Tumor Inhibiting [1,2-Bis(fluorophenyl)ethylenediamine]platinum(II) Complexes

Part II: Biological Evaluation - *in vitro* Studies on the P 388 D_1 Leukemia Cell Line

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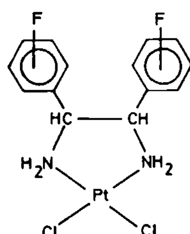
Experiments on the P 388 D_1 cell line (48 h exposure) demonstrate that [1,2-bis-(fluorophenyl)ethylenediamine]platinum(II) complexes are comparably active on the cell number and ^{3}H-thymidine incorporation, irrespective of the position of the fluorine atom (ortho, meta, or para) and the nature of the "leaving group" (Cl^- or H_2O). However, the compounds of the R,R/S,S series are more active than those of the R,S series and comparable to cisplatin. In the "tumor colony forming assay" the R,R/S,S configurated compounds are about ten times as active as cisplatin. The R,R/S,S configurated diaqua[1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) salts reach their half maximum effect more readily ($t_{1/2} \cong 1.6$ h) than their R,S configurated analogues ($t_{1/2} \cong 20$ h). A time limited contact of the cells with R,R/S,S configurated diaqua[1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) salts (~1h) leads to a similar inhibition like a permanent drug exposure indicating a fast uptake of the complex by the tumor cell. In experiments on the *Ehrlich* ascites tumor of the mouse and on the L 1210 leukemia cell line R,R/S,S-[1,2-bis(4-fluorophenyl)ethylenediamine]dichloroplatinum(II) turns out to be equipotent with cisplatin.

Tumorhemmende [1,2-Bis(fluorphenyl)ethylendiamin]platin(II) Komplexe, 2. Teil: Biologische Prüfung - In vitro Studien an der P 388 D_1 Leukämie Zellinie

In Versuchen an der P 388 D_1 Leukämie Zellinie (48 h Wirkstoffinkubation) wird gezeigt, daß [1,2-Bis-(fluorphenyl)ethylendiamin]platin(II) Komplexe unabhängig von der Stellung des Fluoratoms (ortho, meta oder para) und der Art der Abgangsgruppe (Cl^- oder H_2O) eine vergleichbare Wirkung auf Zellzahl und ^{3}H-Thymidin-Einbau aufweisen. Die Verbindungen der R,R/S,S-Reihe sind jedoch wirksamer als die der R,S-Reihe und mit Cisplatin vergleichbar. Die Wirkung der R,R/S,S konfigurierten Verbindungen liegt im "Tumor Colony Forming Assay" um eine Größenordnung über der des Cisplatin. Der halbmaximale Effekt wird bei den R,R/S,S-konfigurierten Diaqua[1,2-bis(4-fluorphenyl)ethylendiamin]platin(II)Salzen ($t_{1/2} \cong 1.6$ h) erheblich schneller als bei ihren R,S-konfigurierten Analogen ($t_{1/2} \cong 20$ h) eingestellt. Ein zeitlich limitierter Kontakt der Zellen mit R,R/S,S-konfigurierten Diaqua[1,2-bis(4-fluorphenyl)ethylendiamin]platin(II)Salzen (~1h) führt zu ähnlichen Hemmwerten wie eine permanente Wirkstoffeinwirkung, ein Indiz für eine schnelle Wirkstoffaufnahme. R,R/S,S-[1,2-Bis(4-fluorphenyl)ethylendiamin]dichloroplatin(II) erweist sich in Versuchen am *Ehrlich* Ascites Tumor der Maus und an der L 1210 Leukämie Zellinie als äquipotent mit Cisplatin.

The antitumor activity of diastereomeric dichloro(1,2-diphenylethylenediamine)platinum(II) complexes is strongly influenced by substituents in para positions of both benzene rings. Among various residues fluorine proved to be of special interest[1]. The R,R/S,S configurated [1,2-bis(4-fluorophenyl)ethylenediamine]-dichloroplatinum(II) complex (**15**) was the most active. In part I of this publication we have described the synthesis of the diastereomeric ortho, meta and para fluoro substituted dichloro(1,2-diphenylethylenediamine)platinum(II) complexes **13** to **18** and of their more water soluble diaquaplatinum(II) salts **25** to **32**[2]. In part II of this publication we investigate the influence of the F-position, the configuration and the leaving group (Cl- or H_2O) on the antitumor activity against P 388 leukemia in vitro.



13 - 18

Compd.	Config.	F-Position	Abbreviation
13	D,L	2	D,L-2F-$PtCl_2$
14	D,L	3	D,L-3F-$PtCl_2$
15	D,L	4	D,L-4F-$PtCl_2$
16	meso	2	meso-2F-$PtCl_2$
17	meso	3	meso-3F-$PtCl_2$
18	meso	4	meso-4F-$PtCl_2$

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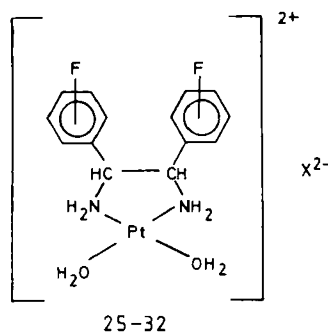


Compd.	Config.	F-Pos.	Counter Ion	Abbreviation
25	D,L	2	SO_4	D,L-2F-$PtSO_4$
26	D,L	3	SO_4	D,L-3F-$PtSO_4$
27	D,L	4	SO_4	D,L-4F-$PtSO_4$
28	meso	2	SO_4	meso-2F-$PtSO_4$
29	meso	3	SO_4	meso-3F-$PtSO_4$
30	meso	4	SO_4	meso-4F-$PtSO_4$
31	D,L	4	NO_3	D,L-4F-$Pt(NO_3)_2$
32	meso	4	NO_3	meso-4F-$Pt(NO_3)_2$

Results and Discussion

The experiments on the P 388 D_1 leukemia cell line (48 h drug incubation) show that the identically configurated [1,2-bis(fluorophenyl)ethylenediamine]dichloroplatinum(II) complexes **13** to **18** and their diaquaplatinum(II) sulfates and nitrates **25** to **32** are comparably active in inhibiting cell proliferation and ^3H-thymidine incorporation regardless of the position of the fluorine atoms. Generally, a stronger activity of the R,R/S,S diastereomers was observed, which are equiactive with cisplatin (Table 1).

In addition to these experiments **13** to **18** were comparatively tested with cisplatin in the in vitro soft agar tumor clonogenic assay (TCA) to determine their effect on the colony forming ability of P 388 D_1 leukemia cells (i.e. the loss of their stem cell function in the presence of drugs).

Tab. 1: Effect of Diastereomeric [1,2-Bis(fluorophenyl)ethylenediamine]dichloroplantinum(II) Complexes and Diaqua-[1,2-bis(fluorophenyl)ethylenediamine]platinum(II) Sulfates and Nitrates on ^3H-Thymidine Incorporation and Cell Proliferation of P 388 D_1 Leukemia Cells, 48 h Drug Incubation.

Conpound (No)	Cell Number % T/C at $1{\cdot}10^{-6}$ M	Cell Number ED_{50} [M]	^3H-Thymidine Incorp. % T/C at $1{\cdot}10^{-6}$ M	^3H-Thymidine Incorp. ED_{50} [M]
meso-2F-$PtSO_4$ (**28**)	93	$2.6{\cdot}10^{-6}$	92	$2.6{\cdot}10^{-6}$
meso-3F-$PtSO_4$ (**29**)	77	$1.8{\cdot}10^{-6}$	77	$2.3{\cdot}10^{-6}$
meso-4F-$PtSO_4$ (**30**)	88	$3.8{\cdot}10^{-6}$	84	$4.0{\cdot}10^{-6}$
meso-4F-$Pt(NO_3)_2$ (**32**)	86	$3.8{\cdot}10^{-6}$	92	$3.4{\cdot}10^{-6}$
meso-2F-$PtCl_2$ (**16**)	69	$2.3{\cdot}10^{-6}$	75	$2.2{\cdot}10^{-6}$
meso-3F-$PtCl_2$ (**17**)	83	$4.1{\cdot}10^{-6}$	93	$4.8{\cdot}10^{-6}$
meso-4F-$PtCl_2$ (**18**)	90	$2.6{\cdot}10^{-6}$	91	$3.4{\cdot}10^{-6}$
D,L-2F-$PtSO_4$ (**25**)	34	$4.3{\cdot}10^{-7}$	8	$3.5{\cdot}10^{-7}$
D,L-3F-$PtSO_4$ (**26**)	32	$5.1{\cdot}10^{-7}$	26	$6.5{\cdot}10^{-7}$
D,L-4F-$PtSO_4$ (**27**)	31	$5.4{\cdot}10^{-7}$	24	$7.2{\cdot}10^{-7}$
D,L-4F-$Pt(NO_3)_2$ (**31**)	-	$4.6{\cdot}10^{-7}$	-	$5.9{\cdot}10^{-7}$
D,L-2F-$PtCl_2$ (**13**)	23	$2.0{\cdot}10^{-7}$	8	$4.8{\cdot}10^{-7}$
D,L-3F-$PtCl_2$ (**14**)	30	$4.4{\cdot}10^{-7}$	14	$4.9{\cdot}10^{-7}$
D,L-4F-$PtCl_2$ (**15**)	31	$4.3{\cdot}10^{-7}$	23	$5.5{\cdot}10^{-7}$
Cisplatin	29	$4.8{\cdot}10^{-7}$	19	$4.5{\cdot}10^{-7}$

Tab. 2: Antitumor Effect of Fluoro Substituted Dichloro-(1,2-diphenylethylenediamine)platinum(II) Complexes on the P 388 D_1 Leukemia Cell Line - Colony Forming Assay

Compd.		Colony Formation 48 h Drug Exposure % Inhibition at 10^{-6} M	48 h Drug Exposure ED_{50} (M)	1 h Drug Exposure % Inhibition at 2 x 10^{-6} M	1 h Drug Exposure ED_{50} (M)
13	(D,L-2F-$PtCl_2$)	100	2 x 10^{-8}	60	1 x 10^{-6}
14	(D,L-3F-$PtCl_2$)	98	5 x 10^{-8}	72	9 x 10^{-7}
15	(D,L-4F-$PtCl_2$)	96	5 x 10^{-8}	70	1 x 10^{-6}
16	(meso-2F-$PtCl_2$)	49	1 x 10^{-6}	27	5 x 10^{-6}
17	(meso-3F-$PtCl_2$)	9	4 x 10^{-6}	18	8 x 10^{-6}
18	(meso-4F-$PtCl_2$)	7	3 x 10^{-6}	0	3 x 10^{-5}
Cisplatin		97	2 x 10^{-7}	30	6 x 10^{-6}

Stem cells (e.g. the hematopoietic stem cell), which are responsible for maintaining the integrity and continued survival of any particular cell population, are capable of an indefinite number of divisions. Unlike to this the differentiated cells lack this unlimited capacity of proliferation[3]. In accordance with the stem cell conception it is assumed that in cancer diseases so called tumor stem cells are responsible for tumor growth or regrowth after treatment and also for the forming of metastases. Therefore, it is the tumor stem cell population that must be eradicated to obtain curative cancer chemotherapy[4]. Clonogenic tumor cells in TCA are considered to be closely related to tumor stem cells in situ, therefore, the predictive value of this assay for a possible in vivo activity should be superior to other in vitro assays.

In the TCA, too, the R,R/S,S configurated compounds **13** to **15** as well as the R,S configurated compounds **16** to **18** proved to be equiactive on the P 388 D_1 leukemia cell line irrespective of the position of the fluorine atoms in the phenyl rings. However, the compounds of the R,R/S,S series are much more active than those of the R,S series. Drug incubation periods of 48 h produced stronger inhibition effects than those of 1 h. ED_{50}-values of the R/S configurated compounds **16** to **18** are comparable to those listed in table 1. It is of interest that the ED_{50}-values of the R,R/S,S configurated compounds **13** to **15** after 48 h drug exposure are one order of magnitude smaller than those achieved in cell culture experiments (see Table 1 and 2). In the TCA compounds **13** to **15** turned out to be markedly superior to cisplatin.

Tab. 3: Effect of Diastereomeric [1,2-Bis(4-fluorophenyl)ethylenediamine]dichloroplatinum(II) Complexes and Diaqua-[1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) Sulfates and Nitrates ($1 \cdot 10^{-5}$ M) on ^{3}H-Thymidine Incorportation and Viability of P 388 D_1 Leukemia Cells.

Compound (No)	Incubation Time [h]	% T/C	% Viability*
D,L-4F-$PtCl_2$	1.5	89.2	98.3
(15)	2.0	69.2	98.5
D,L-4F-$PtSO_4$	1.0	83.5	98.5
(27)	2.0	55.3	98.8
D,L-4F-$Pt(NO_3)_2$)	1.0	63.4	95.0
(31)	2.0	42.6	97.5
meso-4F-$PtSO_4$	10.2	88.3	97.5
(30)	14	71.4	97.5
	18	63.9	97.0
	24	23.5	96.0
	34.3	14.4	96.3
	48	3.2	96.0
meso-4F-$Pt(NO_3)_2$	10.2	75.9	95.5
(32)	14	63.3	97.5
	18	45.5	97.0
	24	17.5	96.0
	34.3	10.3	93.5
	48	2.7	96.8

*: determined by trypan blue exclusion staining

Platinum(II) complexes cause a slowed DNA synthesis phase and a transient G_2 arrest at low drug concentrations, as shown with cisplatin. However, at higher concentrations an irreversible G_2 arrest associated with extensive chromosome damage takes place. This is accompanied by a loss of viability (visible by trypan blue staining), which is detectable 4 days after short drug incubation (2 h) and reaches its maximum after 7 days[5].

In the case of the R,R/S,S configurated [1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) complexes **15**, **27**, and **31** we found no loss of viability of P 388 D_1 cells (drug exposure: 2 h, table 3). Under the same experimental conditions the ^{3}H-thymidine incorporation was reduced at about 50 %.

Tumor cells which had been treated with the R,S configurated compounds **30** and **31** were viable even after 48 h of exposure (table 3). Perhaps the loss of cell membrane integrity (a sign for cell death) is a longer, time dependent process which requires 4 to 8 days as shown in the cisplatin experiment[5]. Also in the TCA, (indicative for cell death, too), the R,S-[1,2-bis(4-fluorophenyl)ethylenediamine]platinum (II) complex was only slightly active (7 % inhibition at 10^{-6} M and 48 h exposure; table 2). The R,R/S,S-[1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) complex, however, leads to a marked reduction of colony numbers (96 % inhibition at 10^{-6} M and 48 h exposure; 70 % inhibition at 2 x 10^{-6} M and 1 h exposure; table 2). Therefore, a longer observation time (compare also table 4) should also lead to a corresponding portion of non-viable cells in the trypan blue exclusion test.

The cytotoxic effect of platinum complexes is not the result of an inhibition of the DNA synthesis in the S phase of the cell cycle (up to now generally accepted as the critical step in cisplatin-induced toxicity) but of a persistent (i.e. irreversible) G2 arrest, as shown by *Eastman* and *Sorenson*[6]. Furtheron it is proposed that the G2 arrest results from the inability of the cells to transcribe genes required for the passage into mitosis[6].

The lack of differences in activity between analogous cis-PtA_2Cl_2 and cis[$PtA_2(H_2O)_2$]X complexes (A_2: 1,2-bis (2-,3-, and 4-fluorophenyl)ethylenediamine; X: SO_4^{2-} or 2 NO^{3-}) is surprising since, as a rule, the change from the nonionic dichloroplatinum(II) complex to the ionic diaquaplatinum(II) complex leads to a decrease in antitumor activity. An example for a drastic change of efficacy is provided by cis-$Pt(NH_3)_2Cl_2$ in which the substitution of Cl by H_2O is accompanied by a loss of activity on the L 1210 leukemia of the mouse[7]. In contrast to this, diaquaplatinum(II) complexes with spacious, lipophilic ligands like cis-[Pt(DAC) $(H_2O)_2$] $(NO_3)_2$ (DAC: 1,2-diaminocyclohexane) show tumor inhibiting properities (L 1210 leukemia, mouse) which are comparable to those of the analogous dichloroplatinum(II) complexes[7].

In these complexes presumably two factors are responsible for the appearance of antitumor activity:

1. The steric shielding of Pt. It impedes the reaction with bionucleophils. Therefore, the inactivation of the complex during its transport to the tumor cell is retarded.

2. The hydrophobic character. It facilitates the penetration of the drug across the plasma membrane of the tumor cell.

Both factors lead to therapeutically active drug levels in the tumor cell.

According to the rate of hydrolysis, two classes of platinum(II) complexes can be observed:

Class 1 complexes in which the leaving group is immediately substituted by H_2O (e.g. [cis-$PtA_2(H_2O)(SO_4)$]·H_2O in which the SO_4 ligand is bonded to the Pt through one of its oxygen atoms; regarding to the coordination of SO_4 to Pt see below).

Class 2 complexes, in which the exchange of the leaving group for H_2O takes 1 to several h (e.g. cis-PtA_2Cl_2).

Compounds of class 1, e.g. **30**, are - as mentioned - only antitumor active, if their amine ligand causes a steric shielding of Pt, thereby hindering the reaction with bionucleophils during the transport to the tumor cell. Unlike to this, compounds of class 2 are also antitumor active, if they contain small amine ligands like NH_3. Class 2 complexes are weakly-reactive prodrugs which are slowly hydrolized into the active drugs (cis[$PtA_2(H_2O)_2$]$^{2+}$). Therefore, the inactivation of class 2 complexes by bionucleophils is impeded during the transport to the tumor cell. They probably pass the cell membrane in the form of non-ionic cis-PtA_2X, followed by an activation in the cell (e.g. exchange of X by H_2O). In the case of cisplatin hydrolysis is supposed to be suppressed under the conditions of the high extracellular Cl^- concentration (0.103 M), while the low Cl^- level (0.004 M) in the cytoplasm facilitates the Cl^-/H_2O exchange.

These findings infer that the kinetic behavior of platinum(II) complexes in the reaction with nucleophils is essential for the antitumor activity, therefore, too fast or too slowly reacting complexes are weakly active.

We think that the development of water soluble and hence therapeutically better applicable platinum(II) complexes with [cis-$PtA_2(H_2O)_2$]X structure is feasible. This requires the choice of appropriate amine ligands which do retard the inactivation of the drug via reaction with bionucleophils during the transport to the tumor cell but still allow the interaction with DNA, which is essential for the antitumor activity.

In the following we describe the influence of the leaving group (H_2O or Cl^-) and the configuration of C-1 and C-2 of the 1,2-bis(4-fluorophenyl)ethylenediamine ligand on the time-dependent inhibition of the ^{3}H-thymidine incorporation in the P 388 D_1 leukemia cell culture.

In the R,R/S,S configurated series a considerably faster onset of action of the equally active diaquaplatinum(II) sulfate (**27**) and nitrate (**31**), $t_{1/2} \cong 1.6$ h (time (h) required for a 50 % inhibition), is observed compared to dichloroplatinum(II)-compound **15**, $t_{1/2} \cong 3.3$ h, (Figure 1). This difference is caused by a slow hydrolysis of **15** to form the active species [cis-$PtA_2(H_2O)_2$]X, which is responsible for the reaction with DNA.

In the case of **31**, whose nitrate residues are directly coordinated with Pt^{2+}, an exchange of NO_3 by H_2O takes place

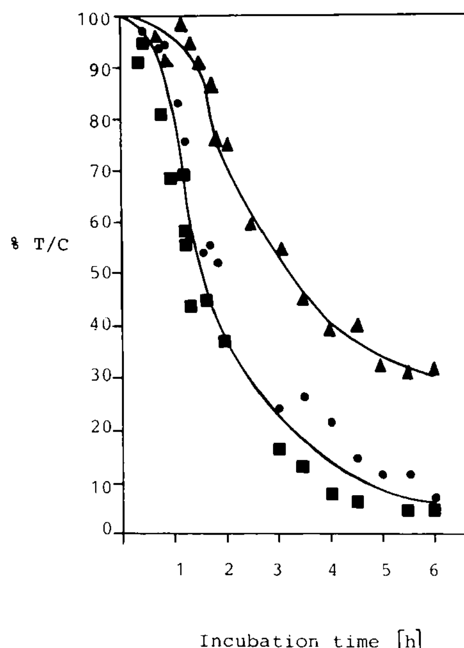


Figure 1: Influence of the Leaving Group (H_2O or Cl^-) of [D,L-1,2-Bis(4-fluorophenyl)ethylenediamine]platinum(II) Complexes on the Time-Dependent Inhibition of the ^{3}H-Thymidin Incorporation in the P 388 D_1 Leukemia Cell Culture. Drug Concentration: $1 \cdot 10^{-5}$ M. Compounds: **15** (D,L-4F-$PtCl_2$), **27** (D,L-4F-$PtSO_4$), **31** (D,L-4F-$Pt(NO_3)_2$). Additional values for **15**: 13.25 h / 26 % T/C; 22.25 h / 5 % T/C; 30.1 h /1 % T/C; 48 h / 1 % T/C.

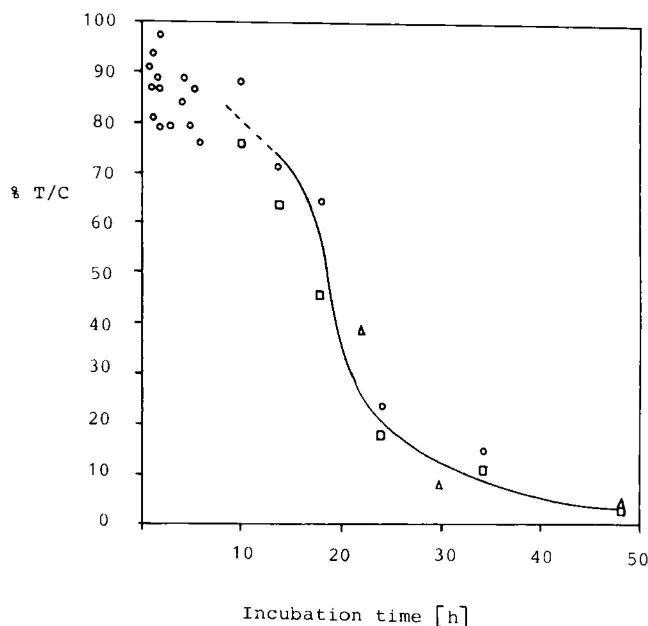
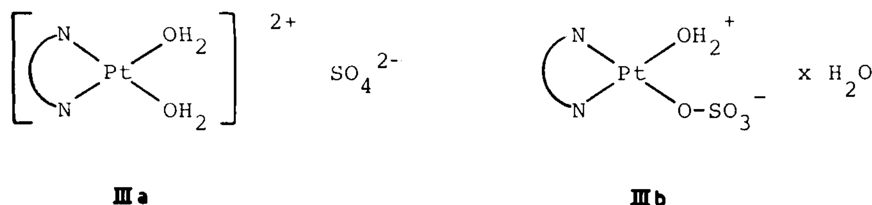


Figure 2: Influence of the Leaving Group (H_2O or Cl^-) of [meso-1,2-Bis(4-fluorophenyl)ethylenediamine]platinum(II) Complexes on the Time-Dependent Inhibition of the ^{3}H-Thymidine Incorporation in the P 388 D_1 Leukemia Cell Culture. Drug Concentrations: $1 \cdot 10^{-5}$ M. Compounds: **18** (meso-4F-$PtCl_2$), **30** (meso-4F-$PtSO_4$) **32** (meso-4F-$Pt(NO_3)_2$)



Formula IIIa and IIIb

immediately after dissolution in water. The same is true for a sulfate residue in compound **27** which is coordinated with Pt.

According to the elemental analysis, **27** contains two molecules of water[2]. Besides the structural formula **IIIa**, structure **IIIb** (unidentate complex) must be discussed (see also Part I of this publication).

A compound of type **IIIb** (structurally analogous to **27**) was obtained by *Rochon* and *Melanson*[8] by the reaction of (N,N'-dimethylethylenediamine)diiodoplatinum(II) with Ag_2SO_4. The structure of [aqua(N,N'-dimethylethylenediamine)sulfatoplatinum(II)]hydrate was confirmed by X-ray analysis[8]. In this molecule the coordinated water forms two strong hydrogen bonds, donating one proton to an oxygen atom of the sulfate ligand and the other proton to the lattice water oxygen. The relationship between the H_2O molecules and the sulfate residue contributes to complex stability.

The question whether **27** is described by formula **IIIa** or **IIIb** is of no importance for the inhibition kinetics of the ^3H thymidine incorporation into DNA. In water the sulfato residue of the unidentate sulfatoplatinum(II) complex **IIIb** is quickly replaced by H_2O molecules, forming the diamine-diaquaplatinum(II) ion **IIIa**. This was proved by us in the case of the analogous compound aqua[meso-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]sulfatoplatinum (II) by an increase in conductance to a constant level within a few min after dissolution of the compound in water[9].

In contrast to the R,R/S,S configurated complexes the time-dependent inhibition of the ^3H-thymidine incorporation by the R,S configurated complexes **18, 30, and 32** is not contingent on the nature of the leaving group (H_2O or Cl^-) as shown by the $t_{1/2}$-values (20 h) (Figure 2).

The great differences between the $t_{1/2}$-values of R,R/S,S and R,S configurated complexes can be explained by a stronger steric hindrance caused by the axially standing phenyl ring of the latter complexes impeding the approach to DNA, as has been discussed elsewhere[10,11]. In the case of the R,R/S,S configurated complexes only the conformation is favored in which both phenyl rings are equatorially arranged[12].

Accordingly, different kinetics have been reported for the reactions of the diastereomeric [1,2-bis(4-fluorophenyl)ethylenediamine]sulfatoplatinum(II) complexes **27** and **30** with salmon testis DNA[13]. In these experiments, DNA synthesis catalysed by *E. coli*-DNA polymerase I was inhibited by the coordination reaction of the platinum(II) complexes with DNA, presumably in N-7 positions of guanines. The kinetics of inhibition followed the formation of monoadducts, and was faster in the case of the racemate **27** than of the meso-form **30**.

Tab. 4: Long-Term Effect of [1,2-Bis(4-fluorophenyl)ethylenediamine] platinum(II) Complexes (1×10^{-5}M) on the ^3H-Thymidine Incorporation in P 388 D_1 Leukemia Cells after Short Drug Incubation Times

Compound (No)	^3H-Thymidine Incorporation % T/C[a]	% T/C[b]
D,L-4F-$PtCl_2$ (**15**)	1.5 h : 87.9	1.5 h[c]/4 h[d] : 73.7
	2 h : 69.2	2 h / 4 h : 63.4
	4 h : 39.4	
D,L-4F-$PtSO_4$ (**27**)	1 h : 82.6	1 h /3 h : 45.4
		1 h / 6 h : 22.8
	2 h : 51.9	2 h / 3 h : 36.0
		2 h / 6 h : 19.3
	3 h : 16.7	
	6 h : 6.4	
D,L-4F-$Pt(NO_3)_2$ (**31**)	1 h : 63.4	1 h / 6 h : 21.0
	6 h : 4.6	

a : % T/C- values for continues treatment (SD ≤ 14 %)
b : % T/C- values from the wash-out experiments (SD ≤ 14 %)
c: Time of drug exposure
d: total incubation time (drug exposure plus incubation in drug-free medium)

Experiments in which P 388 D_1 leukemia cells were exposed to the [1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) comlexes **15**, **27**, and **31** for a short time, washed and reincubated with drug-free medium (Table 4), hint at possible differences in the uptake of ionic and non-ionic species by the tumor cells.

While the dichloroplatinum(II) complex **15** yields only a moderate further decrease in DNA synthesis during the reincubation, a strong effect is observed in the case of the diaquaplatinum(II) complexes **27** and **31**. This finding could be explained by a higher intracellular concentration caused by a faster uptake of the ionic diaquaplatinum(II) complexes **27** and **31** by the tumor cells compared to the non-ionic dichloroplatinum(II) complex **15**[14]. This is even more surprising since an inactivation of the reactive diaquaplatinum(II) species by nucleophils in the medium must be expected. With the dichloroplatinum(II) species this inactivation process is only possible after hydrolysis. Apparently, the reaction of **27** and **31** with nucleophils is strongly retarded due to steric conditions. It may also be that distinct mechanisms are responsible for the permeation of cisplatin and [1,2-bis(4-fluorophenyl)ethylenediamine]-

diaquaplatinum(II) complexes across the cell membrane. Such differences can be the basis of an activity of the [1,2-bis(4-fluorophenyl)ethylenediamine]platinum(II) complexes against cisplatin-resistant tumors, as is pointed out in the following.

The development of resistance toward cisplatin as a result of alterations in drug accumulation has been discussed[15]. Although it is widely accepted that platinum(II) complexes enter the cell by diffusion along a concentration gradient[16], there is evidence that the transport of cisplatin is carrier mediated. *Byfield* and *Calabro-Jones*[17,18] demonstrated that amino acids protect cells against cisplatin, suggesting that the latter is also taken up by an amino acid transport mechanism.

The resistance to cisplatin could stem from somatic mutations that yield cells whose membrane carriers are either fewer in number or have reduced drug affinity[17]. These alterations lead to a minor trans-membrane transport. Hints at this mechanism of resistance are given by uptake experiments with a L 1210 leukemia cell line which show a 6.6 fold resistance to cisplatin. They demonstrated a decreased accumulation of cisplatin in the cells[19]. The observation that a L 1210 leukemia cell line primarily resistant to melphalan is cross-resistant to cisplatin supports also this mode of action, since the melphalan resistance was accompanied by a decrease in the melphalan concentration within the cells[20].

Because of the suggested differences between cisplatin and the diaquaplatinum(II)complexes **27** and **31** concerning the mechanism of their trans-membrane transport, an effect of these new complexes against cisplatin resistant tumors is conceivable. An indication of such a therapeutic applicability is the finding that of mice bearing cisplatin-resistant *Ehrlich* ascites tumors about 70 % are cured by [R,R/S,S-1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum (II) (3 x 5 mg/kg ip), a substance which is structurally related to **27** and **31** (Seeber, unpublished). Compounds **27** and **31** might be "carrier-independent" platinum complexes, which penetrate the tumor cell membrane by simple diffusion. "Carrier-independent" drugs are of therapeutic interest since: 1. they show a reduced tendency to induce resistance[21] and a high antitumor activity in vivo[22] and 2. they are known to lack cross-resistance to "carrier-dependent" platinum(II) complexes like cisplatin[17,23], Treatment schedules in which such "carrier-independent" platinum(II) complexes are combined with cisplatin or another "carrier-dependent" platinum(II) complex are predicted to be most useful, as they would minimize the outgrowth of drug-resistant tumors and, therefore, should elongate the remission period.

In order to verify the lack of cross resistance to cisplatin the R,R/S,S configurated [1,2-bis(fluorophenyl)ethylenediamine]-platinum(II) complexes **13** to **15** were evaluated on the cisplatin-sensitive and -resistant Ehrlich ascites tumor of the mouse. Compounds **13** and **14** were inactive on the cisplatin-sensitive Ehrlich ascites tumor at a dose of 3 x 10 mg/kg and 3 x 20 mg/kg (Figures 3 and 4). Complex **15**, however, cured 80 % of the animals at a dose of 3 x 10 mg/kg (Figure 3). After the elevated dosage of 3 x 20 mg/kg **15** all animals were disease-free (Figure 4). On the cisplatin-resistant Ehrlich Ascites tumor however, **15** was inactive even at an extremely high dosage (Figure 5).

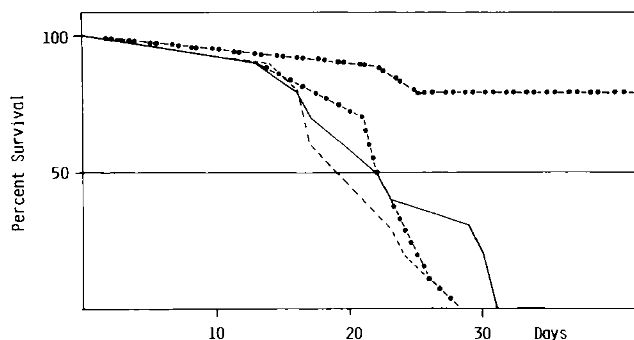


Figure 3: Effect of **13** (2-F; R,R/S,S), **14** (3-F; R,R/S,S) and **15** (4-F; R,R/S,S) on Cisplatin-sensible Ehrlich Ascites Tumor of the Mouse; 3 x 10 mg/kg: **13** ----; **14** -•-•-; **15** -••-••-••-; Control ————

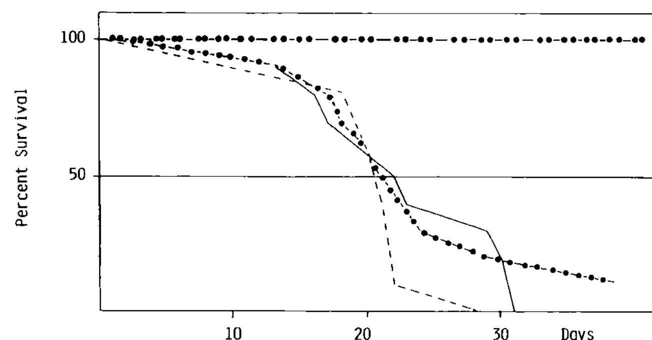


Figure 4: Effect of **13** (2-F; R,R/S,S), **14** (3-F; R,R/S,S) and **15** (4-F, R,R/S,S) on Cisplatin-sensible *Ehrlich* Ascites Tumor of the Mouse; 3 x 20 mg/kg: **13** ----; **14** -•-•-; **15** -••-••-; Control ————

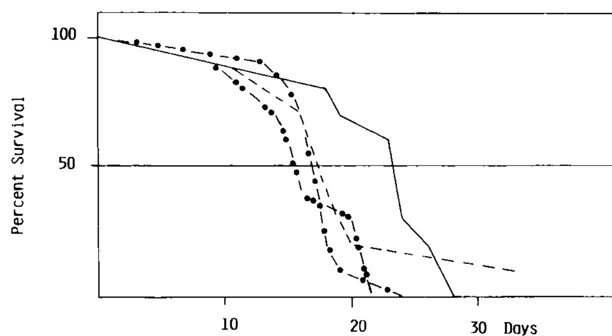


Figure 5: Effect of **15** (4-F; R,R/S,S) on Cisplatin-resistant *Ehrlich* Ascites Tumor of the Mouse; 3 x 10 mg/kg ----; 3 x 20 mg/kg -•-•-•-; 3 x 50 mg/kg -••-••-••-; Control ————

In figure 6 a comparative test with cisplatin on this resistant tumor model is shown.

Compound **15** was also tested on various L 1210 leukemia cell lines which possess resistance against platinum

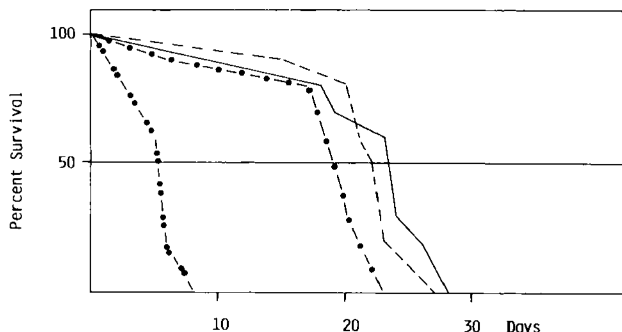


Figure 6: Effect of Cisplatin on a resistant *Ehrlich* Ascites Tumor of the Mouse; 6 mg/kg ----; 8 mg/kg -•-•-•-; 12 mg/kg -••-••-••-; Control ——

complexes. These cell lines have been developed by *Eastman* et. al.[28-30]. Against the wild type of the L 1210 cell line, **15** showed an ED_{50} value (0.2 µ M) which is comparable to that of cisplatin ED_{50} (µ M) = 0.3). In contrast to the results on the cisplatin-resistant *Ehrlich* ascites tumor (Figure 5), **15** caused a marked effect on a L 1210 cell line, which is 100 fold resistant to cisplatin. The determined ED_{50} value of 1,4 (µM) corresponds only to a 7 fold resistance. However, a surprisingly high resistance against **15** (ED_{50} (µM = 37, corresponding to a 185 fold resistance) was found in a DAC-resistant L 1210 cell line. This line is about 40 fold resistant to DAC-Pt(II) complexes. This suggests that **15** is closer related to DAC-Pt(II) complexes than to cisplatin regarding its mode of action.

The resistance of the above described new L 1210 cell lines against platinum(II) complexes has in part been attributed to an enhanced repair of DNA-Pt-lesions[31,32]. However, reduced drug accumulation also seems to play a role. In this connection it is also worthy of note that the DAC resistant L 1210 cells exhibit a pronounced reduction in accumulation of (1,2-diaminocyclohexane)platinum(II) complexes[28]. This feature may be an important factor in the low sensibility of the DAC resistant L 1210 cell line against **15**.

These and other results[27] in the class of (1,2-diphenylethylenediamine)platinum(II) complexes show that the activity against cisplatin-resistant tumors depends on the nature and position of the ring substituents and also of the ligand configuration.

In further publications we well inform on the development of new platinum complexes for the second line therapy of tumor diseases after development of resistance against cisplatin.

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Experimental Part

P 388 D_1 Leukemia cell culture experiments

Standard conditions. The P 388 D_1 cells (ATCC CCL 46) were grown in static suspension culture in RPMI 1640 medium (Gibco) supplement with 10 % heat-inactivated horse serum (Boehringer), 10 mM HEPES buffer (Biochrom), 2 mM glutamine (Biochrom) and $NaHCO_3$ (0.85 g/l) in a humidified atmosphere of 5 % CO_2 in air at 37°C. Stock cultures were grown in 75 cm^2 culture flasks (Falcon). The diaquaplatinum(II) complexes were dissolved in bidistilled water or methanol, the dichloroplatinum(II) complexes in DMF. The test compounds were added as 500- or 1000-fold concentrated stock solutions.

Determination of ED_{50}

2 ml of cell suspension (7-8·10^4 cells/ml) were placed in glass centrifuge tubes sealed with aluminium caps and incubated under standard conditions. After 4 h incubation cells were counted with a Coulter Counter (Coulter Electronics Ldt) and the test compounds were added. 2 h prior to the end of the experiment the cells were labeled with 0.3 µCi ^{3}H-thymidine (40-60 Ci/mmol, New England Nuclear) per tube. After 48 h 0.5 ml of culture were used to determine cell numbers, the remaining portion was centrifuged and washed twice with ice-cold PBS (Phosphate buffered saline: NaCl (8 g), KCl (0.2 g), $Na_2HPO_4 \cdot H_2O$ (0.15 g) and KH_2PO_4 (0.2 g) in 1 L of H_2O). The pellet was resuspended in 1 ml water and the cells were broken up by sonication using a Branson sonifier. To the sonicated cells 4 ml 10 % TCA was added and the precipitate was filtered over 0.45 µm filter (Sartorius). The ^{3}H-thymidine incorporation was determined in 10 ml Quickszint 212 (Zinsser Analytik) in a Beckman LS 1801 liquid scintillation counter. % T/C of cell growth was calculated according to (T-Z)·100/(C-Z) (T: cell number of treated cell culture at the end of incubation, C: cell number of untreated cell culture at the end of incubation, Z: cell number at the beginning of incubation).

Time-dependent of inhibition of ^{3}H-thymidine incorporation

Cell suspension (2 ml) were placed in glass centrifuge tubes sealed with aluminium caps. For incubation times up to 6 h the initial cell concentration was 2.4-2.6 · 10^5 cells/ml. When cell cultures were incubated up to 48 h the initial cell number per ml was 1.1-1.4 · 10^5. The test compounds were added in a final concentration of 1·10^{-5} M and 20 min prior to the end of the experiment the cells were labeled with 1 µCi ^{3}H-thymidine per tube. The ^{3}H-thymidine incorporation was stopped rapidly by shaking the tubes in ice water. The ^{3}H-thymidine was determined as described above. For % T/C calculation control cultures were treated identically.

Long term effect on the ^{3}H-thymidine incorporation after short drug incubation times

Cells were incubated with test compounds for varying periods of time and spun down at 500 g. The pellet was washed with medium and the cells were resuspended and reincubated in drug-free culture medium. The ^{3}H-thymidine labeling procedure was performed as described above.

Count of viable cells[24]

The number of viable cells was determined by means of trypan blue exclusion staining. Technique: trypan blue stock solution (Sigma) was diluted with PBS to an end concentration of 0.16 %. Equal volumes of diluted stain and cell suspension were mixed. The number of cells not being stained (viable cells) was determined microscopically by means of a Neubauer hematocytometer.

Tumor colony forming assay (TCA)[25]

Drug treatment for 1 h. The P 388 D_1 leukemia cells (3 x 10^4 viable cells fo a culture in asynchronous exponential growth) were incubated in 3 ml RPMI 1640 (Biochrom) supplemented with $NaHCO_3$ (0.85 g/l; Merck), HEPES (10 mM, Biochrom), 10 % heat inactivated horse serum (Biochrom) and glutamine (2 mM, Biochrom) for 1 h with several drug concentrations (addition as stock solution in H_2O or DMF). Previous experiments had shown that the DMF itself in final concentration of 0.1 % does not inhibit the growth of the tumor cells. Than the cells were spepatated by centrifugation, washed out and suspended in 3 ml upperlayer.

Drug treatment for 48 h. The volume which contains 3 x 10^4 cells was determined in the control. This volume was used as inoculum as well for the control as for the test. Further conditions c. f. 1 h experiments.

Soft agar cloning method

With slight modifications, the clonogenic assay was performed according to *Hamburger* and *Salomon*[25]. Briefly, the tumor cells were suspended in upperlayer consisting of 0.3 % agar in enriched CMRL 1066 (Gibco) supplemented with 15 % heat inactivated fetal calf serum (FCS; Boehringer), penicillin (final concentration 100 U/ml), streptomycin (100 µg/ml), glutamine (2 mM), $CaCl_2$ (4 mM), ascorbic acid (0.3 mM), bovine insulin (2 U/ml), asparagine (0.5 mM) and mercaptoethanol (50 µM; the latter substances all obtained from Sigma). 1 ml of the resultant mixture was pipetted onto 1 ml underlayer in plastic petri dishes (Falcon plastics); this was done in triplicate for each of at least 3 different drug concentrations. The underlayer consisted of 0.5 % agar in enriched *McCoy's* 5A medium (Gibco) supplemented with 15 % FCS, Napyruvate (final concentration 2 mM); L-serine (0.4 mM), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), tryptic soy broth [0.5 % (w/v)] and asparagine (0.75 mM, the latter substances all obtained from Sigma). The plates were incubated at 37°C in an atmosphere of 5 % CO_2 and 100 % humidity, after microscopical control had confirmed that there were no artificial cell-aggregates. The number of tumor cell colonies (aggregates of more than 32 cells) was counted 10-14 days after plating by means of an inverted microscope. Drug effects were expressed as the percentage of inhibition of colony formation compared to the untreated dishes.

Ehrlich Ascites Tumor[26]

The different resistant *Ehrlich* ascites tumor lines (wild type and Cisplatin-resistant) are maintained by routine passage in female NMRI mice. For the evaluation of the therapeutic activity groups of ten female mice were inoculated in with 10^6 cells. On the following 3 days the animals received ip injections of either platinum complex suspended in polyethylene glycol 400/H_2O 1:1 or of polyethylene glycol 400/H_2O 1:1 alone for control.

L 1210 Leukemia cell culture experiments.

Sensitive and resistant L 1210 cells have been developed and described by *Eastman* et al.[28,29].

Suspension cultures are grown in *McCoy's* 5a (modified) medium supplemented with $NaHCO_3$ (2.2 g/l), penicillin (250 U/ml), streptomycin (250 U/ml), fungizone (lµg/ml) and 16 % calf serum. For growth inhibition 4 ml of cell suspension (approximately 5 x 10^4 cells/ml) were incubated in triplicate with varying concentrations of drug over a 3-day period. The drug was added as a 1000 fold stock solution in DMF or DMSO. Stock solutions especially DMSO solutions were kept no longer than 5-10 min. After 3 days l ml aliquots were counted on a *Coulter* counter and ED_{50} values were determined.

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