses (days 1, 5, 9;  $T/C = 170\%$ ). The effect of DL-3-PtCl<sub>2</sub> is comparable to that of cisplatin (dose:  $3 \times 5 \mu\text{mol/kg}$ , days 1, 5, 9;  $T/C$ : DL-3-PtCl<sub>2</sub> = 170%; cisplatin = 180%). However, DL-3-PtCl<sub>2</sub> is much less toxic than cisplatin. The dosage  $9 \times 13.2 \mu\text{mol/kg}$  DL-3-PtCl<sub>2</sub> produced the strongest antitumor effect ( $T/C = 300\%$ ). At the end of the experiment (day 30) four out of six animals survived. After a further increase of dose, toxic side-effects became evident, which demanded a reduction of the number of doses from nine to five.

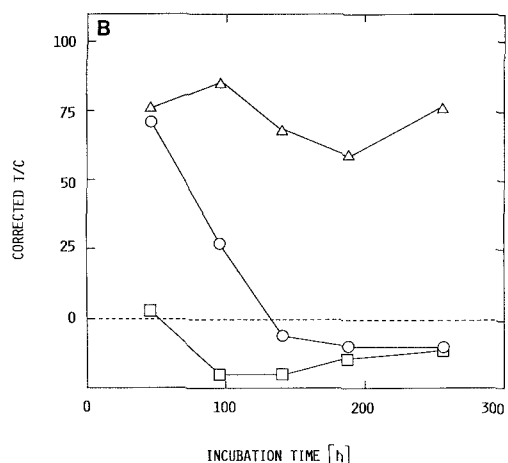
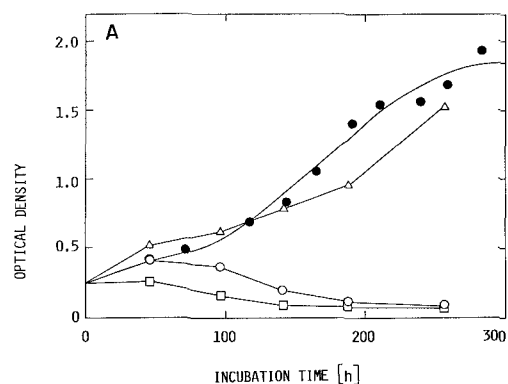
In further experiments the activities of *meso*-3-PtCl<sub>2</sub> and DL-3-PtCl<sub>2</sub> in wild-type (ET<sub>WT</sub>) and cisplatin-resistant (ET<sub>DDP</sub>) Ehrlich ascites tumor were studied. These results are demonstrated in Figs. 4–6. In the case of complex *meso*-3-PtCl<sub>2</sub> (Fig. 4) three daily doses of 5 mg/kg were curative and three of 10 mg/kg were already lethal

**Table 5.** Antitumor effect of *R,S* and *R,R/S,S* [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) on the hormone-independent, human MDA-MB231 breast cancer cell line

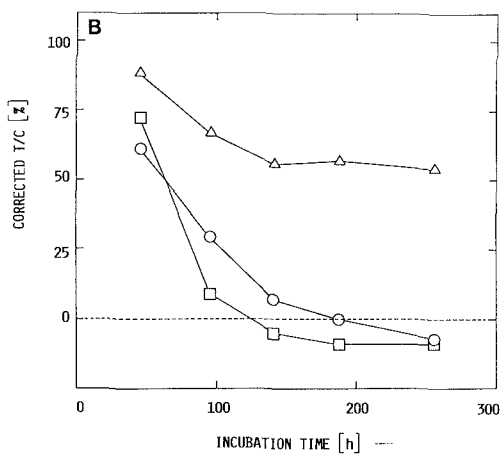
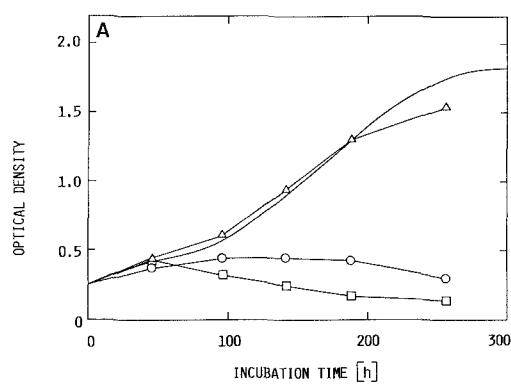
Compound	[ <sup>3</sup> H] Thymidine inhibition at 5 μM (%)	Incorporation ED <sub>50</sub> (μM)	Inhibition of cell growth at 5 μM (%)	ED <sub>50</sub> (μM)
Cisplatin	97.6	0.16	83.9	0.56
DL-3-PtCl <sub>2</sub>	95.3	0.60	85.7	1.5
<i>meso</i> -3-PtCl <sub>2</sub>	59.9	3.7	43.7	6.6

<sup>2</sup> The development of resistance can also be feigned by the decomposition of the drug under the cell culture conditions used. This can happen particularly with highly reactive compounds like cisplatin, which exchanges its chlorine substituents for anionic components of the culture medium

<sup>3</sup> The evaluation of DL-3-PtCl<sub>2</sub> on the P388 leukemia of the mouse proves that single doses of 40 μmol/kg are tolerated by the animals without complications (See also Table 6; animal weight change days 1–5). Therefore it is supposed that under *in vivo* conditions in ovarian cancer cytotoxic DL-3-PtCl<sub>2</sub> levels ( $\geq 2.5 \mu\text{mol/kg}$ ) are achievable. This is especially true for intraperitoneal and intrapleural administration. *meso*-3-PtCl<sub>2</sub> is a less promising drug because of its lower antitumor activity and its higher toxicity (compare Table 6)



**Fig. 1 A, B.** Effect of cisplatin on the proliferation of NIH:OVCAR-3 ovarian cancer cells. **A** Growth curves. **B** Plot of corrected  $T/C$  values versus time of drug exposure, where  $T$  is absorbance of treated cells and  $C$  is that of the controls.  $\Delta$ ,  $0.1 \mu M$ ;  $\circ$ ,  $1 \mu M$ ;  $\square$ ,  $5 \mu M$ ;  $\bullet$ , absorbance of the dimethylformamide control with the curve fitted (Reile et al. 1989)



**Fig. 2 A, B.** Effect of DL-3-PtCl<sub>2</sub> on NIH:OVCAR-3 cells. **A** Growth curves. **B** Corrected  $T/C$  values as a function of time of incubation.  $\Delta$ ,  $1.0 \mu M$ ;  $\circ$ ,  $2.5 \mu M$ ;  $\square$ ,  $5.0 \mu M$ ; —, computed control

**Table 6.** Antitumor effect of  $R,S$  and  $R,R/S,S$  [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) on the P388 leukemia of the CD<sub>2</sub>F<sub>1</sub> mouse

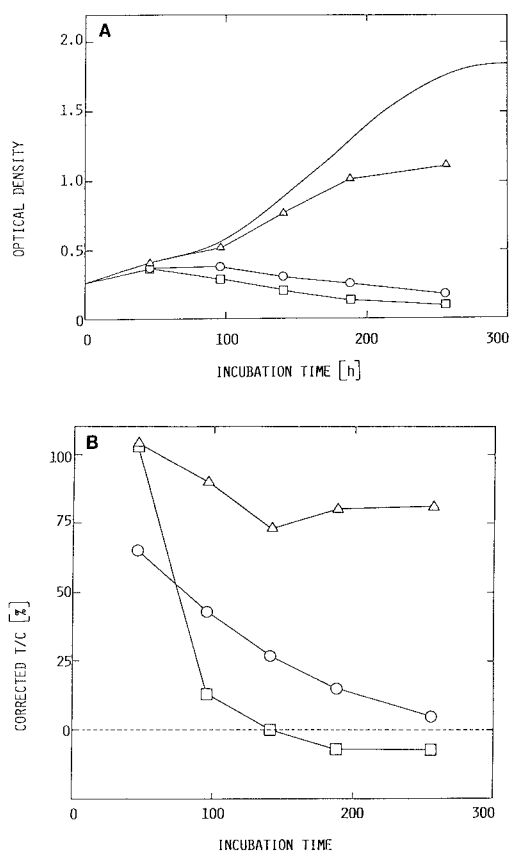
Compound	Day of injection	Animal weight				$T/C$ (%)	
		Single dose <sup>a</sup>		Change days 1-5	Median survival time (range) (days)		
		( $\mu\text{mol/kg}$ )	( $\text{mg/kg}$ )				
Control	1, 5, 9			-1.8	10 (10-12)	100	
	1-9			-1.4	10 (9-11)	100	
Cisplatin	1, 5, 9	5	1.5	1.4	18 (16-21)	180	
	DL-3-PtCl <sub>2</sub>	1, 5, 9	40	20.4	-0.4	22.5 (17-25)	225
		1, 5, 9	20	10.2	-0.4	17 (16-18)	170
	1, 5, 9	10	5.1	-1.0	17 (17-19)	170	
	1, 5, 9	5	2.55	-0.7	17 (17-25)	170	
	1-9	3.3	1.68	-2.1	20.5 (17-25)	205	
1-9	1.7	0.87	1.2	16 (16-18)	160		
Control	1-9			-0.5	10 (9-11)	100	
Cisplatin	1-6	3.3	1.0	3.4	20 (9-30) <sup>b</sup>	200	
	DL-3-PtCl <sub>2</sub>	1-9	6.6	3.4	0.0	21 (20-25)	210
		1-9	13.2	6.8	2.5	30 (24-30) <sup>c</sup>	300
	1-5	26.4	12.6	4.4	28 (8-30) <sup>d</sup>	280	
<i>meso</i> -3-PtCl <sub>2</sub>	1, 5, 9	40	20.4		Toxic		
	1, 5, 9	20	10.2	4.0	13.5 (6-16)	135	
	1, 5, 9	10	5.1	-0.3	11 (10-13)	111	
Control	1-9	6.6	3.37	0.4	14 (10-14)	140	
	1-9	3.3	1.68	1.5	12.5 (12-19)	125	

<sup>a</sup> The compounds were administered intraperitoneally as polyethylene glycol 400/1.8% NaCl solution

<sup>b</sup> Three of ten animals survived the end of the test (day 30)

<sup>c</sup> Four of six animals survived the end of the test (day 30)

<sup>d</sup> Two of six animals survived the end of the test (day 30)

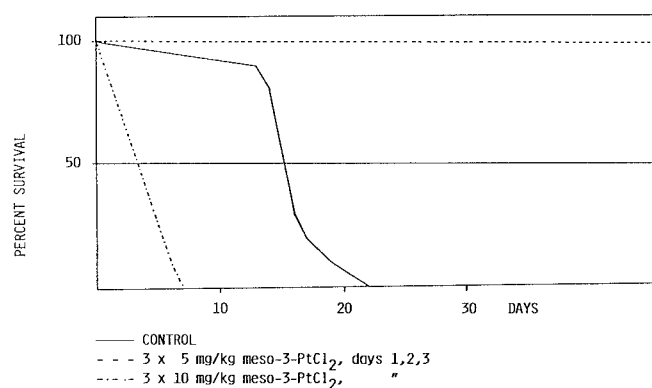


**Fig. 3A, B.** Effect of *meso*-3-PtCl<sub>2</sub> on NIH:OVCAR-3 cells. **A** Growth curves. **B** Corrected T/C values as a function of time of incubation.  $\Delta$  1  $\mu$ M;  $\circ$  2.5  $\mu$ M;  $\square$  5  $\mu$ M; — computed control

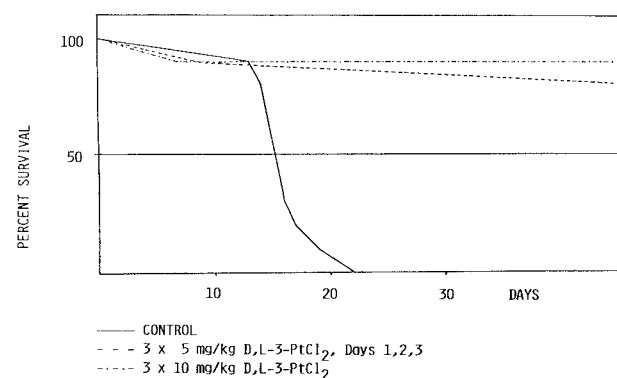
in the wild-type tumor. For complex DL-3-PtCl<sub>2</sub> the therapeutic ratio was improved in the wild-type tumor: as shown in Fig. 5 a dose range of 3  $\times$  5 mg/kg to 3  $\times$  10 mg/kg was curative without any significant toxicity (compare also Table 6). The most important result of this study is demonstrated in Fig. 6. When both complexes *meso*-3-PtCl<sub>2</sub> and DL-3-PtCl<sub>2</sub> were tested in the cisplatin-resistant subline in vivo, 70% of the tumor-bearing animals could be cured by complex DL-3-PtCl<sub>2</sub> as compared to only 20% of the animals when treated with complex *meso*-3-PtCl<sub>2</sub>. This result was substantiated by a repeat experiment and is based on the documentation of in vivo activity in 20 animals altogether.

The lack of complete cross-resistance of DL-3-PtCl<sub>2</sub> with cisplatin, found in the comparative testing on a cisplatin-sensitive and -resistant Ehrlich ascites tumor of the mouse, was also observed on the L1210 leukemia cell line. In these experiments, DL-3-PtCl<sub>2</sub> proved to be markedly more active than cisplatin on the cisplatin-sensitive L1210 cell line as well as on the cisplatin-resistant (25-fold) L1210 subline (Fig. 7). *meso*-3-PtCl<sub>2</sub> was comparably active with cisplatin on the sensitive L1210 cell line and inactive on the resistant L1210 subline (Fig. 7).

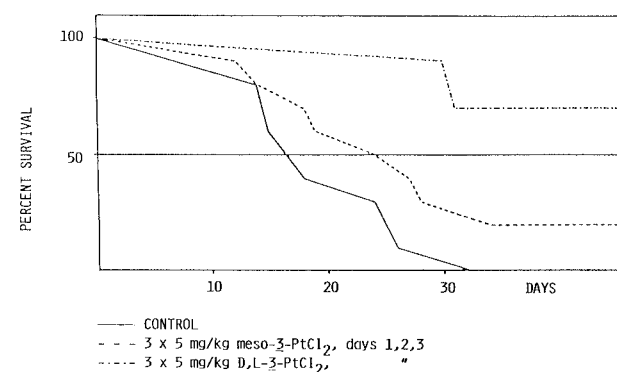
DL-3-PtCl<sub>2</sub> was also tested against a series of resistant L1210 cell lines developed by Eastman et al. (1987). Only little cross-resistance was found against the L1210/DDP<sub>5</sub> and L1210/DDP<sub>10</sub> cells (DDP = cisplatin), which are highly resistant against cisplatin (Table 7). It is of further



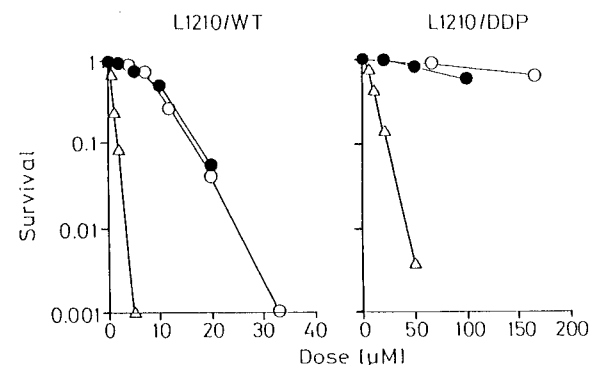
**Fig. 4.** Effect of the platinum complex *meso*-3-PtCl<sub>2</sub> on the cisplatin-sensitive Ehrlich ascites tumor of the mouse



**Fig. 5.** Effect of the platinum complex DL-3-PtCl<sub>2</sub> on the cisplatin-sensitive Ehrlich ascites tumor of the mouse



**Fig. 6.** Effect of the platinum complexes *meso*-3-PtCl<sub>2</sub> and DL-3-PtCl<sub>2</sub> on the cisplatin-resistant Ehrlich ascites tumor of the mouse



**Fig. 7.** Cytotoxic activity of cisplatin (DDP/O), *meso*-3-PtCl<sub>2</sub> (●) and DL-3-PtCl<sub>2</sub> ( $\Delta$ ) on the cisplatin-sensitive (L1210/WT) and on the cisplatin-resistant (L1210/DDP) L1210 leukemia cell line

**Table 7.** Growth-inhibiting effect of DL-[1,2-bis(2-hydroxyphenyl)ethylenediamine]-dichloroplatinum(II) on various L1210 leukemia cell lines (numbers in paranthesis are-fold resistance)

Compound	ED <sub>50</sub> (μM)			
	L1210/0	L1210/DDP <sub>5</sub>	L1210/DDP <sub>10</sub>	L1210/DACH
Cisplatin	0.3	17 (55)	32 (107)	13 (4)
DL-3-PtCl <sub>2</sub>	3.7	13 (3)	24 (6)	17 (4.6)

interest that the L1210/DACH cells, which are 40-fold resistant to (1,2-diaminocyclohexane)dichloroplatinum(II) (DACH), also showed only marginal resistance to DL-3-PtCl<sub>2</sub>.

Owing to its low toxicity<sup>3</sup> and its antitumor effect on various models described here, DL-3-PtCl<sub>2</sub> is of great interest for further development. The effect on the NIH:OVCAR-3 ovarian cancer cell line makes this type of compound a promising candidate for use in the therapy of ovarian cancer. In further publications we will report on attempts to optimize DL-3-PtCl<sub>2</sub>.

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