

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

## II. \*\* Synthesis and preliminary testing of the enantiomeric complexes

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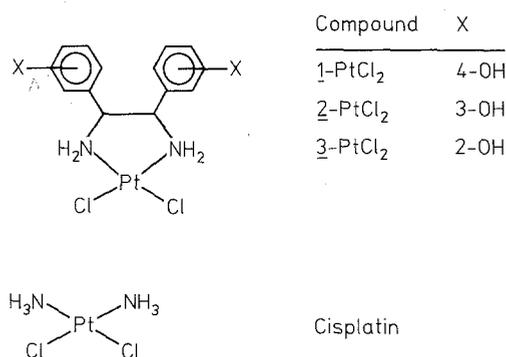
Received 15 July 1991/Accepted 1 November 1991

**Summary.** The enantiomeric [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes were synthesized and their configuration assessed. A preliminary test in the cisplatin-resistant human NIH:OVCAR-3 ovarian cancer cell line, which was previously characterized by its sensitivity against several therapeutically used drugs, showed that both enantiomers produce cytotoxic effects in a concentration of 2.5  $\mu$ M. A difference between the enantiomers became evident from the faster onset of cytotoxic activity of the *S,S*-configured compound.

**Key words:** (*R,R*)- and (*S,S*)-[1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) – Synthesis – NIH:OVCAR-3 ovarian cancer cell line – preliminary testing

### Introduction

Among the 1,2-diphenylethylenediamineplatinum(II) complexes, [( $\pm$ )-1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) (Scheme 1: ( $\pm$ )-3-PtCl<sub>2</sub>) has proved to be a potent cytostatic (Müller et al. 1990). It is noteworthy that ( $\pm$ )-3-PtCl<sub>2</sub> is also active in several cisplatin-resistant tumor models in cell culture as well as in animal experiments. Of special interest are the results of in vitro experiments on the human NIH:OVCAR-3 ovarian cancer cell line, which is described to be resistant to clinically relevant concentrations of Adriamycin, melphalan and cisplatin. In this trial ( $\pm$ )-3-PtCl<sub>2</sub> exhibited a cytotoxic effect at concentrations (2.5 and 5.0  $\mu$ M) that were tolerated without complications in animal experi-



Scheme 1

**Scheme 1.** Structure of 1,2-diphenylethylenediamineplatinum(II) complexes compared to cisplatin

ments. Because of these results we assume that ( $\pm$ )-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 this publication we report attempts to optimize the effect of ( $\pm$ )-3-PtCl<sub>2</sub> on ovarian carcinoma by resolution of the drug into its enantiomers.

### Materials and methods

#### Chemical methods

(-)- and (+)-1,2-bis(2-methoxyphenyl)ethylenediamine [( $\pm$ )-3a and (+)-3a]: *method A.* Compound ( $\pm$ )-3a (10.2 g = 37.5 mmol), dissolved in 40 ml 82% EtOH, was given to a solution of (*R,R*)-(+)-tartaric acid (16.8 g = 112 mmol) in 105 ml 82% EtOH and boiled under reflux for 10 min. Upon slow cooling ( $\pm$ )-3a-tartrate crystallized at room temperature. It was recrystallized from 82% EtOH several times, treated with 5% NaOH and extracted with Et<sub>2</sub>O. The organic layer was washed with H<sub>2</sub>O, dried over MgSO<sub>4</sub>, and evaporated, to leave a colourless powder: m.p. 77–78° C; yield 15%; [ $\alpha$ ]<sub>D</sub><sup>26</sup> = -124°, *c* = 1.0, MeOH; optical purity  $\approx$  100%. From the

\* Dedicated to Professor J. Knabe on the occasion of his 70th birthday

\*\* Part I: Müller et al. (1990)

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filtrates ( $\pm$ )-3a and (+)-3a were isolated and purified in the same manner by crystallization as the (*S,S*)-(-)-tartrate: colourless powder; m.p. 70–72° C, yield 31%;  $[\alpha]_{546}^{26} = +118^\circ$ ;  $c=1.0$ , MeOH; optical purity  $\approx 100\%$ . The enantiomeric purity was determined by  $^1\text{H-NMR}$ -spectroscopy (250 MHz) by means of the formation of diastereoisomeric complexes with (*S*)-(+)-1-(9-anthryl)-2,2,2-trifluoroethanol. The molar ratio of this alcohol to (-)-3a and to (+)-3a, respectively, was 6.3; the solvent was 0.5 ml  $\text{CDCl}_3$  and two drops  $\text{D}_2\text{O}$ . The benzylic proton signals of (-)-3a are shifted to a higher field ( $\delta$ ) = 4.12 ppm than those of (+)-3a. ( $\delta$  = 4.22 ppm) (Fig. 1). The enantiomeric purity of (-)-3a and (+)-3a was established by the integral ratio of the two singlets for the benzylic protons.

(-)-1,2-bis(2-hydroxyphenyl)ethylenediamine [(*-*)-3]: method B. A solution of (-)-3a (793 mg = 2.91 mmol) in 70 ml dry  $\text{CH}_2\text{Cl}_2$  was cooled to -60° C.  $\text{BBr}_3$  (2.91 g = 11.63 mmol), dissolved in 20 ml dry  $\text{CH}_2\text{Cl}_2$ , was added in a  $\text{N}_2$  atmosphere. The reaction mixture was stirred for 3 days under slow warming to room temperature. Then 250 ml 10% NaOH was added and the polymeric side-products were removed by separation of the  $\text{CH}_2\text{Cl}_2$  layer. The aqueous phase was neutralized with HCl (pH 8) and (-)-3 was extracted with ethyl acetate. The organic layer was washed several times with water and dried over  $\text{MgSO}_4$ . The solvent was evaporated under reduced pressure to leave a beige powder: m.p. 156–158° C; yield 61%;  $[\alpha]_{546}^{26} = -122^\circ$ ,  $c=1.0$ , MeOH.

(+)-1,2-bis(2-hydroxyphenyl)ethylenediamine [(+)-3] was obtained as a beige powder: m.p. 151–153° C; yield 49%;  $[\alpha]_{546}^{26} = +125^\circ$ ,  $c=1.0$ , MeOH.

[(*-*)- and (+)-1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) [(*-*)-3-PtCl<sub>2</sub> and (+)-3-PtCl<sub>2</sub>]: method C. Compounds (-)-3-PtCl<sub>2</sub> and (+)-3-PtCl<sub>2</sub> were prepared according to the procedure of Müller et al. (1990) for ( $\pm$ )-3-PtCl<sub>2</sub>. (-)-3-PtCl<sub>2</sub> was obtained as a white powder: yield 77%;  $[\alpha]_{546}^{26} = -174^\circ$ ,  $c=1.0$ , dimethylformamide. (+)-3-PtCl<sub>2</sub> was a white powder: yield 69%;  $[\alpha]_{546}^{26} = +177^\circ$ ,  $c=1.0$ , dimethylformamide.

[( $\pm$ )-1,2-bis(2-hydroxyphenyl)ethylenediamine]diiodoplatinum(II) [( $\pm$ )-3-PtI<sub>2</sub>]. An aqueous solution of  $\text{K}_2\text{PtCl}_4$  (208 mg, 0.5 mmol) and KI (0.83 g, 5 mmol) was stirred for 30 min in the dark at room temperature. ( $\pm$ )-3 (122 mg, 0.5 mmol) was dissolved in 50 ml water by adding 2 M HCl dropwise. The reaction mixture was stirred for 24 h in the dark. The pH was kept between 5.5 and 6.5. The complex was collected by suction filtration, washed with water and dried in vacuo over  $\text{P}_2\text{O}_5$  to leave a yellow powder: yield 72%.

[N,N'-tetradeutero ( $\pm$ )-1,2-bis(2-hydroxyphenyl)ethylenediamine]-sulfatoplatinum(II) [( $\pm$ )-3-PtSO<sub>4</sub>]. ( $\pm$ )-3-PtI<sub>2</sub> (242 mg, 0.35 mmol) and  $\text{Ag}_2\text{SO}_4$  (93 mg, 0.3 mmol) were stirred for 24 h in  $\text{D}_2\text{O}$  at 40° C with protection from light. The precipitated AgI was filtered off and the clear yellow solution was lyophilized to give a colourless powder: yield 83%.

### 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 evaluation of the specific rotation we used a Perkin-Elmer 241 MC polarimeter. The circular dichroism (CD) spectra were obtained with a JASCO J-500 A spectropolarimeter (time constant = 4 s; scan speed = 5 nm/min) and recorded in dimethylformamide at room temperature in 5.0-cm quartz cells. The concentrations were 0.1 mM for (-)-3-PtCl<sub>2</sub> and (+)-3-PtCl<sub>2</sub>. The structure of (-)-3-

PtCl<sub>2</sub> was determined using an Enraf-Nonius CAD4 diffractometer and program package SDP V 3.1.

### Biological methods

*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 medium (Sigma) containing  $\text{NaHCO}_3$  (2 g/l), gentamicin (50 mg/l), 10% Basal Medium Supplement (Seromed) and insulin (10  $\mu\text{g/ml}$ ) (Sigma) in 75-cm<sup>2</sup> flasks at 37° C 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 were plated in 96-well microplates (100  $\mu\text{l/well}$  at a density of about 17–28 cells/microscopic field (Leitz Diavert, 320  $\times$ ) and were allowed to attach. After 48–80 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) or pure solvent. Clinically established chemotherapeutics were dissolved in EtOH, platinum complexes in dimethylformamide. On every plate the rows 5 and 6 ( $n=16$ ) served as controls, whereas two vertical rows ( $n=16$ ) per drug concentration and time point were used. After various times of incubation the cells were fixed with glutardialdehyde and stored under phosphate-buffered saline at 4° C.

All plates were stained with crystal violet simultaneously. The processing procedure and data analysis were performed as described by Reile et al. (1990). In order to save space, the growth curves of the drug-treated cells are not shown. Drug effects were calculated as corrected T/C values according to:  $T/C_{\text{corr}} (\%) = (T - C_0)/(C - C_0) \times 100$  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 (Bernhardt et al. 1992). According to this equation, any growth curve of a drug-treated cell population can be reconstructed from the  $T/C_{\text{corr}}$  profiles (Fig. 10) and the growth curves of the corresponding controls shown in Fig. 9. The experimental errors for  $T/C_{\text{corr}}$  range from approximately  $\pm 20\%$  after short times of incubation (small values for  $T$  and  $C$ ) to  $\pm 5\%$  with prolonged incubation.

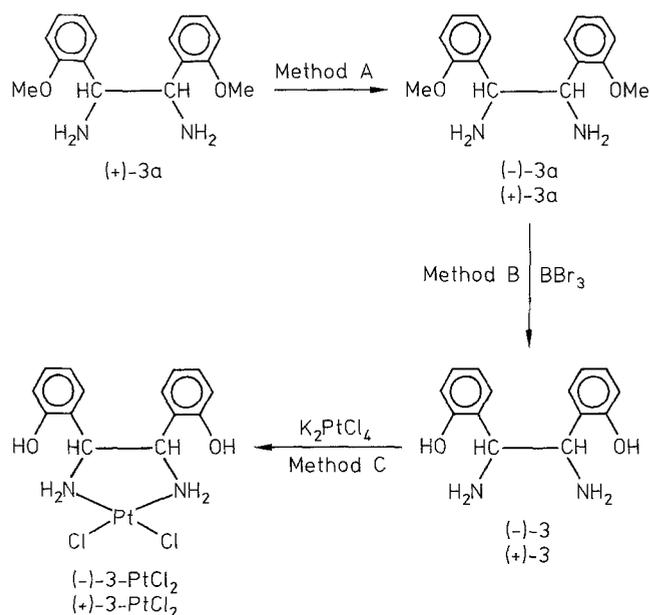
*Cytogenetic analysis.* The cells were grown to about 50% confluence on microscopic slides. The slides were prepared as described elsewhere (Rooney and Czepulkowski 1986). So that spindle formation could be inhibited, the slide chambers were inoculated with colcemid solution (Serva) to a final concentration of 0.04  $\mu\text{g/ml}$  and incubated for 3 h at 37° C. The medium was removed by suction and replaced with 0.075 M KCl. After 30 min of incubation at 37° C an equal volume of cold, freshly made fixative (absolute methanol/glacial acetic acid, 3/1) was added. This hypotonic/fixative mixture was removed immediately and replaced twice with ice-cold, fresh fixative. The slides were removed from the dish and air-dried at 60° C. The chromosomes were stained for 8 min with 10 ml Giemsa plus 90 ml 0.025 M  $\text{KH}_2\text{PO}_4$ , pH 6.8.

*Doubling-time analysis.* Curve fitting of experimental data of the growth curves was accomplished by a polynomial regression fit applying the least-squares method (Reile et al. 1990).

## Results

### Chemistry

The diastereoisomeric [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes ( $\pm$ )-3-PtCl<sub>2</sub>



Scheme 2

**Scheme 2.** Synthesis of enantiomeric 3-PtCl<sub>2</sub> complexes

and *meso*-3-PtCl<sub>2</sub> have been described in our preceding publication (Müller et al. 1990). For the synthesis of the (+) and (-) rotatory [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes [(+)-3-PtCl<sub>2</sub> and (-)-3-PtCl<sub>2</sub>] the resolution into the optically active forms was performed at the stage of (±)-1,2-bis(2-methoxyphenyl)ethylenediamine [(±)-3a; for the synthesis of (±)-3a compare Müller et al. (1990)] (Scheme 2).

The separation into the enantiomers was achieved by fractional crystallization of the diastereoisomeric salts, formed by reaction of (±)3a with optically active tartaric acid in EtOH (method A). This procedure has been successfully applied several times to 1,2-diphenylethylenediamines (von Angerer et al. 1982; Wappes et al. 1984; Jen-

**Table 2.** [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes – analytical data

Compound	C (%)		H (%)		N (%)	
	Calc.	Found	Calc.	Found	Calc.	Found
(-)-3-PtCl <sub>2</sub>	33.0	33.3	3.16	3.28	5.5	5.3
(+)-3-PtCl <sub>2</sub>		33.1		3.19		5.4

nerwein et al. 1989; vom Orde et al. 1990). The complete enantiomeric purity of the diamines (+)-3a and (-)-3a could be proved by means of the formation of diastereoisomeric complexes with (*S*)-(+)-1-(9-anthryl)-2,2,2-trifluoroethanol. They show different  $\delta$  values for the benzylic protons of (+)-3a (4.22 ppm) and (-)-3a (4.12 ppm) in the 250-MHz <sup>1</sup>H-NMR spectra (Fig. 1).

Ether cleavage of the methoxy compounds (+)-3a and (-)-3a with BBr<sub>3</sub> leads to the hydroxy derivatives (+)-3 and (-)-3 (method B). The dichloroplatinum(II) complexes (+)-3-PtCl<sub>2</sub> and (-)-3-PtCl<sub>2</sub> were formed by reaction with K<sub>2</sub>PtCl<sub>4</sub> (method C).

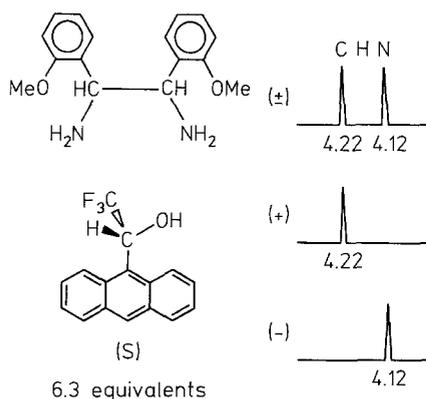
The identity and purity of these compounds were confirmed by <sup>1</sup>H-NMR spectroscopy (Table 1), IR spectroscopy and elemental analyses (Table 2).

The absolute configuration of the enantiomeric [1,2-bis(2-hydroxyphenyl)ethylenediamine] dichloroplatinum(II) complexes (+)-3-PtCl<sub>2</sub> and (-)-3-PtCl<sub>2</sub> was determined by comparison of their CD spectra with those of (+)-(*R,R*)- and (-)-(*S,S*)-dichloro[1,2-diphenylethylenediamine]platinum(II), the structural assignment of which derives from the known absolute configuration of (+)-1,2-diphenylethylenediamine (*R,R*) (Meric and Vigneron 1974). In analogy to the 3- and 4-hydroxy-substituted (±)-dichloro[1,2-diphenylethylenediamine] platinum(II) complexes 2-PtCl<sub>2</sub> (Jennerwein et al. 1989) and *I*-PtCl<sub>2</sub> (Wappes et al. 1984), the dextrorotatory enantiomer of the (±)-[1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) has the *R,R* [compound

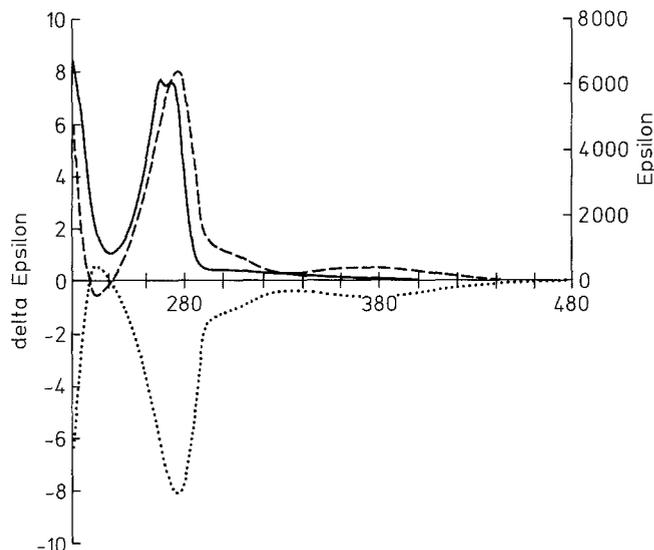
**Table 1.** <sup>1</sup>H-NMR data of [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes and of their ligands [ $\delta$  (ppm), tetramethylsilane (internal)]

Compound	Aromatic H	CH (benzylic)	NH	OH	OCH <sub>3</sub>
(-)-3a <sup>a</sup>	6.75–6.86 (m, 4 H) 7.09–7.16 (m, 2 H) 7.22–7.26 (m, 2 H)	4.46 (s, 2 H)	1.70 (s, 4 H)		3.79 (s, 6 H)
(+)-3a <sup>a</sup>	6.75–6.86 (m, 4 H) 7.09–7.16 (m, 2 H) 7.22–7.26 (m, 2 H)	4.46 (s, 2 H)	1.71 (s, 4 H)		3.79 (s, 6 H)
(-)-3 <sup>b</sup>	6.59–7.47 (m, 8 H)	4.24 (s, 2 H)		5.85 (s, br, 6 H)	
(+)-3 <sup>b</sup>	6.62–7.49 (m, 8 H)	4.23 (s, 2 H)		5.86 (s, br, 6 H)	
(-)-3-PtCl <sub>2</sub> <sup>c</sup>	6.57–6.63 (m, 2 H) 6.85–6.89 (m, 2 H) 7.01–7.08 (m, 4 H)	4.70 (br, 2 H)	5.27 (br, 2 H) 6.09 (br, 2 H)	10.53 (s, 2 H)	
(+)-3-PtCl <sub>2</sub> <sup>c</sup>	6.57–6.63 (m, 2 H) 6.83–6.89 (m, 2 H) 7.01–7.08 (m, 4 H)	4.70 (br, 2 H)	5.28 (br, 2 H) 6.09 (br, 2 H)	10.53 (s, 2 H)	

<sup>a</sup> 250 MHz, CDCl<sub>3</sub><sup>b</sup> 60 MHz, CDCl<sub>3</sub><sup>c</sup> 250 MHz, [D<sub>7</sub>]dimethylformamide



**Fig. 1.**  $^1\text{H-NMR}$  benzyl signals of ligands in  $\text{CDCl}_3$  at 250 MHz in the presence of 6.3 equivalents of an optically active auxiliary and of a small amount of  $\text{D}_2\text{O}$ . *Top:*  $(\pm)3a$  shows singlets for both enantiomers. *Centre:*  $(+)3a$  shows one singlet only, i.e. an enantiomeric purity of approx. 100%. *Bottom:*  $(-)3a$  shows another singlet, exclusively (i.e. purity approx. 100%)

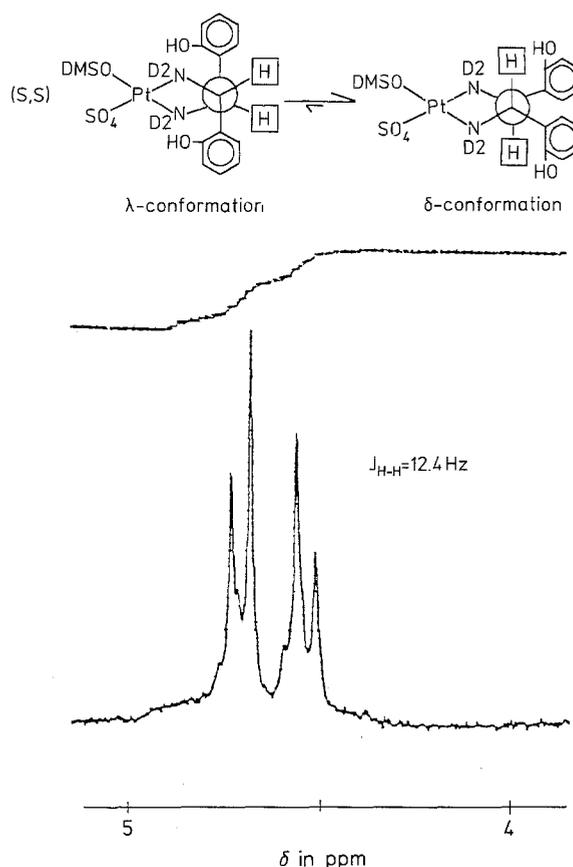


**Fig. 2.** CD spectra of enantiomeric [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes: (...)  $(-)\text{-PtCl}_2$ , (---)  $(+)\text{-PtCl}_2$ ; (—) UV spectrum

$(+)\text{-3-PtCl}_2$ ] and the levorotatory enantiomer the  $S,S$  configuration [compound  $(-)\text{-3-PtCl}_2$ ] (Fig. 2).

The CD spectrum of  $(-)\text{-3-PtCl}_2$  as well as the spectrum of  $(-)\text{-I-PtCl}_2$  (Wappes et al. 1984) showed a negative band at 380 nm with a value of about 0.16, which was assigned to the  $d-d^*$  transition bands (Ito et al. 1967). A comparison of these bands with that of  $(-)\text{-}(S,S)\text{-dichloro}[1,2\text{-diphenylethylenediamine}]$ platinum(II) suggested a predominant  $\delta\text{-gauche}$  conformation of these complexes (Noji et al. 1984). Their mirror-image CD spectra imply a  $\lambda\text{-gauche}$  prevailing conformation (see Fig. 2).

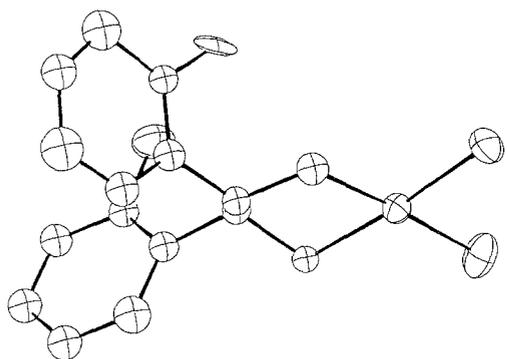
Conformational information for platinum(II) five-membered chelate rings can also be obtained from  $^1\text{H-NMR}$ -spectra. In an earlier  $^1\text{H-NMR}$  study (Gust et al. 1990) we had demonstrated that in [1-(2,6-dichloro-4-hydroxyphenyl)-2-phenylethylenediamine]diiodoplatinum(II) complexes the coupling constant between the ben-



**Fig. 3.** 250-MHz  $^1\text{H-NMR}$  spectrum of the tetradeuterated  $(\pm)\text{-3-PtSO}_4$  in  $[\text{D}_6]$  DMSO. An AB pattern with a  $J_{\text{H-H}} = 12.4$  Hz indicates an angle between the H-C-C-H protons of about  $180^\circ$  representing the  $\delta$  conformation with predominantly equatorially oriented phenyl rings

zylic protons possesses a Karplus-type angle dependence. Platinum complexes with *threo*-configured ligands have stable conformations with equatorially orientated phenyl rings and a coupling constant  $J_{\text{H-H}} = 12.2$  Hz for the chemically non-equivalent benzylic protons. In the case of  $(\pm)\text{-3-PtCl}_2$  this procedure could not be applied for conformation analysis, since the symmetrical complex gives only a single signal for both benzylic protons. This was overcome by using the sulfatoplatinum(II) derivative of  $(\pm)\text{-3-PtCl}_2$ . In dimethylsulfoxide (DMSO) as solvent one DMSO molecule and the  $\text{SO}_4$  residue coordinated to platinum. These two different leaving groups rendered the platinum(II) complex asymmetrical, giving rise to a diastereoisomeric splitting of the benzylic protons in the  $^1\text{H-NMR}$  spectra (compare Fig. 3). The signals were also split by coupling with the amino protons. When the amino groups were tetradeuterated before dissolution in  $[\text{D}_6]$  DMSO the H-C-C-H protons gave an AB pattern with a  $J_{\text{H-H}} = 12.4$  Hz, which indicated a dihedral angle between the protons of about  $180^\circ$ . This meant that the phenyl rings were predominantly equatorially orientated in solution ( $\delta$  conformation Fig. 3). A simultaneous presence of axially orientated phenyl rings was not observed.

The structure discussed above was also found for  $(-)\text{-3-PtCl}_2$  in crystalline form. The X-ray structural



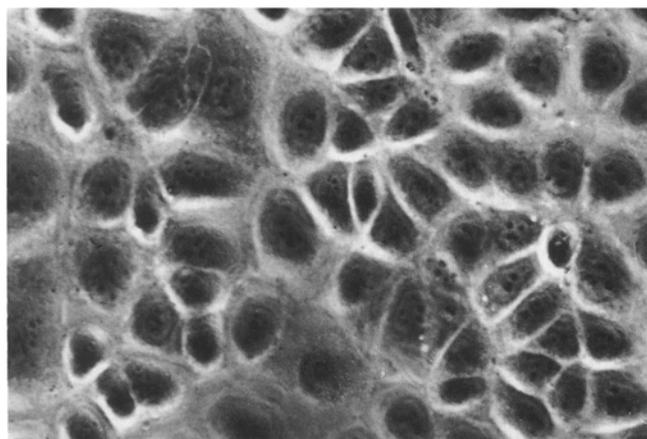
**Fig. 4.** Molecular structure of  $(-)-3\text{-PtCl}_2$  determined by X-ray diffraction analysis

analysis of  $(-)-3\text{-PtCl}_2$  is illustrated in Fig. 4.  $(-)-3\text{-PtCl}_2$  crystallized in the  $\delta$ -*gauche* conformation with equatorially orientated phenyl rings. The aromatic rings were arranged with a dihedral angle of  $70.13^\circ$  and both hydroxy groups placed above the five-membered chelate ring. The chelate ring is puckered with coordinated N atoms lying on the platinum coordination plane and with the benzylic C-atoms lying  $\pm 0.358$  nm out of plane. From this crystal structure the *S,S* configuration can be assigned to  $(-)-3\text{-PtCl}_2$ . A detailed description of these results is given in the following publication.

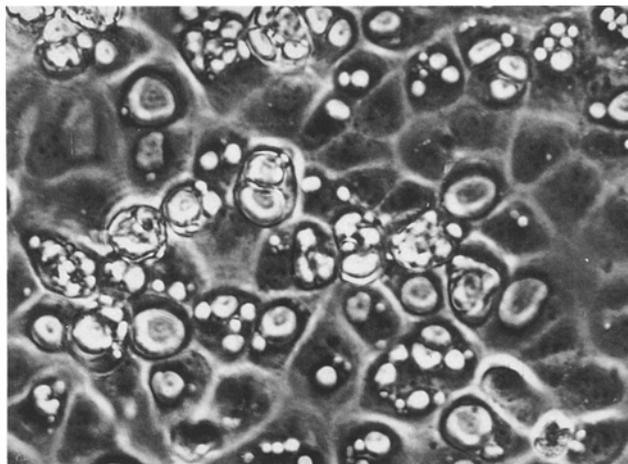
### Biology

For characterization of the NIH:OVCAR-3 ovarian cancer cell line cytogenetic analysis and investigation of growth kinetics and of the sensitivity against therapeutically used drugs were performed.

The epithelial ovarian cancer cell line forms cobblestone-like monolayers (Fig. 5). Several hours after confluence strikingly large vacuoles appear (Fig. 6). In addition, it is noted that cultures left at confluence for extended periods develop foci of "piled-up" cells. These foci seem morphologically more complex than simple monolayers. The detection of mucin, glycogen and lipids by Mucicarmine, periodic acid/schiff and Sudan IV staining was unsuccessful.



**Fig. 5.** Confluent NIH:OVCAR-3 cells in culture (41th passage). Typical cobblestone-like monolayer (phase contrast,  $320\times$ )

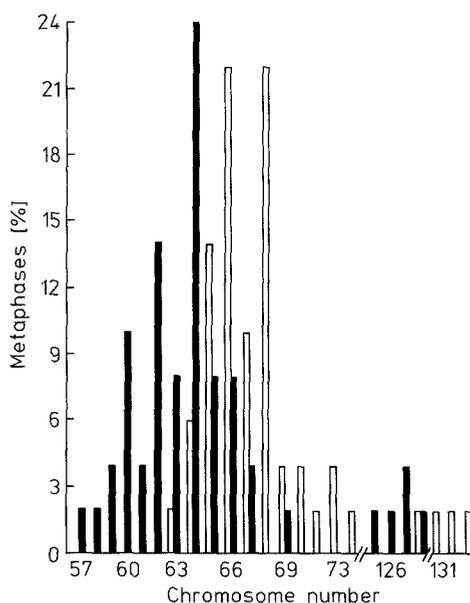


**Fig. 6.** Formation of intracellular vacuoles in an "over-confluent" culture of NIH:OVCAR-3 (passage 41, phase-contrast,  $320\times$ )

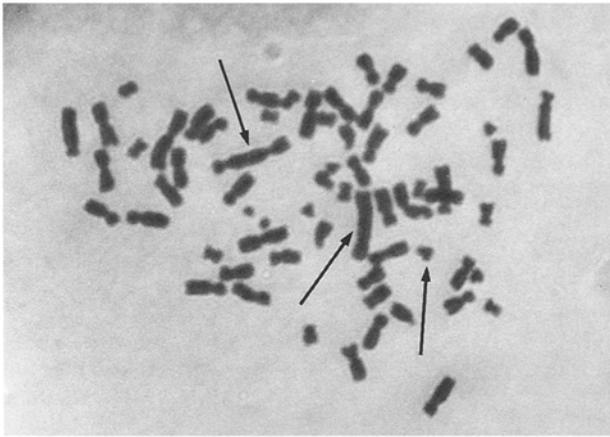
The cytogenetic analysis demonstrated that the NIH:OVCAR-3 line includes cells with a hypotriploid to a hypohexaploid karyotype (Fig. 7). The modal chromosome number amounts to 63. A karyogram of the NIH:OVCAR-3 cell line, which shows several characteristic chromosome anomalies, is presented in Fig. 8.

The morphological and cytogenetic characteristic proved to be constant over 22 passages and an observation period of about 30 weeks.

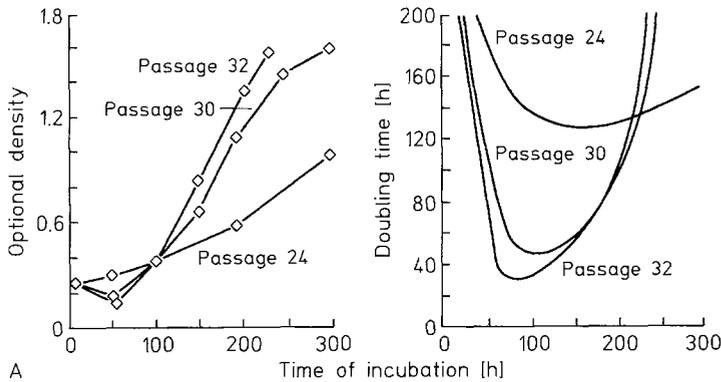
Figure 9 A, B illustrates the growth curves and the corresponding doubling times at any time of incubation for the NIH:OVCAR-3 cell line in the passages 24, 30, 32 (A), and 37, 49 (B). Under the given experimental conditions the logarithmic phase covers only a small fraction of the overall growth curve (Skehan and Friedman 1984). In a plot of doubling time versus time of incubation, exponential growth is characterized by a parallelism of the graphs with the x-axis. The NIH:OVCAR-3 cell line



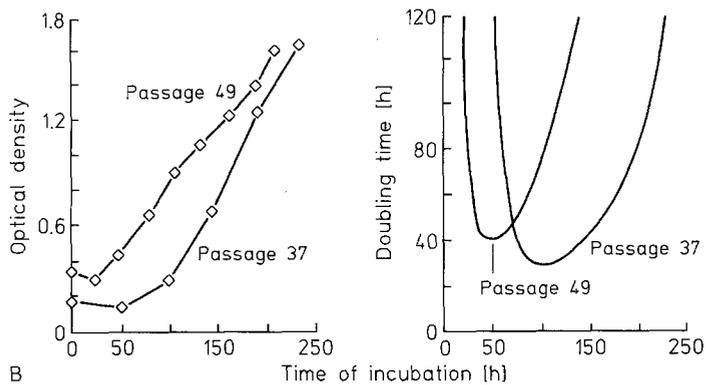
**Fig. 7.** Chromosome distribution of NIH:OVCAR-3 cells.  $\square$ , 29th passage from origin;  $\blacksquare$ , 51th passage from origin. The cells were passaged weekly. For each passage the chromosomes from 50 well-spread metaphase plates were counted



**Fig. 8.** Spread metaphase of NIH:OVCAR-3 cells (passage 26) with 66 chromosomes; obvious chromosome anomalies: bivalent chromosomes, deletions on both arms (magnification 1200 ×, Giemsa chromosome staining)



A

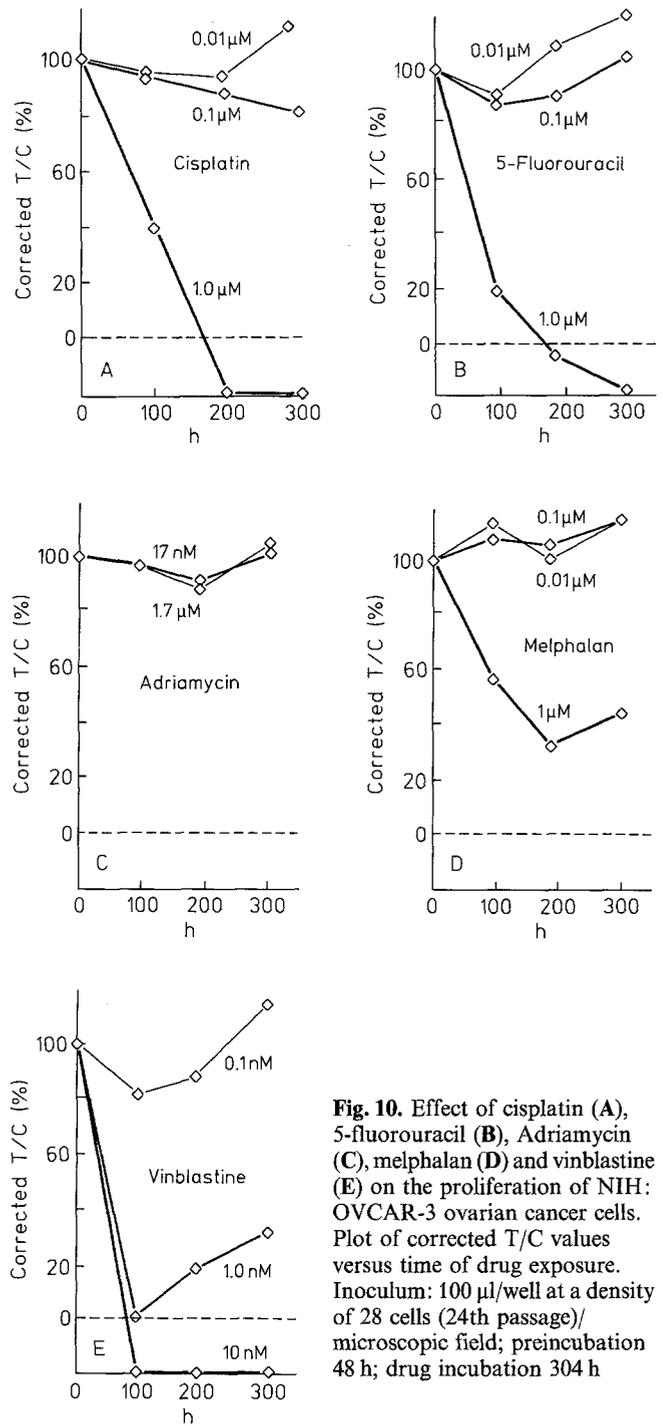


B

**Fig. 9.** Growth curves and corresponding doubling times of the NIH:OVCAR-3 cell line as a function of the passage number. Inoculum: 100µl/well at a density of 28, 19, 25, 17, 22 cells/microscopic field for passages 24, 30, 32, 37, 49

grows exponentially for a maximum of two generations. In the 24th passage the minimal doubling time of the cells amounts to 125 h, which is relatively long. In the following passages, however, the minimal doubling times approximate a constant value of about 35 h.

In order to gain information on the sensitivity of the NIH:OVCAR-3 cell line against the frequently used drugs cisplatin (Fig. 10A), 5-fluorouracil (Fig. 10B), Adriamycin (Fig. 10C), melphalan (Fig. 10D), and vin-



**Fig. 10.** Effect of cisplatin (A), 5-fluorouracil (B), Adriamycin (C), melphalan (D) and vinblastine (E) on the proliferation of NIH:OVCAR-3 ovarian cancer cells. Plot of corrected T/C values versus time of drug exposure. Inoculum: 100 µl/well at a density of 28 cells (24th passage)/microscopic field; preincubation 48 h; drug incubation 304 h

blastine (Fig. 10E), the cells were incubated with these compounds at therapeutically relevant concentrations for 304 h.

Only at the highest concentration (1 µM) did cisplatin, the most active drug in the therapy of ovarian cancer, show a cytotoxic effect, which we consider essential for a curative effect in vivo. It is important, however, that the NIH:OVCAR-3 cells are in contact with this drug for a relatively long period in order to kill the cells (compare part III of this publication series). Owing to the marked toxic side-effects and the pharmacokinetics of cisplatin it is difficult to meet this demand in vivo.

Adriamycin (Fig. 10C) and melphalan (Fig. 10D), which are used in combination with cisplatin in the therapy of the advanced ovarian cancer, showed no cytotoxic activities in the concentrations used. Our results with Adriamycin, cisplatin and melphalan are in accordance with those of Hamilton et al. (1983), who also describe a resistance of the NIH:OVCAR-3 cell line against these drugs. 5-Fluorouracil, which is reported to act synergistically with cisplatin in a human ovarian cancer cell line (Scanlon et al. 1986), showed cytotoxic activity in our experiment only at the highest concentration (1  $\mu$ M). A clinical study in a group of previously untreated patients, however, demonstrated a lower activity of 5-fluorouracil in comparison to melphalan (Smith and Ruthledge 1975; compare Omura 1989).

Among the five common drugs tested on the NIH:OVCAR-3 cell line vinblastine proved to be the most active one. At the very low concentration of 1 nM vinblastine initially leads to a complete inhibition of cell proliferation. However, after 100 h the cells recover from the initial damage (Fig. 10E). By increasing the concentration to 10 nM a cytotoxic effect is brought about. Though in vitro data show vinblastine to be one of the most active drugs against ovarian cancer, it is ineffective as "salvage" therapy (Surwit et al. 1987).

In preliminary experiments we could show that at a concentration of 2.5  $\mu$ M (duration of drug incubation 256 h) the enantiomeric [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes (+)-3-PtCl<sub>2</sub> and (-)-3-PtCl<sub>2</sub> produce equal effects ( $T/C_{\text{corr}} = 10\%$ ) on the NIH:OVCAR-3 cell line. However, a difference between the enantiomers regarding the inhibition kinetics was seen. For (-)-3-PtCl<sub>2</sub> a faster onset of the cytotoxic effect was detected. These experiments will be described in detail in part III of this communication.

## Discussion

In part I of this publication series we have described the synthesis and testing of [(±)-1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) [(±)-3-PtCl<sub>2</sub>], which proved to be a very promising new platinum complex for the therapy of cisplatin-resistant ovarian cancer (Müller et al. 1990). In order to improve the antitumor activity of (±)-3-PtCl<sub>2</sub> a resolution into the optically active forms [(+)- and (-)-3-PtCl<sub>2</sub>] was attempted (compare Scheme 2). This was achieved at the stage of the (±)-1,2-bis(2-methoxyphenyl)ethylenediamine (3a) by fractional crystallization of the diastereoisomeric salts of 3a with optically active tartaric acid in EtOH. The complete enantiomeric purity of (+)-3a and (-)-3a could be proved by <sup>1</sup>H-NMR spectroscopy using (S)-(+)-1-(9-anthryl)-2,2,2-trifluoroethanol as the optically active solvent (see Fig. 1). After ether cleavage of the enantiomeric methoxy compounds [(+)-3a and (-)-3a] [(+)- and (-)-1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) [(+)- and (-)-3-PtCl<sub>2</sub>] were obtained by reaction with K<sub>2</sub>PtCl<sub>4</sub> (Scheme 2). The absolute configuration of the enantiomers (+)-3-PtCl<sub>2</sub> (*R,R*) and (-)-3-PtCl<sub>2</sub> (*S,S*) was determined by CD spectroscopy (Fig. 2).

As substances for comparison, (*R,R*)- and (*S,S*)-dichloro(1,2-diphenylethylenediamine)platinum(II) were used.

<sup>1</sup>H-NMR spectroscopic studies, which were performed with *N,N,N',N'*-tetradeuterated [(±)-1,2-bis(2-hydroxyphenyl)ethylenediamine]sulfatoplatinum(II) [(±)-3-PtSO<sub>4</sub>] dissolved in [D<sub>6</sub>]DMSO, gave insight into the conformation of (±)-3-PtCl<sub>2</sub>. On dissolution in [D<sub>6</sub>]DMSO (±)-3-PtSO<sub>4</sub> forms an asymmetric complex, in which both SO<sub>4</sub><sup>2-</sup> and DMSO are coordinated to platinum. This Pt(DMSO)(SO<sub>4</sub>) derivative shows a diastereoisomeric splitting of the non-equivalent benzylic protons in the <sup>1</sup>H-NMR spectrum giving an AB pattern with a  $J_{\text{H-H}} = 12.4$  Hz (Fig. 3). This dihedral angle between the two protons amounts to about 180°, which signifies an axial orientation of the two protons and two equatorial phenyl rings ( $\delta$  conformation in Fig. 3). The same three-dimensional structure is observed by X-ray structural analysis, which also confirms the *S,S* configuration of (-)-3-PtCl<sub>2</sub> (Fig. 4). For the evaluation of the enantiomeric [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes [(+)- and (-)-3-PtCl<sub>2</sub>] the human ovarian cancer cell line NIH:OVCAR-3, which is described to be resistant against cisplatin and other standard drugs (Hamilton et al. 1983), was used as test model. In order to recognize even small differences in activity we studied the drug-induced changes in cell proliferation kinetics. For this purpose we applied a computer-aided microtechnique that allows the registration of growth curves of the NIH:OVCAR-3 cells in monolayer cultures by large-scale spectrophotometric measurements after crystal violet staining using 96-well microtitration plates (Reilein et al. 1990). The NIH:OVCAR-3 cell line was characterized by cytogenetic analysis (Fig. 7 and 8), by its growth kinetics (Fig. 9) and by its sensitivity against therapeutically used drugs (Fig. 10). The last-mentioned experiments confirmed the low sensitivity of the NIH:OVCAR-3 cell line against therapeutically relevant concentrations of cisplatin, Adriamycin and melphalan described by Hamilton et al. (1983). It is of interest that 5-fluorouracil (cytotoxic at 1  $\mu$ M) and especially vinblastine (cytotoxic at 10 nM) produce a marked inhibition of the NIH:OVCAR-3 cell line in concentrations that can be achieved under in vivo conditions.

In clinical studies, however, the superiority of 5-fluorouracil and vinblastine compared to cisplatin, Adriamycin and melphalan in the therapy of ovarian cancer was not confirmed (Omura 1989; Surwit et al. 1987).

Two mechanisms responsible for the multidrug resistance phenotype in ovarian cancer are discussed (compare Ozols et al. 1989):

1. The ability of the malignant cell to synthesize a membrane glycoprotein (P-170), which is part of a complex system counteracting the drug accumulation in the cancer cell. By this mechanism structurally unrelated chemotherapeutic agents, e.g. *Vinca* alkaloids and Adriamycin, are transported out of the cell.
2. The ability of the malignant cell to elevate its content of glutathione, which inactivates drugs that react with nucleophilic targets of the tumor cell (e.g. DNA). This

mechanism is consistent with the observed cross-resistance between cisplatin and alkylating agents like melphalan<sup>1</sup>.

Further preclinical studies with 5-fluorouracil and vinblastine will be required to evaluate their usefulness in the treatment of ovarian cancer.

The experiments with enantiomeric [1,2-bis-(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes [(+)- and (-)-3-PtCl<sub>2</sub>] on the NIH:OVCAR 3 cell line revealed no differences concerning the maximum antitumor effect. At a concentration of 2.5 μM both enantiomers produce T/C<sub>corr</sub> values of about -10%. With (-)-3-PtCl<sub>2</sub>, however, a faster onset of the cytotoxic effect was detectable.

*Acknowledgements.* The technical assistance of E. Aichinger, L. Gottswinter, D. Krisam, S. Paulus, P. Pistor, and P. Richthammer is gratefully acknowledged. Thanks are also due to the Deutsche Forschungsgemeinschaft (SFB 234), the Matthias-Lackas-Stiftung für Krebsforschung and the Fonds der Chemischen Industrie for financial support.

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<sup>1</sup> In the case of resistance against cisplatin the ability of tumor cells to enhance DNA repair (i.e. cisplatin-nucleoside adduct removal) is thought to play a major role (see Lai et al. 1988 for references). (±)-3-PtCl<sub>2</sub> proved not to be cross-resistant to cisplatin (Müller et al. 1990)