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New synthetic route to [bis-1,2-(aminomethyl)benzene]dichloroplatinum(II) complexes, screening for cytotoxic activity in cisplatin-sensitive and resistant human cancer cell lines, and reaction with glutathione

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Dedicated to Prof. Dr. Dr. P. Pflegel, Greifswald on the occasion of his 65th birthday

A new synthetic route to [bis-1,2-(aminomethyl)benzene]dichloroplatinum(II) complexes is described. *o*-Xylene and the 4-methoxy substituted derivative were used as starting points for the synthesis: benzylic bromination with *N*-bromosuccinamide/benzoylperoxide followed by the substitution of the benzyl bromides for azide and finally a catalytic hydrogenation with Pd/C of the diazides gave the desired diamines ligands. An attempt to synthesize the 4,6-dimethoxy derivative was unsuccessful due to the bromination of the aromatic ring. The diamines were complexed with K_2PtCl_4 to give the target Pt(II) complexes: [1,2-bis(aminomethyl)benzene]dichloroplatinum(II) (**4a**) and [1,2-bis(aminomethyl)-4-methoxybenzene]dichloroplatinum(II) (**4b**). Screening for cytotoxic activity was done in comparison to cisplatin in a panel of eight human cancer cell lines; in all cases, the 4-methoxy derivative **4b** was less active than the unsubstituted analog, **4a.** In four cell lines **4a** was as potent as cisplatin, while in the other four lines cisplatin was considerably more potent then **4a**. The 5637 bladder cancer cell line was made 4–5 fold resistant to either cisplatin or [*d,l-trans*-1,2-diaminocyclohexane]dichloroplatinum(II); **4a** showed some cross resistance (2–3 fold) to both resistant cell lines. The reactivity of **4a** towards substitutions with glutathione (GSH), a biological thiol involved in intrinsic and acquired resistance to Pt-complexes, was measured by a RP-HPLC method. It was found that the second-order rate constant for the reaction of **4a** with GSH was similar to that that reported for CDDP, indicating that reactivity towards GSH does not explain the different levels of cross resistance.

1. Introduction

Therapy of cancer with platinum complexes (i.e., cisplatin (CDDP), carboplatin and oxaliplatin) is frequently unsuccessful in the long term because of inherent and acquired resistance. For example, the median duration of response of esophageal cancer to regimens containing CDDP is only 7 months [1]. Similarly, while 60% of women with ovarian cancer respond to the initial therapy with platinum complexes, after one and two year remissions the response rates to the same therapy drop to 17 and 50%, respectively [2]. Thus, the development of new platinum antitumor agents with little or no cross resistance to the present generation of Pt-cytostatics is of great interest [3].

In previous work we reported that [bis-1,2-(aminomethyl)benzene]dichloroplatinum(II) has comparable *in vitro* cytotoxic activity to CDDP on two human breast cancer cell lines (i.e., MDA-MB-231 and MCF-7) [4] (see Table 1). These encouraging results prompted us to investigate this class of platinum complexes in more detail. Specifically, we are interested in developing an alternative synthesis of bis-1,2-(aminomethyl)benzenes that would be more amendable to analog development, in particular the synthesis of analogues with various substitution patterns in the aromatic ring would be of great interest for structure-activity-relationship studies. Another goal is to investigate the cytotoxic properties of this class of Pt-complexes on a wider selection of cancer cell lines, including esophageal cancer, and to compared these activities with cisplatin. Additionally, for the purposes of investigating cross-resistance to CDDP as well as the oxaliplatin analog [*d,l-trans*-1,2-diaminocyclohexane]dichloroplatinum(II) (DACH), resistant cell lines from the human bladder cancer cell line 5637 have been developed by progressively increasing the doses of the later two compounds over two months of treatment. The results of these studies are reported herein.

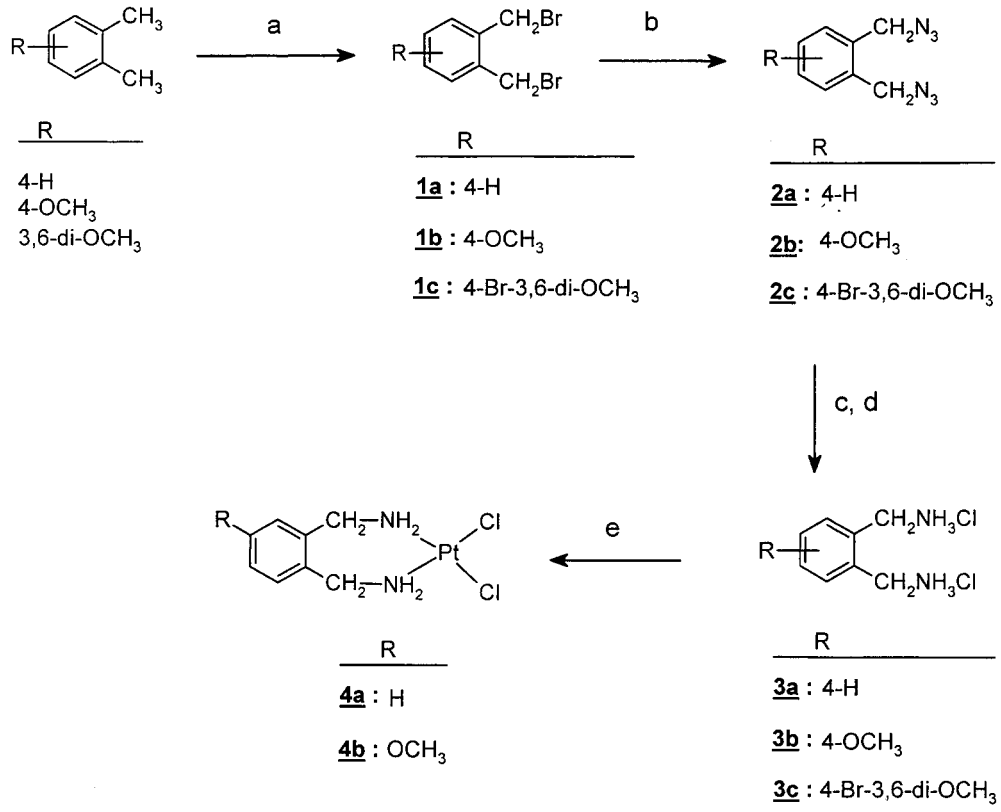
2. Investigations and results

2.1. Synthesis

Bis-1,2-(aminomethyl)benzene (**3**), the diamine ligand used in the synthesis of the title Pt(II)-complex **4**, was previously prepared according to a published method [5], which utilizes the catalytic reduction of phthalazine with H_2/Raney-Ni. Another possibility is to start with *o*-xylenes as shown in Scheme 1; the bromination of both methyl groups followed by substitution by azide and finally a catalytic reduction to the diamines appeared to be a versatile approach that could yield a variety of interesting analoges.

Both the synthesis of bis-1,2-(bromomethyl)benzene (**1a**), beginning from *o*-xylene [6], and that of bis-1,2-(azidomethyl)benzene (**2a**), beginning from either **1a** [7] or bis-1,2-(chloromethyl)benzene [8], have been described; however, the conversion of **2a** to the diamine **3a** has not. The bromination of *o*-xylene with *N*-bromosuccinamide (NBS) in the presence of the radical initiator benzoylperoxid gave the desired product **1a** in acceptable yields. The substitution of both bromides for azides took place with modest yields, resulting in **2a**. The clean reduction of **2a** to **3a** was achieved by hydrogenation in the presence of Pd-C; in contrast, reduction with $LiAlH_4$ in THF gave a complex product mixture. The complexation with K_2PtCl_4 yielded the Pt-complex **4a** in an overall 2.8% yield starting from *o*-xylene; the product showed identical ^{1}H NMR- and IR-spectra and a HPLC-retention time to the compound prepared previously via the phthalazine route [4].

Scheme 1



(a) NBS/benzoylperoxide/CCl_4/reflux; (b) NaN_3/acetone/reflux; (c) H_2/Pd−C; (d) HCl; (e) K_2PtCl_4/H_2O/pH 6–7

An analogous synthesis beginning with 4-methoxy-*o*-xylene yielded the desired 4-methoxy substituted analog **4b**, but only in a 0.75% overall yield (Scheme 1). An attempt to synthesize the 3,6-dimethoxy analog failed at the bromination step, however. Based on ^{1}H NMR and elemental analysis, it was concluded that the bromination of 3,6-dimethoxy-*o*-xylene with NBS/benzoylperoxide gave the tribrominated product **1c**, whereby one of the aromatic hydrogens was replaced by a bromine atom. When the reaction was done at room temperature, only starting material was isolation. Compound **1c** was reacted further with NaN_3 to yield **2c**; the azido functionality was observable by IR ($\nu = 2092\ cm^{-1}$). However, several attempts to produce **3c** by catalytic reduction of **2c** failed to give an isolatable product.

2.2. Biology

To evaluate the *in vitro* cytotoxic potency of **4a** and **4b**, an established microtiter plate assay was used with eight human cancer cell lines: three esophageal squamous cancer (KYSE-70, KYSE-510 and KYSE-520), two lung cancer (A-427 and LCLC-103H), two urinary bladder carcinoma (5637 and RT-112) and a cervix adenocarcinoma (SISO). The assay measures the degree of cell growth inhibition over a 4 d continuous exposure to drug by staining the adherent cells with crystal violet; after washing out the non-bond dye, the cell-bound dye is redissolved in 70%-ethanol/water and the optical density (O.D.) of the wells is measured with a plate reader [9]. The O.D. is proportional to the number of cells in the well. The assay was validated with the A-427 cell line by comparing the IC_{50} values obtained for cisplatin, either by direct cell counting or by the microtiter assay. Fig. 1 shows that the dose response curves obtained by the two methods are comparable; the estimated IC_{50} values are 2.36 μM with cell counting and 4.04 μM with the microtiter assay.

Table 1 contains the IC_{50} values estimated for **4a** and **4b** and compares them with the values obtained for CDDP. Data from the literature [4] are included for comparison. The nonsubstituted compound is more potent than the 4-methoxy analog in all cases where testing was done; differences in potency range from 2 fold in the 5637 cell line to 4 fold in the LCLC-103H and KYSE-70 cell lines. Similar activity to CDDP is seen in the A-427, RT-112, KYSE-520 and KYSE-70 cell lines, while CDDP is more

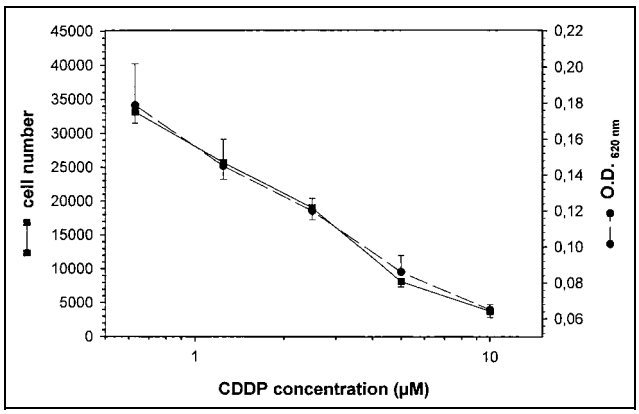


Fig. 1: Dose-response curves for CDDP in the A427 lung cancer cell line, obtained either by direct cell counting (cell number, ■) or by the microtiter method (O.D., ●). Each data point is the average of either 6 (cell number) or 96 (microtiter) determinations. Error bars represent standard devivations

Table 1: IC_{50} values

Cell line	IC_{50} (μM)[a]		
	CDDP	**4a**	**4b**
5637	0.31 ± 0.02[b]	1.06 ± 0.09	5.02
RT-112	2.14 ± 0.64	3.63 ± 1.20	n.d.[c]
KYSE-70	1.49 ± 0.25	1.31 ± 0.13	4.12
KYSE-510	0.88 ± 0.10	5.20 ± 1.72	11.4
KYSE-520	5.07 ± 2.20	3.52 ± 1.96	15.7 ± 4.3
LCLC-104H	1.63 ± 0.50	3.33 ± 1.48	12.6 ± 1.8
A-427	3.30 ± 1.17	4.77 ± 1.58	14.4
SISO	0.20 ± 0.07	1.09 ± 0.19	n.d.
MCF-7	0.74 ± 0.14 [d]	1.3 ± 0.4 [d]	n.d.
MDA-MB-231	1.7 ± 0.7 [d]	1.8 ± 0.2 [d]	n.d.
MEL-24[e]	0.37 ± 0.08 [d]	4.4 ± 0.8 [d]	n.d.

[a]) Values are averages of 2–4 independent determinations; [b]) 1 standard deviation; [c]) not determined; [d]) data from ref. [4]; [e]) human melanoma cell line

potent in the LCLC-103H, KYSE-510, SISO and 5637 lines. Interestingly, a 24 fold difference in potency for CDDP was seen between the most sensitive (SISO) and the least sensitive (KYSE-520) cell lines while for **4a** the difference was only about 5 fold between the least (KYSE-510) and the most sensitive (5637) cells.

Mean graphs have been used to identify differences in patterns of sensitivity of anticancer drugs across panels of cell lines, and they are the basis of the COMPARE program at the NCI, which uses 60 cell lines in the analysis [10]. Positive delta values indicate that the IC_{50} value of a compound in a particular cell line is less than the average IC_{50} value over all cell lines (i.e., is more potent than the average potency), while negative values are obtained when the IC_{50} value is greater than the average IC_{50} (i.e., is less potent than the average potency). Fig. 2A presents the data from Table 1 in the form of a mean graph for CDDP and **4a**. In 7 of the 11 cell lines, CDDP and **4a** gave delta values with the same sign: in 3 cell lines both compounds were more potent than the average potency while in 4 cell lines both compounds were less potent than the average. In the remaining lines (Mel-24, MDA-MB-231, KYSE-510 and KYSE-70), the signs of the delta values are opposed, indicating a different spectrum of activity. A linear regression analysis of the data (Fig. 2B) indicates a weak correlation ($r^2 = 0.25$) between the delta values of CDDP and **4a**.

To explore the level of cross resistance of compound **4a** to CDDP and DACH, the 5637 bladder cancer cell line was made resistant to CDDP and DACH, respectively. This was done by increasing the weekly exposure to Pt-complex over a 2 month period (see experimental). The cells were then allowed to grow in the absence of Pt complex for 1–2 weeks before being cryo-preserved. Fig. 3 shows the level of resistance in the resistant cell lines, 5637-CDDP and 5637-DACH, to CDDP, DACH and **4a** as a function of the time after the preserved cells were re-cultivated. In the early phase, both cell line were 4–5 fold resistant to the platinum complex used to generate resistance. The 5637-CDDP and 5637-DACH cell lines gradually lost some of that resistance with each passage (Fig. 3). In the beginning, both cell lines were only 2–3 fold resistant to **4a**, and this level of resistance changed little over the next 40–50 days of cultivation. This trend can also be seen in the 5637-CDDP cells with respect to DACH, and to a lesser extend in the 5637-DACH cells for

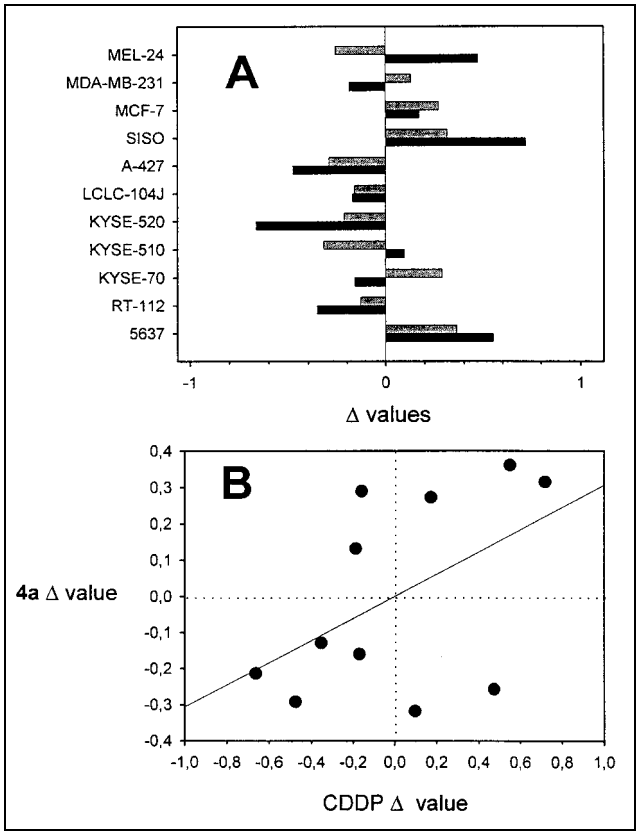


Fig. 2: A: Mean graphs of CDDP (solid bars) and 4a (gray bars) with 11 cell lines; whereby delta values (Δ) = log (mean IC_{50}) − log (IC_{50}). B: Linear correlation between delta values for CDDP and 4a in the 11 cell lines, with a correlation coefficient $r^2 = 0.25$

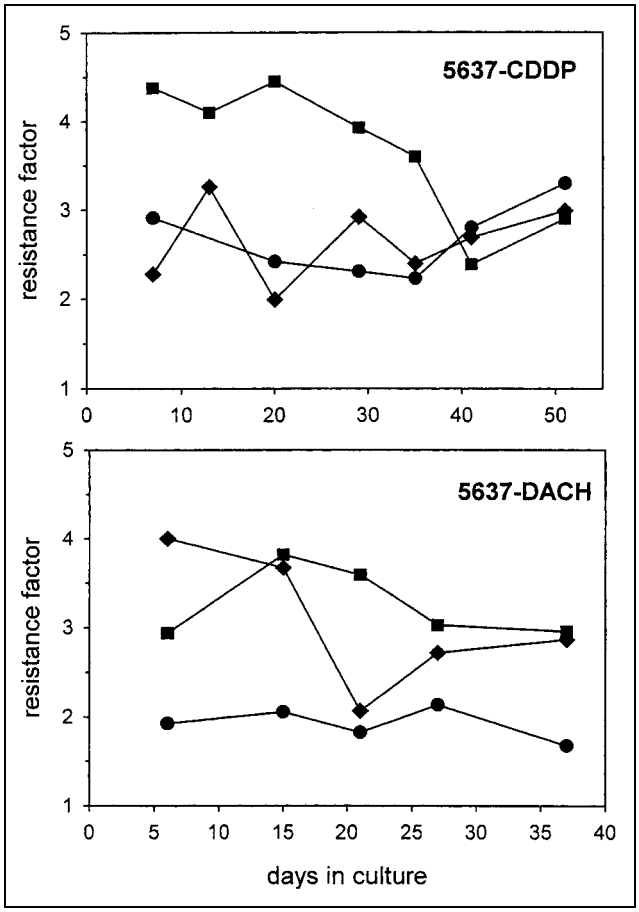
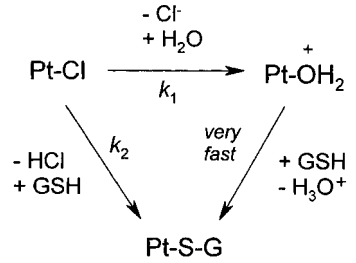


Fig. 3: Resistance factors vs. the time in culture for CDDP (■), DACH (◆) and 4a (●) in the 5637-CDDP (upper panel) and 5637-DACH (lower) cell lines

Scheme 2



Two-term substitution mechanism at square-planar Pt(II)

CDDP. At the end of the 50 d cultivation, the 5637-CDDP cells showed similar levels of resistance to all three compounds.

One mechanism of resistance commonly found with CDDP is an elevation of intracellular GSH levels, which leads to an increased rate of drug inactivation. To characterize the reactivity of **4a** towards GSH, the reaction rate constants were determined with a RP-HPLC method. Substitution reactions at square-planar Pt(II) centers generally obey a two term rate law [11]: $k_{obs} = k_1 + k_2 \cdot [\text{nucleophile}]$ (Scheme 2). Fig. 4 shows the resulting plot of k_{obs} versus GSH concentration at pH 7.0 and 37 C; from this data the pseudo-first order hydrolysis rate constant k_1 and the second-order reaction rate constant k_2 for the direct substitution of GSH are derived. Table 2 contains the values of the rate constants and compares them with those previously reported for the reaction of GSH and CDDP [12]; this analysis indicates that **4a** reacts with GSH at nearly the same rate as CDDP. (Different pH values were used in these two studies, but increases in pH are known to increase the reactivity of thiols to Pt(II) diamines [12]; thus it is expected that **4a** will reacted slightly faster with GSH at pH 7.4).

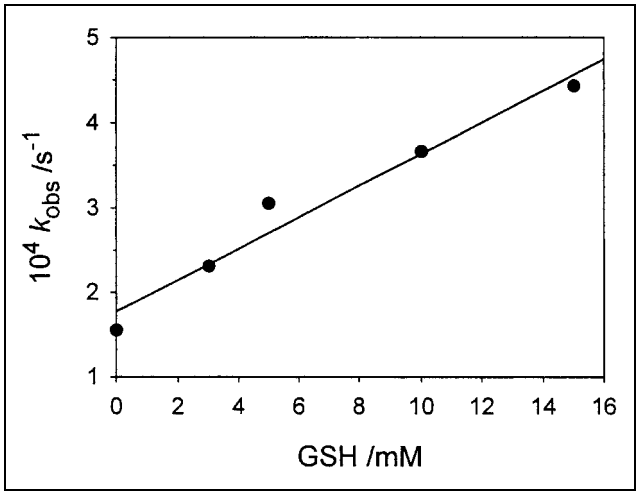


Fig. 4: Plot of the k_{obs} vs. GSH concentration for the loss of **4a** (100 μM initial concentration) from reactions done in 0.3 M $NaNO_3$ and 50 mM PIPES (pH 7.0) at 37 °C. The data fits the equation $k_{obs} = k_1 + k_2$ [GSH], $r^2 = 0.963$

Table 2: k_1 And k_2 rate constants for the reaction of GSH with either CDDP or 4a at 37 °C

Compd.	10^5 k_1/s^{-1}	10^2 $k_2/M^{-1} \cdot s^{-1}$
CDDP[a]	1.17	1.32
4a[b]	1.77	1.86

[a]) data from ref. [23] at pH 7.4; [b]) data from this work at pH 7.0

3. Discussion

Overcoming inherent and acquired resistance to platinum complexes is an important goal of analog development. Here, platinum complexes bearing a bis-1,2-(aminomethyl)benzene ligand were chosen as basis for analog synthesis because of the good *in vitro* cytotoxic activity of the parent compound in two breast cancer cell lines and the seemingly wide variety of substitutions that could be made in the aromatic ring. Unfortunately, finding a common synthetic route that would yield a range of analogs was not successful here. The route attempted, which relies on a bromination of the benzylic methyl groups of *o*-xylene derivatives, is applicable for the synthesis of the parent compound and a monomethoxy derivative. However, with 3,6-dimethoxy-*o*-xylene, bromination of the aromatic ring also took place to give **1c**. The mechanism of allyl bromination by NBS is radical in nature, with traces of Br_2 serving as the source of Br• [13]. In the presence of a sufficiently activated aromatic ring, aromatic electrophilic substitution can also occur under these conditions.

In the screening for cytotoxic activity, **4a** and **4b** were compared with CDDP in an *in vitro* microtiter cell-based assay. In all cases, the 4-methoxy analog **4b** is less active than the parent compound. The activity of CDDP appears to be more selective than **4a** because the difference in potency for CDDP between the most sensitive and least sensitive cell lines was 5 times greater than for **4a**. The results form the mean graph analysis suggest that **4a** may have a different spectrum of activity than CDDP (Fig. 2), but the number of cell lines (11) used here is too small for an accurate prediction. In a 60 cell line-based study, statistically significant differences were found between the activity patterns of oxaliplatin and CDDP [14].

To study the cross resistance of **4a** to CDDP and DACH, we chose the 5637 bladder carcinoma cell line because it is relatively sensitive to all three compounds. To simulate the clinical development of resistance, a two month treatment schedule with exposures to relatively low drug concentrations every 7 to 14 d was used. The cells thus reached a 4–5 fold level of resistance to the respective Pt-complex; this is a level of resistance that has been discussed for clinical settings [15]. Cross resistance to CDDP and DACH was found for **4a** in the 5637 bladder cancer cell line, however, the level of resistance was not as great as either CDDP or DACH at the beginning of the re-cultivation period (Fig. 3). Interestingly, both 5637-CDDP and 5637-DACH cells lost some of the resistance they had acquired to CDDP and DACH, respectively, with each subsequent passage (Fig. 3). Similar observations have been reported by others for CDDP acquired resistance, e.g. in L1210 cells [16], and in human colon adenocarcinoma [17] and human ovarian cell lines [18]. Thus, **4a** circumvents to a greater extent the protective mechanisms developed by 5637 cells to CDDP, but some of these resistance features are later lost in the absence of drug anyway. In the 5637-DACH cell line, **4a** showed less cross-resistance than did CDDP, but CDDP was still more active than **4a** in this cell line.

It is not yet known which mechanisms of resistance the 5637 cells acquire: increased GSH and/or metallothionine levels [19], decreased drug uptake rates [19], increased nucleotide excision repair [20], decreased mismatch repair [21], HMG domain proteins [22] and defective P53 [23] have all been implcated in the development of resistance to CDDP. Increases in the intracellular levels of GSH, resulting in an accelerated inactivation of the drug, have

been observed in many cases. Recently, a sterically hindered Pt(II) complex, *cis*-amminedichloro(2-methylpyridine)platinum(II) (ZD0473), which is chemically less reactive than CDDP, has been reported to overcome resistance to CDDP, possibly by a GSH-dependent mechanism [3, 24]. Thus, we measured the reaction rate of GSH with **4a** and compared it with that published for CDDP. This analysis shows that **4a** reacts with GSH at a similar rate as does CDDP (Table 2), indicating that the reactivity towards GSH probably does not explain the lower level of cross resistance.

In conclusion, the development of bis-1,2-(aminomethyl)benzene-Pt(II) analogs was met with only limited success because of side reactions during the benzyl bromination of *o*-xylenes with activated aromatic rings. A new synthetic route must be compatible with a variety of aromatic functionalities in compounds that are commercially available. Nevertheless, it could be shown with just two analogs that variation at one position in the aromatic ring leads to noticeable changes in biological activity. Finally, the establishment of CDDP- and DACH-resistant cell lines to the 5637 bladder carcinoma will be useful in future drug screening studies.

4. Experimental

4.1. Materials

o-Xylene, *N*-bromosuccinimide, benzoylperoxide, e.p. NaN_3, p.a. $NaNO_3$, 3,4-dimethylphenol, dimethylsulfate, silica gel 60 (0.063–0.200 mm), TLC plates (silica gel 60 F_{254}) and crystal violet were from Merck (Darmstadt, FRG), 2,3-dimethylhydroquinone and *trans-d,l*-diaminocyclohexane were from Aldrich (Taufkirchen, FRG). Pd-C (10%) and K_2PtCl_4 were gifts of the Degussa AG (Hanau, FRG). Cisplatin was from Heraeus (Hanau, FRG). 4-Methoxy-*o*-xylene and 3,6-dimethoxy-*o*-xylene were prepared from 3,4-dimethylphenol and 2,3-dimethylhydroquinone, respectively, by reaction with dimethylsulfate in 10% KOH. All other reagents were commercially available, synthesis-grade chemicals. RPMI-1640 medium, fetal calf serum (FCS), trypsin-EDTA-solution (1x), $NaHCO_3$ and GSH were from Sigma (Taufkirchen, FRG), and glutaraldehyde (50%) was from Fluka (Deisenhofen, FRG). Plastic cell culture materials were from Sarstedt (Nümbrecht, FRG). Cell lines were obtained from the DSMZ (Braunschweig, FRG). Cell counting was done mechanically with a Coulter Counter Z2 (Krefeld, FRG) fitted with a 100 µm aperture. Water was deionized to 18.2 MΩ · cm by means of an Elix/Milli-Q-biocell Pure Water System (Millipore, Eschborn, FRG). Melting points were obtained with an Meltemp 2 and are uncorrected. Proton NMR spectra were recorded with a Bruker DPX 200 instrument (operated at 200.13 MHz), IR spectra with a Perkin-Elmer 1600 and elemental analysis with either a Perkin-Elmer 2400 or a Leco 932.

4.2. Synthetic Methods

4.2.1. Synthesis of 1,2-bis(bromomethyl)benzene (**1a**) [**6**]

In 300 ml dry CCl_4, 32 g (301 mmol) *o*-xylene, 100 g (618 mmol) NBS and 150 mg (0.62 mmol) benzoylperoxide were carefully warmed to reflux. The exothermic reaction was refluxed for 30 min, then cooled, filtered and the filtrate concentrated on the rotary evaporator. **Caution: the product is an extreme lachrymator!** Upon addition of pet. ether the product crystallized out, the colorless crystals were collected by suction filtration, washed with pet. ether and dried, giving 34,92 g (40% yield). M.p. 98–99 °C (Lit. 98–99 °C). TLC (8 : 2 : 1 pet.ether-Et_2O-EtOAc): $R_f = 0.83$. ^1H NMR ($CDCl_3$): δ [ppm] = 7,32 (m, 4 H, Ar-H), 4.66 (s, 4 H, CH_2).
$C_8H_8Br_2$: C, H

4.2.2. Synthesis of 1,2-bis(bromomethyl)-4-methoxybenzene (**1b**)

In 50 ml dry CCl_4, 3.0 g (25 mmol) 4-methoxy-*o*-xylene, 10.9 g (61 mmol) NBS and 80 mg (0.33 mmol) benzoylperoxide were allowed to react 15 h under reflux. The reaction was cooled, filtered and the filtrate concentrated. The crude product was purified by CC with silica gel (1 : 4 CH_2Cl_2-cyclohexane) to give 2.1 g (29% yield) of a yellow oil. IR (neat): ν [cm^{-1}] = 3006 w (C−H, arom), 2940 w (C−H, aliph), 2847 w (C−H, aliph), 1698 w, 1596 m (C=C), 1493 s (C=C), 1461 m, 1385 m, 1274 s (C−O−C), 1202 m (C−Br), 1153 w, 1048 s (C−O−C), 794 w, 719 w, 608 w, 495 w. ^1H NMR ($CDCl_3$): δ [ppm] = 6.71–7.54 (m, 3 H, Ar.-H), 4.59 (s, 2 H, CH_2), 4.57 (s, 2 H, CH_2), 3.88 (s, 3 H, CH_3).

4.2.3. Synthesis of 1,2-bis(bromomethyl)-4-bromo-3,6-dimethoxybenzene (**1c**)

In 50 ml dry CCl_4, 4.29 g (25.8 mmol) 3,6-dimethoxy-*o*-xylene, 11.5 g (64.5 mmol) NBS and 150 mg (0.62 mmol) benzolyperoxide were allowed to react under refluxed for 24 h. The reaction was cooled, filtered and the filtrate concentrated under reduced pressure. The crude product (4.47 g) was purified by CC with silica gel (1 : 3 CH_2Cl_2-cyclohexane) to give 0.8 g (10 % yield) of a yellow oil. ^1H NMR (DMF-d_7): δ [ppm] = 7.07 (s, 1 H, Ar-H), 4.73 (s, 2 H, CH_2), 4.69 (s, 2 H, CH_2), 3.94 (s, 3 H, CH_3), 3.87 (s, 2 H, CH_3).
$C_{10}H_{11}Br_3$: C, H

4.2.4. Synthesis of 1,2-bis(azidomethyl)benzene (**2a**)

In 125 ml acetone, 5.0 g (19 mmol) **1a** and 2.47 g (38.5 mmol) NaN_3 were allowed to react for 5 h under reflux, after which a TLC showed that the reaction had gone to completion. The reaction mixture was filtered, to the filtrate was added 100 ml H_2O and was extracted three times with Et_2O. The organic phases were combined, washed once with water, dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to give 2.53 g (71 % yield) of a yellow oil. The instable product was used without further purification. TLC (8 : 2 : 1 pet.ether-Et_2O-EtOAc): $R_f = 0.77$. IR (neat): ν [cm^{-1}] = 3301 w, 3068 w (C−H, arom), 3026 w (C−H, arom), 2940 m (C−H, aliph), 2887 m (C−H, aliph), 2461 w, 2096 s (−N_3), 1491 w (C=C), 1456 s (C=C), 1346 s, 1252 s, 1178 w, 1106 w, 1049 w, 883 m, 756 s, 678 m, 559 m. ^1H NMR ($CDCl_3$): δ [ppm] = 7.38 (s, 4 H, Ar-H), 4.42 (s, 4 H, CH_3).

4.2.5. Synthesis of 1,2-bis(azidomethyl)-4-methoxybenzene (**2b**)

In 100 ml acetone, 2.08 g (7.1 mmol) **1b** and 1.15 g (17.8 mmol) were heated under reflux for 5 h. After cooling, 40 ml H_2O were added and then extracted 3x with Et_2O, the organic phases were combined, washed with H_2O, dried over Na_2SO_4 and filtered. The filtrate was concentrated on a rotary evaporator to give 0.88 g (57 % yield) of a yellow oil. The instable azide was used without further purification. IR (neat): ν [cm^{-1}] = 3344 w, 3007 w (C−H, arom), 2941 s (C−H, aliph), 2846 w (C−H, aliph), 2469 w, 2096 s (−N_3), 1599 s (C=C), 1566 w, 1495 m (C=C), 1462 m (C=C), 1387 m, 1279 s (C−O−C), 1203 m, 1141 m, 1051 s (C−O−C), 958 w, 871 m, 845 m, 715 cm^{-1}. ^1H NMR ($CDCl_3$): δ [ppm] = 6.73–7.66 (m, 3 H, Ar.-H), 4.44 (2, 2 H, CH_2), 4.32 (2, 2 H, CH_2), 3.94 (s, 3 H, CH_3).

4.2.6. Synthesis of 1,2-bis(azidomethyl)-4-bromo-3,6-dimethoxybenzene (**2c**)

In 40 ml acetone, 0.8 g (2.5 mmol) **5** and 0.41 g (6.25 mmol) NaN_3 were made to react under reflux for 5 h. After cooling, the reaction mixture was filtered, the filtrate diluted with 30 ml H_2O, extracted 3x with Et_2O, the organic phases combined, dried over Na_2SO_4 and filtered. The filtrate was concentrated on the rotary evaporator to give 0.217 g (35 % yield) of a yellow oil. IR (neat): ν [cm^{-1}] = 3308 w, 3091 w, 3004 m (C−H, arom), 2941 s, 2838 m, 2465 w, 2092 s (−N_3), 1574 s (C=C), 1470 s (C=C), 1401 s, 1232 s (C−O−C), 1182 m, 1097 m, 1059 m (C−O−C), 992 m, 932 m, 909 w, 844 m, 772 m, 736 m, 649 w, 612 w, 553 w. ^1H NMR (DMF-d_7): δ [ppm] = 7.14 (s, 1 H, Ar-H), 4.49 (s, 2 H, CH_2), 4.42 (s, 2 H, CH_2), 3.87 (s, 3 H, CH_3), 3.85 (s, 3H CH_3).

4.2.7. Synthesis of 1,2-bis(aminomethyl)benzene dihydrochloride semihydrate (**3a**)

As 1.34 g (7.13 mmol) **2a** and 0.15 g Pd-C stirred in 50 ml abs. EtOH under an atmosphere of N_2, an excess of H_2 was introduced by means of a rubber balloon. The reduction was allowed to progress overnight, the catalyst removed by filtration and the solvent evaporated under reduced pressure. The product was dissolved in a minimum of EtOAc and HCl-staturated EtOAc was added. The precipitate was collected by filtration. To decolorize the product, the precipitate was dissolved in MeOH, activated charcoal was added, the suspension was filtered and the solvent removed under reduced pressure to give 0.26 g (27 % yield) of a colorless powder. IR (KBr): ν [cm^{-1}] = 3448 s (H_2O), 3150–2300 s (C−H, $-NH_3^+$), 1966 w ($-NH_3^+$), 1601 m (C=C), 1588 s (C=C), 1526 m, 1515 w, 1496 m (C=C), 1457 w, 1373 m, 1223 w, 878 w, 770 m, 750 m. ^1H NMR of the free base ($CDCl_3$): δ [ppm] = 7.16–7.34 (m, 4 H, Ar-H), 3.92 (2, 4 H, CH_2), 2.02 (s, 4 H, NH_2).
$C_8H_{12}N_2 \cdot 2\ HCl \cdot 0.5\ H_2O$: C, H, N

4.2.8. Synthesis of 1,2-bis(aminomethyl)-4-methoxybenzene dihydrochloride trihydrate (**3b**)

As 0.88 g (4 mmol) **2b** and 0.10 g Pd-C stirred in 30 ml abs. EtOH under an atmosphere of N_2, an excess of H_2 was introduced by means of a rubber balloon and the reaction was allowed to progress overnight. The work-up was as described above for **3a** and gave 100 mg (15 % yield) of colorless powder. IR (KBr): ν [cm^{-1}] = 3454 s (H_2O), 3200–2400 s (C−H,

$-NH_3^+$), 1927 w ($-NH_3^+$), 1612 s (C=C), 1590 s (C=C), 1515 m, 1485 s (C=C), 1372 m (methyl), 1336 w, 1304 m, 1260 s (C−O−C), 1174 m, 1138 m, 1020 m (C−O−C), 956 w, 857 m, 832 m 710 w. ^{1}H NMR (CD_3OD): δ [ppm] = 7.50 (d, ^3J = 8.5 Hz, 1 H, Ar-H_a), 7.15 (d, ^3J = 2.6 Hz, 1 H, Ar-H_c), 7.07 (q, ^3J = 8.5 and 2.7 Hz, 1 H, Ar-H_b), 4.29 (s, 2H, CH_2),), 4.24 (s, 2 H, CH_2), 3.86 (s, 3 H, CH_3).
$C_9H_{14}N_2O \cdot 2\ HCl \cdot 3\ H_2O$: C, N, H calcd. 7.51; found 7.00

4.2.9. Attempted synthesis of 1,2-bis(aminomethyl)-4-bromo-3,6-dimethoxybenzene dihydro-chloride (**3c**)

The reduction 0.97 g (3.9 mmol) **2c** was carried out under the same conditions as described above for **3a**, however, the subsequent work-up failed to yield a collectable product.

4.2.10. Synthesis of [1,2-bis(aminomethyl)benzene]dichloroplatinum(II) (**4a**)

To a solution of 0.415 g (1 mmol) K_2PtCl_4 in 5 ml 0.2 N HCl was added 218 mg (1 mmol) **3a** in 5 ml 0.2 N HCl. The pH of the reaction solution was adjusted to between 6 and 7 with 1 N NaOH and reaction allowed to stir in the dark at RT for a week. Each day, new precipitate was collect by suction filtration on a glass G4 fritted filter, the solid washed with ice-cold 0.2 N HCl, cold EtOH, and finally Et_2O. The combined precipitates were dissolved in DMF, filtered through a G4 fritted filter and the product reprecipitated by addition of Et_2O. The product was collected by filtration, washed with Et_2O and dried to give 0.144 g (36% yield) of a cream colored powder. IR (KBr): ν [cm^{-1}] = 3264 m, 3229 s, 3183 s, 3117 m (N−H), 2923 w, (C−H, aliph.) 2359 w (N−H),1631 w, 1582 m (C=C), 1477 w, 1454 w, 1378 m, 1290 w, 1221 m (C−N), 1160 w, 1097 w, 984 w, 798 e, 758 w, 524 w (Pt-N). ^{1}H NMR (DMF-d_7): δ [ppm] = 7.39 (s, 4 H, Ar-H), 5.32 (broad s, 4 H, NH_2), 4.02 (t, 4 H, CH_2).
$C_8H_{12}N_2Cl_2Pt$: C, N, H

4.2.11. Synthesis of [1,2-bis(aminomethyl)-4-methoxybenzene]dichloroplatinum(II) (**4b**)

The same procedure as described above for **4a** was used to synthesize **4b,** starting from **3b** and K_2PtCl_4. A cream colored powder was obtained in a 30% yield. IR (KBr): ν [cm^{-1}] = 3220 m, 3193 m, 3127 m (N−H), 2959 w (C−H, arom.), 2832 w, 2360 w, 1614, m, 1578 m (C=C), 1503 m, 1427 w, 1379 w, 1266 s (C−O−C), 1212 w, 1108 m, 1036 m, 993 w, 856 w, 826 w, 793 w, 709 w, 587 w (Pt-N). ^{1}H NMR (DMF-d_7): δ [ppm] = 7.32 (d, ^3J = 8.3 Hz, 1 H, Ar-H_a), 7.01 (d, ^3J = 5.5 Hz, 1 H, Ar-H_c), 6.94 (q, ^3J = 8.3 and 5.5 Hz, 1 H, Ar-H_b), 5.29 (broad d, 4 H, NH_2), 3.96 (m, 4 H, CH_2), 3.85 (s, 3 H, CH_3).
$C_9H_{14}N_2OCl_2Pt$: C, H, N calcd. 6.48; found 7.76

4.2.12. Synthesis of [d,l-trans-1,2-diaminocyclohexane]dichloroplatinum(II) (DACH)

To a solution of 0.557 g (5 mmol) *trans-d,l*-diaminocyclohexane in 5 ml H_2O was added a solution of 2.075 g (5 mmol) $K_2[PtCl_4]$ in 50 ml H_2O in 50 µl increments over 2 h. The reaction was stirred an additional 2 h, during which time the pH was continually adjusted to 7-8 with 1 N NaOH. The precipitate was collected by suction filtration, washed with H_2O, EtOH, Et_2O and dried *in vacuo* over P_2O_5 to give 1.56 g (82 %) of a yellow powder. IR (KBr): ν [cm^{-1}] = 3270 s (N−H), 3194 s (N−H), 2932 s (C−H, aliph.), 2862 m, 2359 w, 1564 s, 1450 w, 1385 w, 1211 w, 1160 m, 1125 m, 1063 m, 1030 m 756 s, 436 w. ^{1}H NMR (DMF-d_7): δ [ppm] = 4.9–5.8 (broad dd, 4 H, NH_2),), 2.1–2.4 (dd, 2 H, CH), 1.0–1.8 (m, 8 H, CH_2).
$C_6H_{14}N_2Cl_2Pt$: C, H, N

4.3. Cytotoxicity studies

The culture medium used in all work was RPMI-1640 medium containing 2 g/l HCO_3, and 10% FCS. Cells were incubated in a humid atmosphere of 5% CO_2 at 37 °C and passaged shortly before becoming confluent. For the cytoxicity studies, 100 µl of a cell suspension were seeded into 96-well microtiter plates at a density of 500–1000 cells/well, except for the LCLC-103H cell line, which was plated out at 250 cells/well. One day after plating, cells were treated with test substance at five concentrations per compound: 1000 fold concentrated stock solutions in DMF were serially diluted by 50% in DMF to give the feed solutions, which were diluted 500 fold into culture medium. The controls received just DMF. Each concentration was tested in 8 wells, with each well receiving 100 µl of the medium containing the Pt-complex. Cells were then incubated 96 h, after which time the medium was removed and replaced with a 1% glutaraldehyde/PBS solution for 20 min. Cells were stored at 4 °C under PBS. Staining with crystal violet was done as previously described [9]. O.D. was measured at λ = 620 nm with an Anthos 2010 plate reader (Salzburg, Austria). Corrected T/C values were calculated by the equation:

$$(T/C)_{corr}\,(\%) = \frac{O.D._{\cdot T} - O.D._{\cdot c,0}}{O.D._{\cdot c} - O.D._{\cdot c,0}} \cdot 100$$

where $O.D._{\cdot T}$ is the mean absorbance of the treated cells, $O.D._{\cdot c}$ the mean absorbance of the controls and $O.D._{\cdot c,0}$ the mean absorbance at the time drug was added. The IC_{50} values were estimated by a linear least-squares regression of the T/C_{corr} values versus the logarithm of the substance concentration; only concentrations that yielded T/C_{corr} values between 10 and 90% were used in the calculation.
In the cytotoxicity studies with the A-427 line where direct counting of the cells was done, the cells were plated into 6-well plates in 3 ml/well at a density pro cm^2 corresponding to the density in the 96-well plates. The treatment procedure was analogous to that described above for the microtiter plates. The detachment of the cells 96 h following the addition of substance was done by first incubating with a 10 fold diluted trypsin-EDTA solution for 2–3 min at 37 °C, then the solution was removed by aspiration, 4 ml PBS/10% FCS was added and the cells were suspended by repeated rinsing the floor of the wells with a Pasteur pipette. A 500 µl aliquot was removed and the cells were counted mechanically.

4.4. Development of resistance to CDDP and DACH in the 5637 cell line

5637 cells were treated once every 7 to 14 d with either CDDP or DACH over a period of 2 months with increasing Pt-concentrations, beginning at 1.0 µM and ending at 2.5 and 5.0 µM for CDDP and DACH, respectively. The cells were then expanded over the next 2 weeks and cryo-preserved. The new cell lines created by pretreatment with CDDP and DACH were named 5637-CDDP and 5637-DACH, respectively. The growth curves of both resistance cell lines were comparable to that of the wild-type (data not shown).
The resistance factor was calculated as follows:

$$\text{Resistance factor} = \frac{IC_{50}\text{ of resistant cells}}{IC_{50}\text{ of wild-type cells}}$$

The IC_{50} values of the wild-type cells for three compounds, which were always determined parallel to the resistant cell lines, showed little variation over the 40–50 days of testing: CDDP (n = 7): 0.329 ± 0.033; DACH (n = 5): 0.87 ± 0.19; **4a** (n = 5): 1.05 ± 0.084 µM.

4.5. Determination of observed rate constants by RP-HPLC

The observed rate constants (k_{obs}) were determined by quantifying with RP-HPLC the remaining fraction of **4a** present in reaction mixtures of GSH, as has been previously described [12]. The chromatography system used here consisted of a L-7100 pump controlled by a D-7000 interface (Merck, Darmstadt, Germany) and a 7125 Rheodyne sample injector fitted with a 500 µl injection loop. Detection was done at λ = 264 nm with an L-4500 diode array detector (Merck). A 0.4 × 25 cm Nucleosil-100 RP-18 column with a 0.4 × 1.1 cm precolumn (Macherey-Nagel, Düren, Germany), maintained at 30 °C by means of a L-7360 column oven, was used for chromatography. A 9 : 1 mixture of phosphate buffer (20 mM, pH 3.3)/MeOH was used as the eluant. At a flow rate of 0.7 ml/min, **4a** eluted with a retention time of 12.1 min. Quantification was done by measuring peak height; for injection volumes of 500 µl the response was linear with respect to the concentration of Pt-complex between 100 and 10 µM.

Acknowledgements: We thank Frau K. Portner for the synthesis of [*d,l-trans*-1,2-diaminocyclohexane]dichloroplatinum(II). The technical assistance of Ms. E. Böttcher and Mr. Boubukari are gratefully acknowledged. This work was supported in part by the Fonds der Chemischen Industrie (FCI).

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Received May 7, 2001
Accepted June 21, 2001

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