Dichloro-[1-(hydroxyphenyl)-2-phenylethylenediamine]platinum(II) Complexes: Testing on the Human Ovarian Cancer Cell Lines NIH: OVCAR 3 and SK OV 3*)

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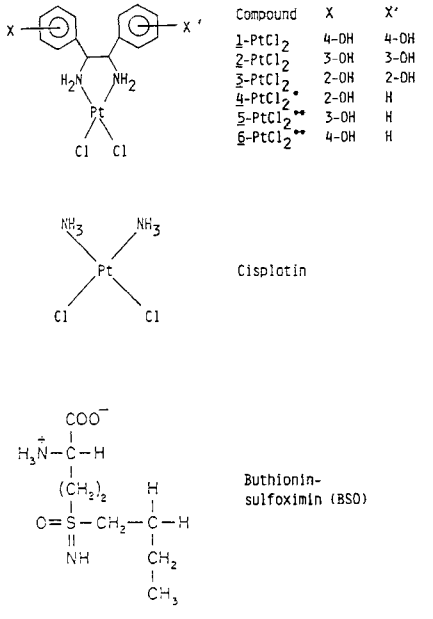
The diastereoisomeric dichloro-[1-(2-, 3- and 4-hydroxyphenyl)-2-phenylethylenediamine]platinum(II) complexes were tested on two human ovarian cancer cell lines NIH: OVCAR-3 and SK-OV-3, both resistant against cisplatin. Dichloro-[*threo*-1-(3-hydroxyphenyl)-2-phenylethylenediamine]platinum(II) (*threo*-**5**-$PtCl_2$) proved to be the most active representative of the new series, producing cytocidal effects at a concentration range of 2.5 to 5.0 µM on the NIH: OVCAR-3 cell line. On the more resistant SK-OV-3 cell line, *threo*-**5**-$PtCl_2$ was only moderately active, while in combination with BSO, a GSH level lowering compound, *threo*-**5**-$PtCl_2$ showed a strong synergistic effect.

Dichloro-[1-(hydroxyphenyl)-2-phenylethylendiamin]platin(II)-Komplexe. Prüfung an den menschlichen Ovarialcarcinom-Zellinien NIH: OVCAR-3 und SK-OV-3

Die diastereomeren Dichloro-[1-(2-, 3- und 4-hydroxyphenyl)-2-phenylethylendiamin]platin(II)-Komplexe wurden an zwei cisplatinresistenten, menschlichen Ovarialcarcinom-Zellinien, NIH: OVCAR-3 und SK-OV-3, getestet. Dichloro-[*threo*-1-(3-hydroxyphenyl)-2-phenylethylendiamin]platin(II) (*threo*-**5**-$PtCl_2$) erwies sich als der aktivste Vertreter der Reihe. *Threo*-**5**-$PtCl_2$ ruft im Konzentrationsbereich 2.5 - 5.0 µM an der NIH: OVCAR-3 Zellinie cytocide Effekte hervor. An der resistenten SK-OV-3 Zellinie war *threo*-**5**-$PtCl_2$ nur mäßig wirksam; es zeigte aber in Kombination mit BSO, einer GSH-Spiegel emiedrigenden Substanz, stark synergistische Effekte.



* Available as threo form

** Available as threo and erythro form

Scheme 1

In recent publications[1-4] we have shown that stereoisomeric dichloro-(1,2-diphenylethylenediamine)platinum(II) complexes with hydroxy groups in the 4-, 3- or 2-positions of both benzene rings (Scheme 1, compounds **1**-$PtCl_2$ to **3**-$PtCl_2$) are of interest as antitumor agents. D,L-**2**-$PtCl_2$ and especially D,L-**3**-$PtCl_2$ led to a marked inhibition of the cisplatin-resistent *Ehrlich* ascites tumor of the mouse. In this experiment 70% of the tumor-bearing animals were cured by complex D,L-**3**-$PtCl_2$ but only 20% by complex *meso*-**3**-$PtCl_2$[3].

This result prompted us to a thorough study of the stereoisomeric [1,2-bis(2-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) complexes (*meso*-, D,L-, (+)-, and (-)-**3**-$PtCl_2$) on the NIH : OVCAR-3 ovarian cancer cell line, which exhibits a multidrug resistance phenotype (compare ref.[5] and footnote 1). The NIH : OVCAR 3 cell line was established from a patient with ovarian cancer who was treated unsuccessfully with cisplatin, adriamycin and cyclophosphamide[5]. Among the studied stereoisomeric **3**-$PtCl_2$ complexes the *(S),(S)*-configurated, levorotatory compound proved to be the most active one. Since non-toxic concentrations of this compound produces cytocidal effects on the NIH: OVCAR 3 cell line, it is of interest for further evaluation for the therapy of ovarian cancer.

This publication will report on the testing of the structurally related diastereomeric dichloro-[1-(hydroxyphenyl)-2-

1. Cisplatin based combination chemotherapy regimens produce complete remissions in 60-80% of patients with advanced stage ovarian cancer. However, their effectiveness is limited by the development of acquired resistance[6]. Therefore, the development of new platinum complexes, which overcome the cisplatin resistance, is of great interest: (a) in combination with cisplatin for first-line treatment of ovarian cancer to avoid or retard the development to resistance, (b) for the second-line therapy of ovarian cancer after development of resistance against cisplatin.

* Dedicated to Professor Dr. Dr. *E. Mutschler* on the occasion of his 60th birthday.

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phenylethylenediamine]platinum(II) complexes on the human epithelial ovarian cancer cell lines NIH: OVCAR-3 and SK-OV-3.

Derivatives which are OH-substituted in 2-, 3-, and 4-position (**4**-$PtCl_2$, **5**-$PtCl_2$, and **6**-$PtCl_2$; Scheme 1) were used in this study. With the exception of *erythro*-**4**-$PtCl_2$, the synthesis which was not successful (cf. ref.[7]), all compounds were available as *threo*- and *erythro*-forms.

For characterization of both cell lines a cytogenetic analysis as well as an investigation of growth kinetics and of the sensitivity against the therapeutically most used drug cisplatin were performed.

The cytogenetic analysis demonstrated that the NIH: OVCAR-3 cell line included cells with a hypotriploid to a hypohexaploid karyotype and the SK-OV-3 cells with a hypodiploid to a hypotetraploid karyotype. The modal chromosome numbers amount to 63 for NIH: OVCAR-3 (Fig. 1) and to 43 and 83 for SK-OV-3 (Fig. 2). The karyograms of both cell lines show numerous chromosome anomalies. Their morphological and cytogenetic characteristic proved to be constant in the course of the study.

Fig. 3A and B illustrate examplarily the growth curves and the corresponding doubling times at any time of incubation for the NIH: OVCAR-3 cell line in the passages 30 and 32 (see footnote 2)). Under the given experimental conditions the logarithmic phase covers only a small fraction of the overall growth curve. In a plot 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 grows exponentially for about two generations. The same is true for the SK-OV-3 cell line in the passages 38 to 40, which were used for the evaluation of

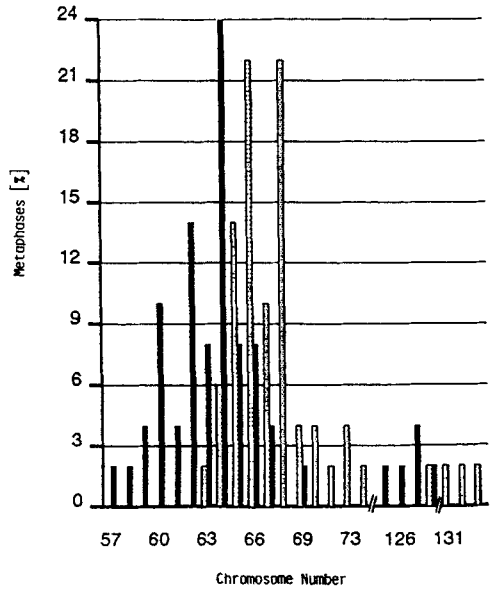

Fig. 1: Chromosome distribution of NIH-OVCAR 3 cells. (□) 29th passage from origin, (■) 51th passage from origin: The cells were passaged weekly. For each passage the chromosomes from 50 well-spread metaphase plates were counted.

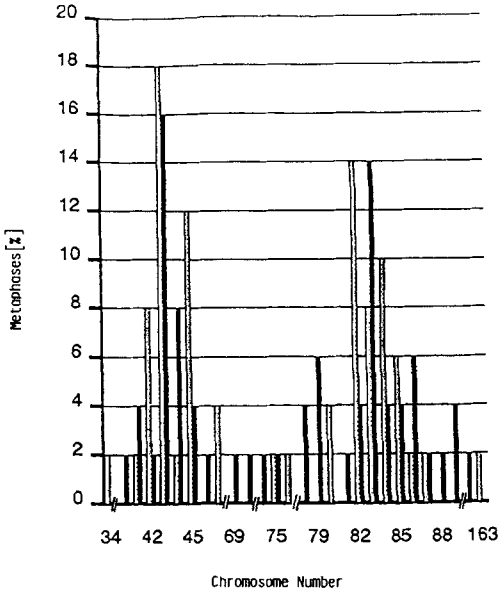

Fig. 2: Chromosome distribution of SK-OV-3 cells, (□) 32th passage from origin, (■) 42th passage from origin: The cells were passaged weekly. For each passage the chromosomes from 50 well-spread metaphase plates were counted.

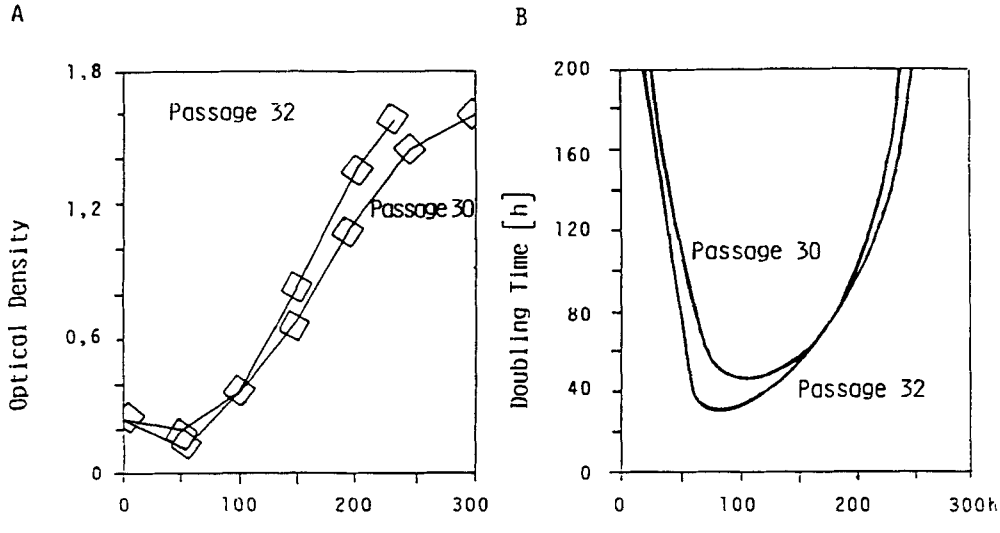

Fig. 3: Growth curves and corresponding doubling times of the NIH: OVCAR-3 cell line in dependence on the passage number. Inoculum: 100 µl/well at a density of 19, 25 cells/microscopic field for passages 30, 32.

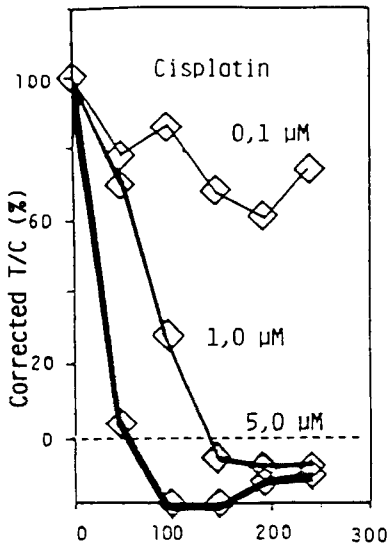

Fig. 4A: Effect of cisplatin on the proliferation of NIH: OVCAR-3 ovarian cancer cells. Plot of corrected T/C values *versus* time of drug exposure. Inoculum: 100 µl/well at a density of 19 cells (30th passage) / microscopic field; preincubation 73.5 h, drug incubation 256 h.

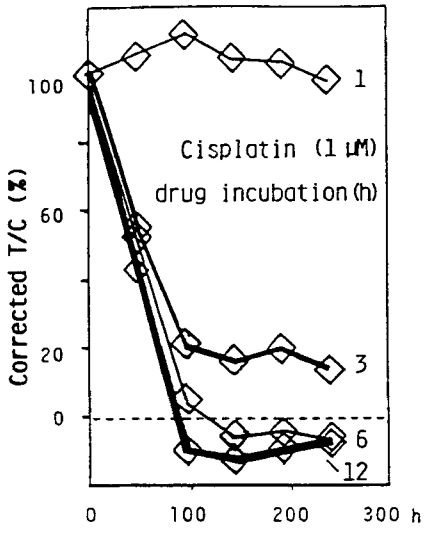

Fig. 4B: Variation of time of drug exposure: Effect of cisplatin (A: 1 µM) on the proliferation of NIH: OVCAR-3 ovarian cancer cells. Plot of corrected T/C values versus time; Inoculum: 100 µl/well at a density of 17 cells (37th passage)/microscopic field; preincubation 61 h; drug incubation 1, 3, 6, and 12 h; final incubation without drug; duration of the experiment 244 h.

the antitumor activity of the new platinum complexes. A doubling time of about 35 h was estimated for the NIH: OVCAR-3 line and one of about 28 h for the SK-OV-3 line.

In order to get informations on the sensitivity of the NIH: OVCAR-3 cell line against cisplatin, the most active drug in the therapy of ovarian cancer, the cells were incubated with this compound at therapeutically relevant concentrations for 256 h. Only at the highest concentrations (1 and 5 mM) a cytocidal effect, which we consider essential for a curative effect *in vivo*, could be detected (Fig. 4A). However, a relatively long period of contact between cells and cisplatin (6 h) is required to achieve cell kill (compare Fig. 4B). Due to the marked toxic side effects and the pharmacokinetics of cisplatin it is difficult to meet this demand. The SK-OV-3 ovarian cancer cell line proved to be less sensitive against cisplatin than the NIH: OVCAR-3 line (Fig. 5). In a concentration of 2.5 µM, which produced cytocidal effects on the NIH: OVCAR-3 cell line, cisplatin showed merely cytostatic activity on the SK-OV-3 cell line. After an incubation period of about 140 h development of resistance of the SK-OV-3 cells occurred.

In long term experiments (256 h drug incubation) the diastereoisomeric dichloro-[1-(hydroxyphenyl)-2-phenylethylenediamine]platinum(II) complexes showed a strong concentration-dependent activity on the cisplatin-resistant NIH: OVCAR-3 cell line (Fig. 6-10).

In the *threo* series the 2- and 3-hydroxy-substituted complexes (*threo*-**4**-$PtCl_2$ and *threo*-**5**-$PtCl_2$) produced cytocidal effects at a concentration of 5 µM (Fig. 6 and 7). The 4-hydroxy-substituted complex (*threo*-**6**-$PtCl_2$) was merely cytostatically active at this concentration (5 µM, Fig. 9). Comparison of the activity of *threo*-**4**-$PtCl_2$ and *threo*-**5**-$PtCl_2$ at lower concentrations reveals the superiority of *threo*-**5**-$PtCl_2$.

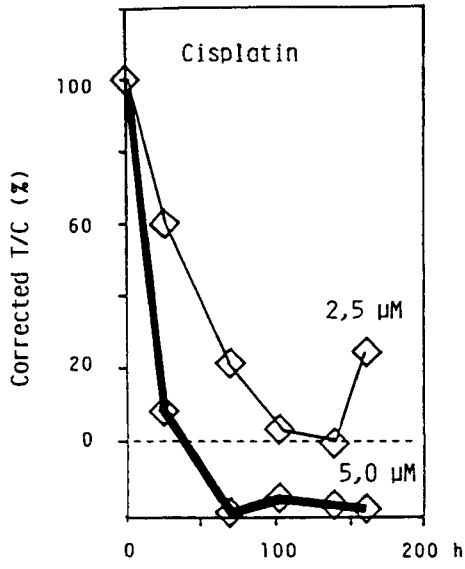

Fig. 5: Effect of cisplatin on the proliferation of SK-OV-3 ovarian cancer cells. Plot of corrected T/C values *versus* time of drug exposure. Inoculum: 100 µl/well at a density of 7 cells (38th passage) / microscopic field; preincubation 77 h, drug incubation 162 h.

Erythro-**5**-$PtCl_2$, which also brought about cytocidal effects at the highest concentration (5 µM), was less active than its diastereoisomer *threo*-**5**-$PtCl_2$ (compare Fig. 7 and 8). The antitumor activity of *erythro*-**5**-$PtCl_2$ was comparable with that of *threo*-**4**-$PtCl_2$. In contrast to the diastereoisomeric pair **5**-$PtCl_2$ (*threo*-**5**-$PtCl_2$ is more active than *erythro*-**5**-$PtCl_2$) the *erythro*-**6**-$PtCl_2$ proved to be somewhat more active than its counterpart *threo*-**6**-$PtCl_2$ (compare Fig. 9 and 10). The comparison of the test results of *threo*-**5**-$PtCl_2$, the most active representative of the new series, with those of (-)-**3**-

2. Comparable growth kinetics were also observed for the higher passage numbers of the NIH: OVCAR-3 cell line which were used in the test series.

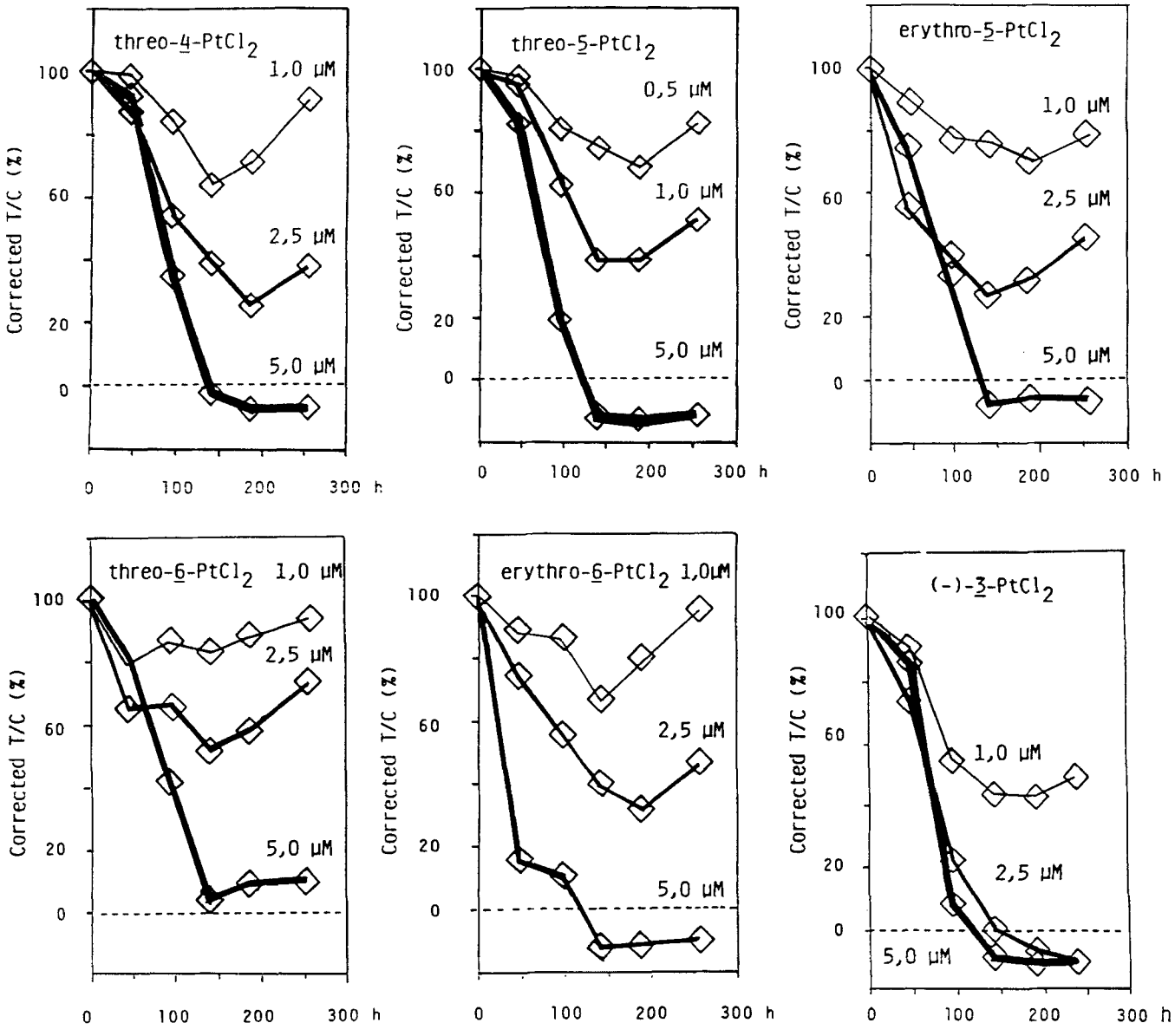

Fig. 6-11: Effect of *threo*-4-$PtCl_2$, *threo*-5-$PtCl_2$, *erythro*-5-$PtCl_2$, *threo*-6-$PtCl_2$, *erythro*-6-$PtCl_2$, and (-)-3-$PtCl_2$ on the proliferation of NIH: OVCAR-3 ovarian cancer cells. Plot of corrected T/C values *versus* time of drug exposure. Inoculum: 100 µl/well at a density of 19 cells (30th passage) / microscopic field; preincubation 73.5 h; drug incubation 265 h.

$PtCl_2$, which we used as positive control, showed a higher potency of the latter (compare Fig. 7 and 11).

To find out the time which is necessary to achieve a cytocidal effect of *threo*-5-$PtCl_2$, the most active representative of the new platinum complexes, the NIH: OVCAR-3 cells were preincubated with this drug at concentrations of 2.5 and 5.0 µM for 1, 3, 6, and 12 h, respectively, and the growth curves were registered after removal of the drug by medium exchange (duration of the experiment 244 h). In a concentration of 5.0 µM *threo*-5-$PtCl_2$ produced cytocidal effects after a drug-cell-contact of at least 3 h (Fig. 12A). However, at a concentration of 2.5 µM only a drug incubation time of 12 h led to cytocidal effects (Fig. 12B).

Comparative experiments with (-)-3-$PtCl_2$ (which was used as control) revealed that at a drug concentration of 2.5 µM incubation times of ≥ 3 h were necessary to bring about cytocidal effects (Fig. 13). From this experiment, too, the superiority of (-)-3-$PtCl_2$ compared with *threo*-5-$PtCl_2$ becomes evident.

On the SK-OV-3 ovarian cancer cell line, which showed a marked resistance against cisplatin, only the most active representative of the new series, *threo*-5-$PtCl_2$ was testet. Even at the highest concentration (5 µM) only a weak cytostatic effect was obvious (Fig. 14). The comparison compound (-)-3-$PtCl_2$ displayed a similar low activity, which was even limited in time. After a drug incubation of 150 h, cells grow unlimited (Fig. 15).

In the development of the resistance of ovarian cancer against cisplatin the ability of malignant cells to elevate its content of glutathione (GSH) is supposed to be of great importance[5]. Glutathione inactivates drugs like cis-

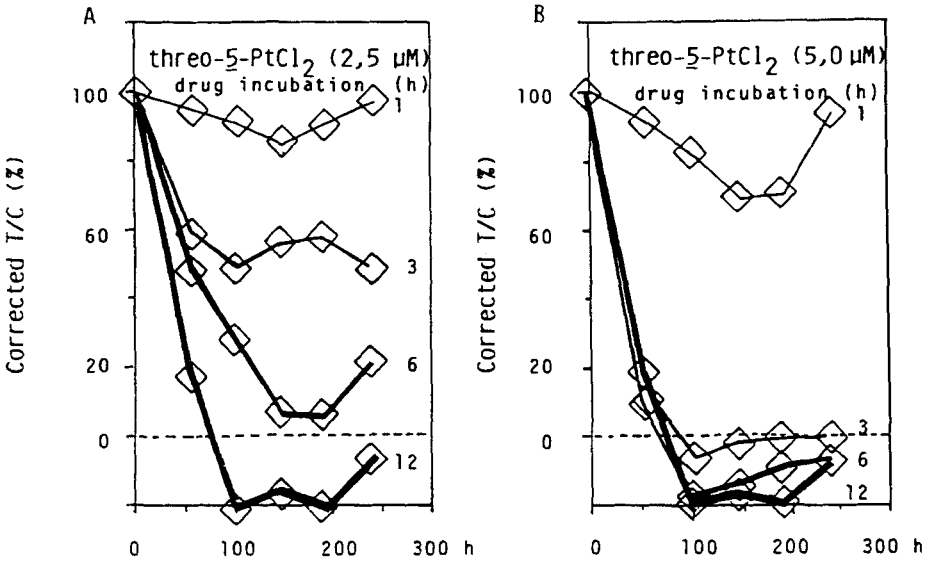

Fig. 12: Variation of the time of drug exposure: Effect of *threo*-5-$PtCl_2$ (A: 2.5 µM; B: 5.0 µM) on the proliferation of NIH: OVCAR-3 ovarian cancer cells. Plot of corrected T/C values *versus* time. Inoculum: 100 µl/well at a density of 17 cells (37th passage) / microscopic field, preincubation 61 h; drug incubation 1, 3, 6, and 12 h; final incubation without drug; duration of the experiment 244 h.

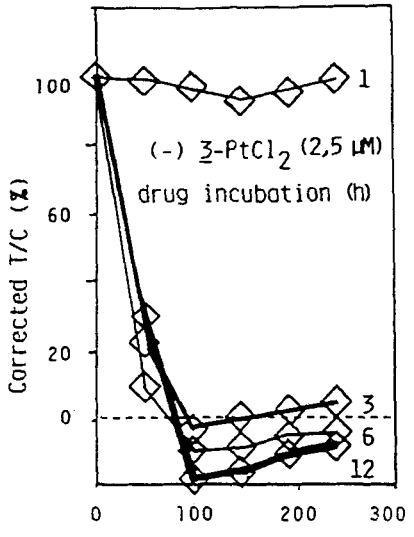

Fig. 13: Variation of the time of drug exposure: Effect of (-)-3-$PtCl_2$ (2.5 µM) on the proliferation of NIH: OVCAR-3 ovarian cancer cells. Plot of corrected T/C values *versus* time; Inoculum: 100 µl/well at a density of 17 cells (37th passage)/microscopic field; preincubation 61 h; drug incubation 1, 3, 6, and 12 h; final incubation without drug; duration of the experiment 244 h.

platin, which produce their effect by reaction with nucleophilic targets of the tumor cell (*e.g.* DNA). Therefore, drugs causing a GSH reduction in the cells should lead to a potentiation of the effect of platinum complexes. In this context buthionine sulfoximine (BSO), an inhibitor of the γ-glutamylcsteine synthetase, the key enzyme of GSH biosynthesis, has met great interest. At a concentration range of 15 to 25 µM it produces a reduction of the GSH level of about 85% in NIH: OVCAR-3 cells and, as a consequence, markedly elevates the inhibitory effect of cisplatin on this cell line[5].

Due to these findings the combined administration of BSO and platinum complexes is being thoroughly studied. Our experiments on the SK-OV-3 cell line revealed a strong effect of *threo*-5-$PtCl_2$ in a concentration of 2.5 µM after preincubation with BSO (50 µM, 48 h; Fig. 17).

This is a remarkable finding, since both substances, *threo*-5-$PtCl_2$ (2.5 µM) and BSO (50 µM) are ineffective when applied singly (compare Fig. 14 and 16). (-)-3-$PtCl_2$ was markedly less active under the same conditions (Fig. 18). It causes only weak cytostatic effects.

Also with cisplatin, the antitumor effect was only slightly increased by BSO. At the therapeutically relevant cisplatin concentration (0.5 µM) a combination with BSO (50 µM) gave no cytocidal effects.

Only at therapeutically non-relevant cisplatin concentrations (2.5 µM), which are themselves markedly cytostatic, the additional treatment with BSO ends up in a vast cell kill (Fig. 19). The strongly synergistic effect of the *threo*-5-$PtCl_2$/BSO combination could be of great interest in the therapy of the cisplatin resistent ovarian cancer.

Further investigations using *in vitro* and *in vivo* ovarian cancer models are planned.

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Biological methods

NIH : OVCAR-3 ovarian cancer cell line. The NIH: OVCAR-3 (ATCC no. HTB 161), a human adenocarcinoma of the ovary[8], was obtained from the American Type Culture Collection in passage 17. Cell-line-banking and quality control were performed according to the "see stock concept" reviewed by *Hay*[9]. The cells were maintained in RPMI-1640 (Sigma) containing $NaHCO_3$ (2 g/l), gentamicin (50 mg/l), 10% BMS (Seromed) and insulin (10 µg/ml) (Sigma) in 75-cm^2 flasks at 37°C in a H_2O-saturated atmosphere of 95% air and 5% CO_2. The cells were serially passaged weekly following trypsinization using trypsin/EDTA (Boehringer). For chemosensitivity testing the cells (in passage 24, 30, 32, 37, and 49, respectively, were plated in 96-well microplates (100 µl/well at a density of about 17-28 cells/microscopic field (Leitz Diavert, 320x)) and were allowed to attach. After 48-80 h, the medium was removed by suction and replaced with

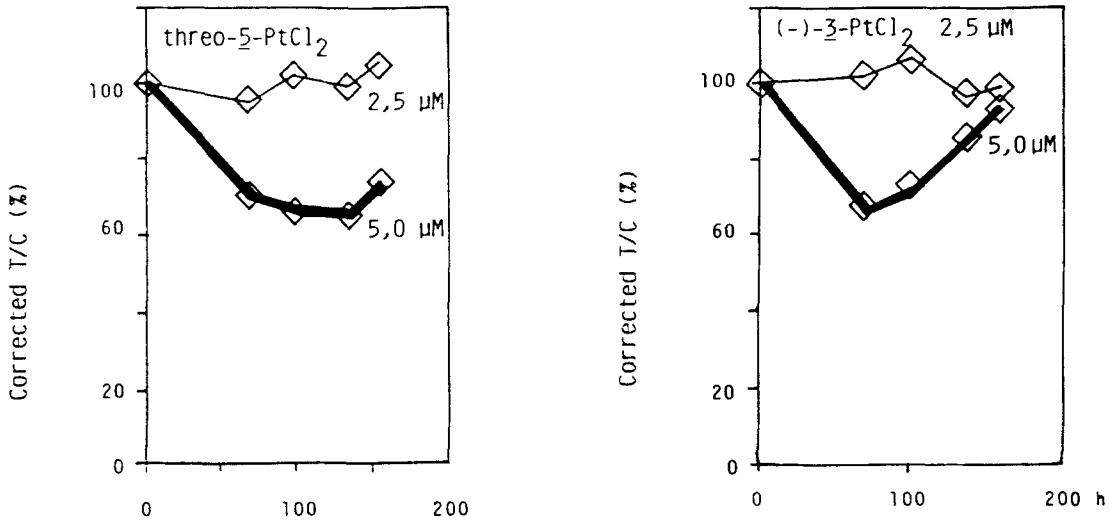

Fig. 14 and 15: Effect of *threo*-5-$PtCl_2$ and (-)-3-$PtCl_2$ on the proliferation of SK-OV-3 ovarian cancer cells. Plot of corrected T/C values *versus* time of drug exposure. Inoculum: 100 µl/well at a density of 7 cells (38th passage) / microscopic field; preincubation 77 h; drug incubation 162 h.

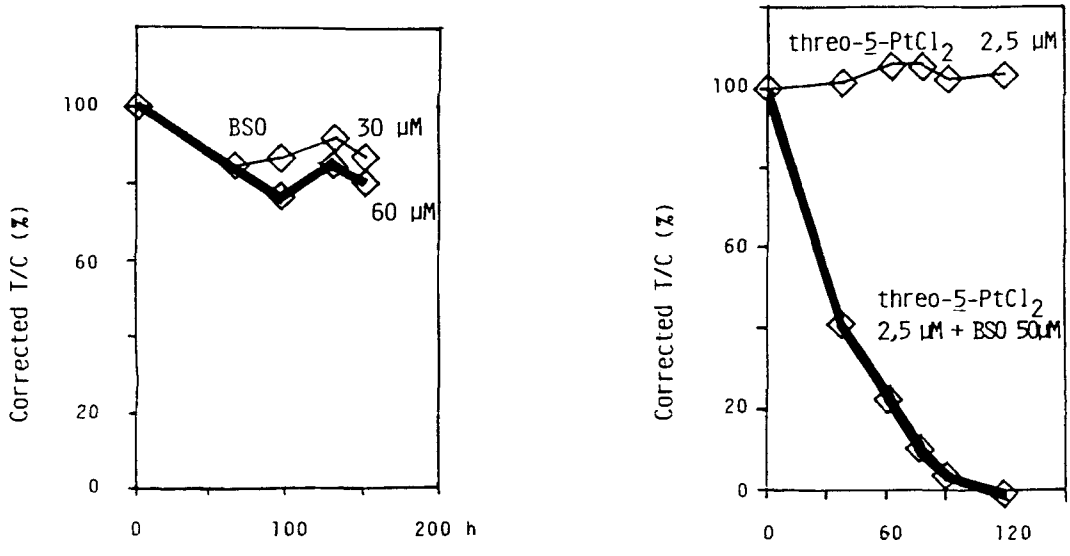

Fig. 16: Effect of BSO (30 and 60 µM) on the proliferation of SK-OV-3 ovarian cancer cells. Plot of corrected T/C values *versus* time of drug exposure. Inoculum: 100 µl/well at a density of 100 cells (39th passage) / microscopic field; preincubation 44 h, drug incubation 160 h.

Fig. 17: Effect of *threo*-5-$PtCl_2$ (2.5 µM) on the proliferation of SK-OV-3 ovarian cancer cells after preincubation with BSO (50 µM, 48 h). Plot of corrected T/C values *versus* time. Inoculum: 100 µl/well at a density of 10 cells (39th passage) / microscopic field; preincubation without drugs 44 h; duration of the experiment 120 h.

fresh medium (200 µl/well) containing drug (drugs were added as a 1000-fold stock solution) or pure solvent. The platinum complexes were dissolved in DMF. On every plate two rows (n = 16) acted 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 glutaraldehyde and stored under PBS 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.[10]. Drug effects were calculated as corrected T/C values according to: $T/C_{corr} = (T - C_0)/(C - C_0)\ 100\ [\%]$, 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. The experimental errors for T/C_{corr} range from approximately ± 20% after short times of incubation (small values for T and C compared to C_0) to ± 5% with prolonged incubation.

SK-OV-3 ovarian cancer cell line. The experiment with the SK-OV-3 cell line (ATCC no. HTB 77)[12] were performed in the same manner as with NIH: OVCAR-3. Contrary to this procedure cells in passage 38 were plated at a density of about 7 cells/microscopic field (Leitz Diavert, 320x) for chemosensitivity testing. After 77 h the medium was replaced by fresh medium containing drug or pure solvent (control).

Cytogenetic analysis. The cells were grown to about 50% confluence on microscopic slides. The slides were prepared

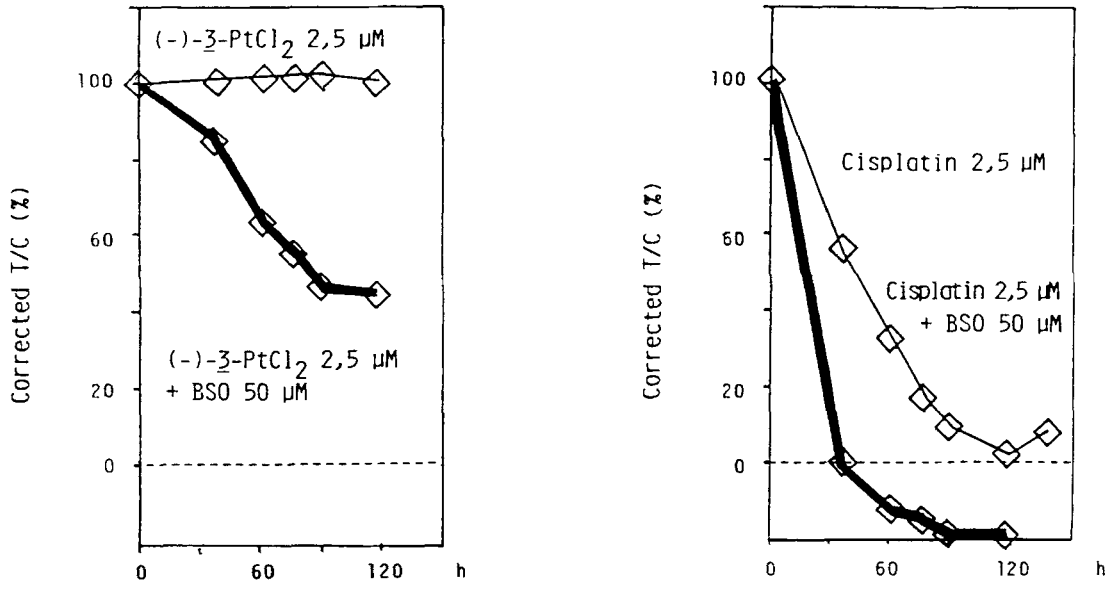

Fig. 18 and 19: Effect of (-)-3-$PtCl_2$ (2.5 µM) and cisplatin (2.5 µM) on the proliferation of SK-OV-3 ovarian cancer cells after preincubation with BSO (50 µM, 48 h). Plot of corrected T/C values *versus* time of drug exposure. Inoculum: 100 µl/well at a density of 10 cells (39th passage) / microscopic field; preincubation without drugs 44 h; duration of the experiment 120 h.

as described[11]. So that spindle formation could be inhibited, the slide chambers were inoculated with colcemid solution (Serva) to a final concentration of 0.04 µg/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 (absol. methanol/glacial acetic acid, 3/1) was added. This hypotonic/fixative mixture was removed immediately and replaced twice by 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 of 0.025 M KH_2PO_4, pH 6.8.

Doubling time analysis. Curve fitting of experimental data of the growth curves is accomplished by a polynomal regression fit applying the least-squares method[10].

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