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Differential cytotoxicity, uptake and DNA binding of tetraplatin and analogous isomers in sensitive and resistant cancer cell lines

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Some platinum complexes contain 1,2-diaminocyclohexane (DACH) as a stable carrier ligand, which can exist as the *R,R*-, *S,S*- and *cis*-isomers. Tetraplatin, for instance, is a mixture of *R,R*- and *S,S*-DACH-Cl_4-Pt(IV). We have examined each of the three individual isomers of DACH-Cl_4-Pt(IV) with respect to cytotoxicity, uptake of platinum and total DNA–platinum in three murine leukemia L1210 (cisplatin-sensitive L1210/0, 50-fold cisplatin-resistant L1210/DDP and 36-fold tetraplatin-resistant L1210/DACH) and human ovarian carcinoma A2780 (cisplatin-sensitive) and A2780cp (8-fold cisplatin-resistant) cell lines. Against A2780, A2780cp and L1210/DDP cell lines, the *R,R*-isomer was the most potent followed by the *S,S*-isomer and then the *cis*-isomer. However, the three isomers demonstrated similar IC_{50} values against the L1210/0 and L1210/DACH cell lines. The *cis*-isomer demonstrated cross-resistance (9- to 20-fold) to cisplatin in L1210/DDP and A2780cp cell lines. On the other hand, *R,R*- and *S,S*-isomers demonstrated minimal (2- to 4-fold) cross-resistance against these tumor models. Intracellular platinum accumulation over a 2 h period at 40 μM drug concentration was significantly ($p < 0.05$) greater for the *R,R*-isomer than the *cis*-isomer in L1210/0 (122 versus 101 ng Pt/mg protein) and L1210/DDP (73 versus 50) cell lines, while no difference was observed in L1210/DACH cells (55 versus 56). In L1210/DDP cells, total DNA-bound platinum was significantly ($p < 0.05$) greater for the *R,R*-isomer compared with the *cis*-isomer (10.3 versus 7.5 ng Pt/mg DNA). No significant differences ($p > 0.05$) were found in levels of platinum bound to DNA between *R,R*- and *cis*-isomers in either the L1210/0 or L1210/DACH cell lines. These data suggest that isomeric differences in the L1210 models may be explained, in part, by differential drug handling at the cellular and/or DNA level.

Key words: DNA–platinum binding, drug uptake, isomers, tetraplatin.

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Introduction

Cisplatin (*cis*-diamminedichloroplatinum (II); CDDP) has been widely used in cancer chemotherapy alone or in combination with other anticancer drugs for the treatment of several human cancers, including those of the ovary, testes and bladder.[1] The interaction of cisplatin with DNA has been implicated as the major cytotoxic action of the drug.[2,3] Its therapeutic efficacy, however, is limited due to significant side effects including nephrotoxicity, nausea and vomiting, myelosuppression, and ototoxicity,[4] and to the development of resistance.[5,6] Among the mechanisms proposed for the observed resistance are altered accumulation of platinum by the cell,[7,8] increased levels of GSH[9,10] or metallothionein,[11,12] and increased DNA repair.[13,14]

Analogs of cisplatin containing the *trans*-*R,R*-, *trans*-*S,S*- or *cis*-*R,S*–*S,R*-isomer of 1,2-diaminocyclohexane (DACH) coordinated to the central platinum atom are of interest because of their potential ability to overcome cisplatin resistance in tumor cells.[15–18] One such compound is the newly developed tetraplatin (*trans*-*R,R*–*S,S*-DACH-Cl_4-Pt-(IV)), which is currently in clinical trials. Tetraplatin used clinically is a mixture of two *trans*-(*R,R*- and *S,S*-) stereoisomers, while the analogous *cis*-isomer can also be synthesized. In order to satisfy chemical reproducibility requirements and pharmaceutical formulation needs, one of these isomers may eventually be selected for continued clinical development. For this reason, examination of possible differences in biological activity between tetraplatin isomers is critical.

The *R,R*- and *S,S*-isomers of DACH-Cl_4-Pt(IV) have been previously examined *in vitro*,[19] and shown to display differential cytotoxicities in some tumor systems. The reported data, however, did not

include the *cis*-isomer, which *in vivo* has significant activity against the L1210 model.[15] Moreover, Bhuyan *et al.*[19] demonstrated only a marginal advantage for tetraplatin over cisplatin with regard to the degree of cross-resistance in the cisplatin-resistant human ovarian A2780 tumor model, while others[16,20] consistently reported a 4-fold advantage in this system. These contrasting data raise concern about the potential clinical utility of tetraplatin and other DACH-containing platinum complexes undergoing development. More importantly, no biochemical or pharmacological mechanisms have been identified which may explain the relative differences in antitumor activities of the isomers.

In this study, we have examined the relative cytotoxicities of tetraplatin and analogous *R,R*-, *S,S*- and *cis*-isomers against murine and human cell lines sensitive and resistant to either cisplatin or tetraplatin, and have investigated the roles of cellular drug accumulation and DNA binding in the observed isomeric effects.

Materials and methods

Chemicals

Cisplatin, tetraplatin and DACH-Cl_4-Pt(IV) isomers (Figure 1) were synthesized according to published procedures.[15] Solutions were prepared by dissolving the drugs in saline immediately before use. RPMI 1640 culture medium was obtained from Gibco (Grand Island, NY), Agar noble from Difco (Detroit, MI), fetal bovine serum (FBS) from Whittaker M.A. Bioproducts (Walkersville, MD).

Cell lines and culture conditions

Sensitive and resistant murine leukemia L1210 cell lines were provided by Dr Alan Eastman (Department of Pharmacology, Dartmouth Medical School, Hanover, NH).[7,21] Sensitive and resistant human ovarian carcinoma A2780 cell lines were obtained from Dr Thomas C Hamilton (Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA).[16] All cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μM/ml streptomycin, 0.3 mg/ml L-glutamine and with (A2780 cell lines) or without (L1210 cell lines) 0.25 U/ml insulin. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air. Stocks of the original cell lines were stored at −70°C and fresh batches recultured every 3–4 months. The characteristics of these cell lines are summarized in Table 1.

Growth inhibition assays

Growth inhibition assays were performed by the method of Eastman.[21] Briefly, on day −1, 1×10^5 A2780 cells were plated in six-well plates (Falcon) and allowed to attach overnight. On day 0, the medium was aspirated and fresh medium was added to the wells, prior to drug addition. For L1210 cells, 1×10^5 cells were plated in six-well plates on day 0 and the drug added immediately. After 3 days, the number of cells per well was determined by Coulter Counter (Coulter Electronics, Hialeah, FL). Results are expressed as a percentage of control growth, calculated as:

$$\frac{\text{treated cells/well day 3} - \text{treated cells/well day 0}}{\text{control cells/well day 3} - \text{control cells/dish day 0}} \times 100$$

IC_{50} values were defined as the drug concentration (μM) inhibiting cell growth by 50% compared with the control cells.

Clonogenic assays

To evaluate the effect of the drug on cell viability in terms of proliferation competency, we employed

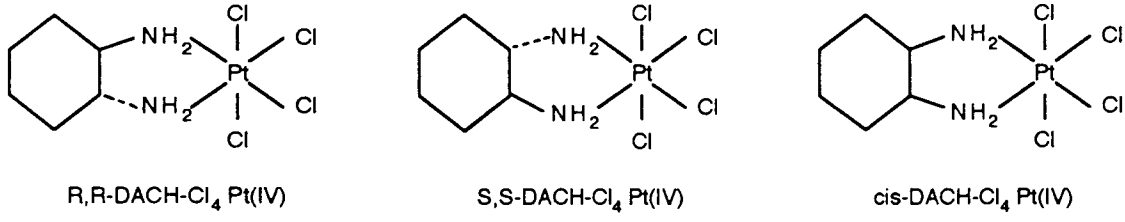

Figure 1. Structures of *R,R*-, *S,S*- and *cis*-DACH-Cl_4-Pt(IV).

Table 1. Characteristics of the cell lines

Cell line	Type of cells	Doubling time (h)	Cloning efficiency[a]	IC_{50} of cisplatin (μM)[b]
L1210/0	mouse leukemia	14.5	93.4 ± 9.9	0.15 ± 0.02
L1210/DDP	mouse leukemia	20.0	89.7 ± 12.5	7.40 ± 0.50
L1210/DACH	mouse leukemia	17.3	86.1 ± 10.6	0.83 ± 0.40
A2780	human ovarian cancer	13.5	49.3 ± 6.7	0.41 ± 0.07
A2780cp	human ovarian cancer	23.2	53.0 ± 6.6	3.40 ± 0.60

[a]Number colonies per tube/number cells plated. Mean ± SD.
[b]IC_{50} values were determined from the growth inhibition assay (see Materials and methods). Mean ± SD.

a colony-formation assay.[22] Briefly, exponentially growing cells were treated with drug for 2 h at 37°C and washed twice with PBS. Between 10^2 and 10^5 cells were suspended in 3 ml of media containing 0.1% Noble agar in closed tubes (triplicate) and colonies were counted after 10–14 (L1210) or 20 days (A2780) of incubation. Colony formation efficiencies are indicated in Table 1.

Cell treatment for drug uptake and DNA binding studies

For these studies, L1210 cell lines were treated with 40 μM of *R,R*-isomer or *cis*-isomer for 2 h. Cellular DNA platinum levels were estimated by flameless atomic absorption spectrophotometry (FAAS) (Varian, model AA300/GTA-96, Mulgrave, Victoria, Australia) using conditions described previously (detection limit = 100 pg Pt).[23,24] Exponentially growing cells were resuspended to 1×10^6 cells/ml in 250 cm^2 flasks with 100 ml complete RPMI 1640 medium. Aliquots (30 ml) were incubated with the drug at 37°C. After 2 h, duplicate 1.5 ml portions were transfered to micro-centrifuge tubes for assessment of drug accumulation, and the remaining cells were used to evaluate DNA-bound platinum. The cells were washed twice with ice cold PBS and the pellets were stored at −20°C until assayed. For determination of cellular platinum and the amount of platinum bound to DNA, at least five independent experiments were performed.

Determination of platinum uptake in cells

Cell pellets following drug incubation (see above) were resuspended in 0.1 ml saline and digested in 0.2 ml 2 M NaOH in an oven at 55–60°C for 2 h. The protein concentration of an aliquot (100 μl) of the digested cell sample was determined by the method of Lowry *et al.*[25] A separate aliquot (180 μl) was acidified with 60 μl of 4 M HCl and platinum determined by FAAS.

Determination of platinum bound to DNA

High molecular weight DNA was isolated from cell pellets following drug incubation (see above) according to standard procedures.[26] Briefly, pellets were lysed at 37°C overnight using extraction buffer (10 mM Tris, 100 mM EDTA, 20 μg/ml RNase, 0.5% SDS, pH 8.0), then treated with proteinase K (100 μg/ml, 50°C, 3 h), and the DNA extracted with phenol three times, precipitated with ethanol and dissolved in H_2O (200 μl). DNA content was assessed by absorption at 260 nm and the amount of platinum in the sample determined by FAAS.

Statistical analysis

Statistical significances were determined by paired Student's *t*-test; values of $p < 0.05$ were considered to be significant.

Results

Chemosensitivity studies

Cytotoxicities of *trans*-*R,R*-, *trans*-*S,S*- and *cis*-*R,S*-*S,R*-isomers of DACH-Cl_4-Pt(IV) were evaluated using both a growth inhibition assay (72 h exposure) and a clonogenic assay (2 h exposure). Tetraplatin, a mixture of the *R,R*- and *S,S*-isomers, was included for comparison. Dose-response curves for tetraplatin and the analogous individual isomers for murine leukemia L1210/0, L1210/DDP and L1210/DACH cells are shown in Figure 2, and the IC_{50} values for these and the A2780 cell lines

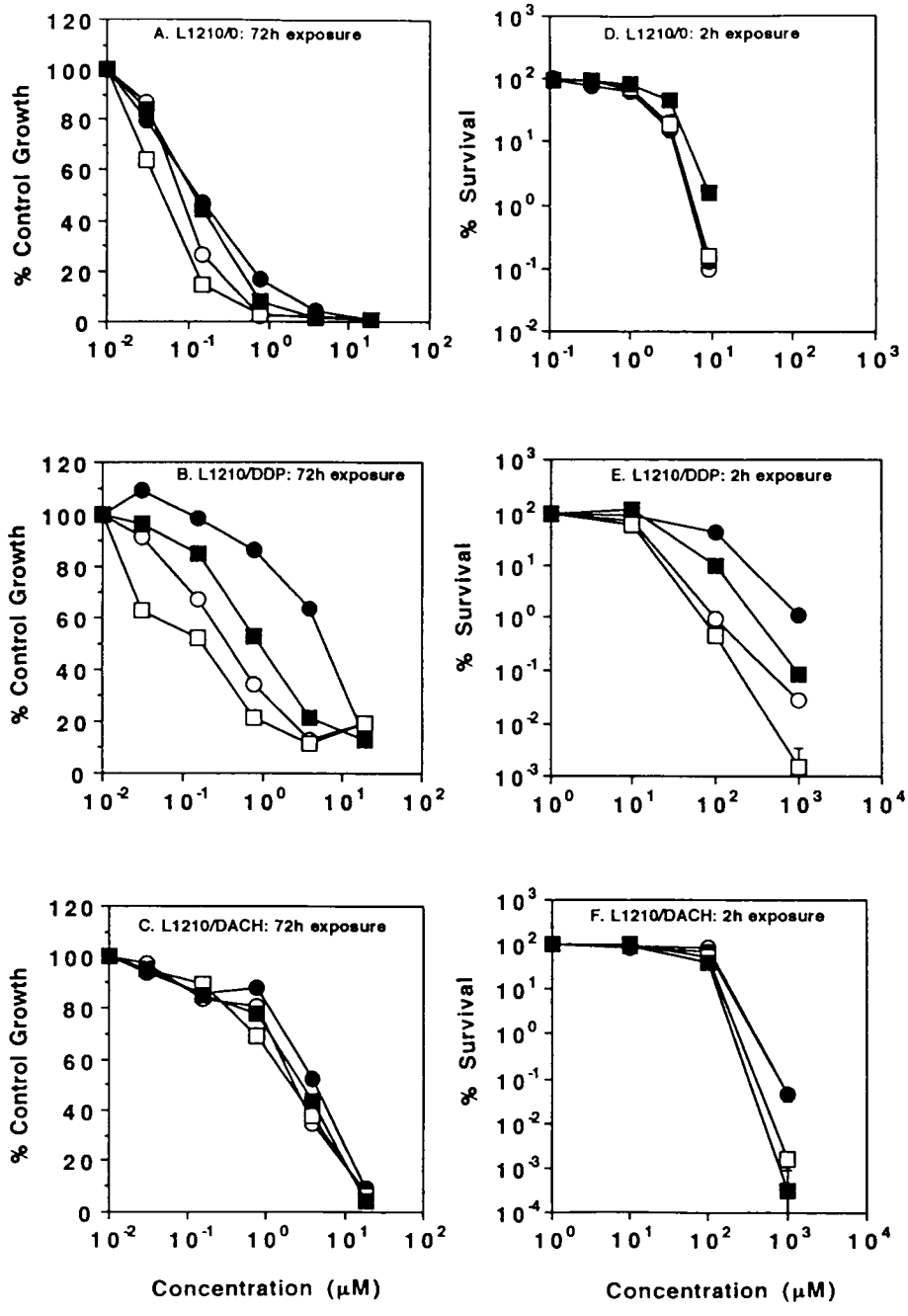

Figure 2. Sensitivity of L1210/0 (A,D), L1210/DDP (B,E) and L1210/DACH (C,F) cells to isomeric DACH-Cl_4-Pt(IV) complexes. (A)–(C) As assessed by growth inhibition assays. (D)–(F) As assessed by clonogenic assays. Each data point represents mean ± SD ($n = 3$); error bars are shown if larger than the size of symbols. ○, Tetraplatin; ●, *cis*; □, *R,R*; ■, *S,S*.

summarized in Table 2. In either of the cytotoxicity assays for cisplatin-sensitive L1210/0 and tetraplatin-resistant L1210/DACH cells, tetraplatin and the three isomers demonstrated similar potencies compared with each other, with the IC_{50} ratio of about 3 between the most potent and the least potent compound. Against cisplatin-sensitive human ovarian A2780 and cisplatin-resistant A2780cp and L1210/DDP cell lines, this ratio was about 6–8 in the clonogenic assay and about 7–31 in the growth inhibition assay. The molar order of the potency of the isomers in these cell lines was in

Table 2. IC_{50} values (μM) of tetraplatin and isomers of DACH-Cl_4-Pt(IV)

Cell line	Clonogenic assay (2 h exposure)				Growth inhibition assay (3 day exposure)			
	R,R	*S,S*	*cis*	tetraplatin	*R,R*	*S,S*	*cis*	tetraplatin
A2780	0.38 (1.0)[a]	0.68 (1.8)[b]	2.3 (6.0)[b]	0.81 (2.1)[b]	0.057 (1.0)	0.19 (3.3)[b]	0.39 (6.8)[b]	0.09 (1.6)
A2780cp	2.4 (1.0)	7.6 (3.2)[b]	18.5 (7.9)[b]	4.6 (2.0)	0.16 (1.0)	0.62 (4.0)[b]	3.3 (21.3)[b]	0.35 (2.3)
L1210/0	1.45 (1.0)	3.4 (2.3)[b]	1.7 (1.2)	1.21 (0.83)[b]	0.058 (1.0)	0.18 (3.1)	0.20 (3.4)	0.084 (1.5)
L1210/DDP	10.0 (1.0)	26.3 (2.6)[b]	56.9 (5.7)[b]	6.42 (0.64)	0.13 (1.0)	0.71 (5.6)[b]	3.9 (30.8)[b]	0.22 (1.8)
L1210/DACH	102.7 (1.0)	91.2 (0.89)[b]	125.7 (1.22)[b]	148.7 (1.45)[b]	3.9 (1.0)	4.6 (1.2)	5.5 (1.4)	3.0 (0.75)

[a]Numbers in parentheses indicate relative IC_{50} compared with the *R,R*-isomer, which was arbitarily assigned a value of 1.
[b]$p < 0.05$ versus the *R,R*-isomer.

general *R,R*- > tetraplatin > *S,S*- > *cis*-isomer. Cross-resistance evaluations for the isomers using data from the growth inhibition assay are presented in Figure 3. The *cis*-isomer demonstrated cross-resistance (9- to 20-fold) to cisplatin in L1210/DDP and A2780cp cell lines. On the other hand, *R,R*- and *S,S*-isomers demonstrated minimal (2- to 4-fold) cross-resistance against these cells. The L1210/DACH cells, on the other hand, showed 26- to 36-fold resistance to tetraplatin, *S,S*- and *cis*-isomers, and 67-fold resistance to the *R,R*-isomer.

Uptake and DNA binding study

Uptake of tetraplatin, expressed on the basis of cell number or intracellular protein content, was linear

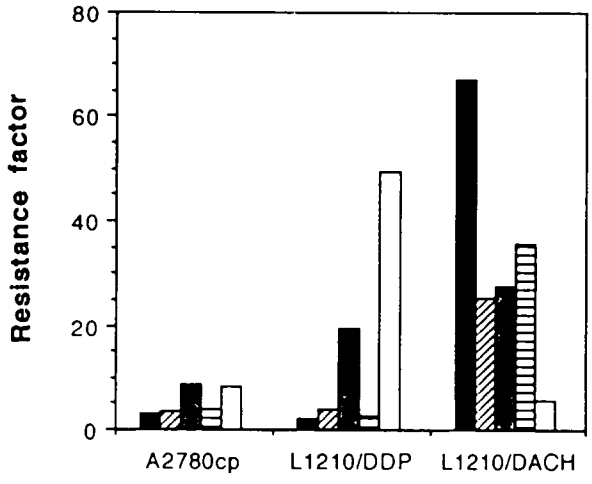

Figure 3. Cross-resistance profile for tetraplatin and isomeric DACH-Cl_4-Pt(IV) complexes in cisplatin and tetraplatin resistant cell lines. Mean values derived from three experiments. ■, *R,R*; ▨, *S,S*; ■, *cis*; ▤, tetraplatin; □, cisplatin.

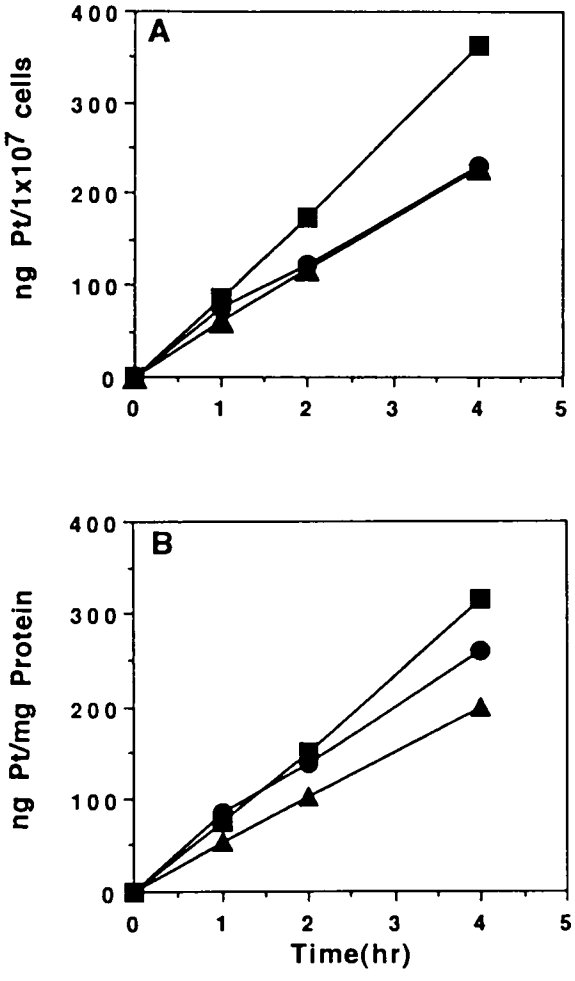

Figure 4. Accumulation of platinum from *trans*-DACH-Cl_4-Pt(IV) in L1210 cell lines. Cells at a density of 1×10^6 cells/ml were incubated in RPMI 1640 supplemented with 10% FBS at 37°C with 40 μM drug and the time-dependent accumulation of platinum was determined by atomic absorption spectrophotometry. (A) Data expressed as ng Pt/1×10^7 cells. (B) Data expressed as ng Pt/mg protein. Data represents mean values of three experiments. ■, L1210/0; ●, L1210/DDP; ▲, L1210/DACH.

Table 3. Comparison of cytotoxicity, uptake and DNA binding between *R,R*- and *cis*-isomers of DACH-Cl_4-Pt(IV)

	L1210/0		L1210/DDP		L1210/DACH	
	R,R	*cis*	*R,R*	*cis*	*R,R*	*cis*
IC_{50} (μM) (clonogenic assay)	1.45 ± 0.03[a]	1.7 ± 0.20	10.0 ± 0.20	56.9 ± 11.5[c]	102.7 ± 1.15	125.7 ± 23.1[c]
Uptake[b] (ng Pt/mg protein)	122.0 ± 27.8	101.1 ± 19.3[c]	72.7 ± 15.4	49.5 ± 13.5[c]	54.7 ± 23.1	56.2 ± 19.0
DNA binding[b] (ng Pt/mg DNA)	22.6 ± 9.9	16.5 ± 3.9	10.3 ± 2.7	7.5 ± 1.5[c]	13.1 ± 7.3	11.1 ± 5.9
Extrapolated uptake[d] (ng Pt/mg protein)	4.42 ± 1.01	4.30 ± 0.82	18.18 ± 3.85	70.41 ± 19.20[c]	140.44 ± 59.31	176.61 ± 59.71
Extrapolated DNA binding[d] (ng Pt/mg DNA)	0.82 ± 0.36	0.70 ± 0.17	2.58 ± 0.68	10.67 ± 2.13[c]	33.63 ± 18.74	34.88 ± 18.54

[a]Results are presented as mean ± SD (n = 3–5).
[b]Uptake and DNA binding studies were conducted at 40 μM drug concentration and 2 h incubation.
[c]$p < 0.05$ versus the *R,R*-isomer.
[d]Data were extrapolated by multiplying the respective values by IC_{50} and dividing by 40 (concentration at which uptake and DNA binding studies were conducted).

as shown in Figure 4, and was typical for the compounds under investigation in both sensitive and resistant cell lines over a 4 h incubation time and extracellular concentration up to 80 μM. A 2 h incubation and a 40 μM drug concentration were, therefore, chosen to examine the role of intracellular accumulation and DNA binding in the relative differences in potency/cross-resistance between the *R,R*- and *cis*-isomers in the L1210 tumor models. The data were expressed as ng Pt/mg protein to take into consideration cell volume differences between the cell lines.[7] As shown in Table 3, significantly greater cellular uptake of platinum (21–46%) was seen for the *R,R*-isomer than for the *cis*-isomer in L1210/0 and L1210/DDP, while accumulations of the two isomers were identical in L1210/DACH cells. Significant difference between the *R,R*- and *cis*-isomer in DNA-bound platinum was observed only in L1210/DDP cells, with 40% more of the *R,R*-isomer bound to DNA. Table 3 also shows uptake and DNA binding data extrapolated to the IC_{50} concentration for comparative purpose. The extrapolated data shows no differences between the two isomers in uptake and DNA binding in either the L1210/0 or L1210/DACH cells, while in L1210/DDP cells the intracellular platinum content and DNA-bound platinum for the *cis*-isomer were both about four times those for the *R,R*-isomer. The combined extrapolated data from the three cell lines and the two isomers provide good correlation between drug uptake and DNA binding ($r = 0.986$, $p < 0.0003$).

Discussion

In this study, we have demonstrated that large variations (up to 31-fold) can be seen in potency between stereo-isomers of DACH-Cl_4-Pt(IV). In general, the *trans*-isomers (*R,R*- and *S,S*-) were the most potent, tetraplatin intermediate between these two isomers and the *cis*-isomer least potent (Table 2). However, there was a large dependency on the tumor model; for instance, the A2780, A2780cp and L1210/DDP cells gave a 6- to 31-fold spread in IC_{50} between the isomers, while the L1210/0 and L1210/DACH cells demonstrated minor variations only. Bhuyan *et al.*[19] reported an 11-fold difference in potencies between tetraplatin, *R,R*- and *S,S*-isomers in the cisplatin-sensitive murine L1210 cell line in a growth inhibition assay, whereas our study using identical conditions and the same cell line demonstrated only a 3-fold difference. At this stage, we do not know the reason for this inconsistency. It is worth noting, however, that the range of potencies for the complexes against the human parental A2780 line reported by us and these authors[19] was similar. Furthermore, we have made the important observation in the present study that for platinum complexes data and conclusions are similar using either murine L1210 or human A2780 tumor models and either growth inhibition or clonogenic assay.

Data of Behrens *et al.*[16] and Perez *et al.*[20] confirmed the potential value of tetraplatin in the ovarian A2780cp model. In contrast, Bhuyan *et al.*[19] reported only a minimal advantage of tetraplatin

over cisplatin in cisplatin-resistant A2780 lines. Our data, however, agree with those of Bhuyan *et al.*[19] with this cell line, and demonstrate that the degree and pattern of cross-resistance varies between cell lines. We have found, for instance, that the L1210/DDP line is almost non-cross-resistant to tetraplatin. The degree of cross-resistance was also isomer-dependent, with the *R,R*- and *S,S*-isomers demonstrating a lower level of cross-resistance to cisplatin than the *cis*-isomer.

The substantial difference in potency between *trans*- and *cis*-isomers has not been previously investigated, and may be due to several factors, including differences in drug uptake, intracellular distribution, formation and repair of DNA–platinum adducts. Jannerwein *et al.*[27] studied kinetics of reaction of DNA with isomers of DACH-SO_4-Pt(II) and concluded that the qualitative and quantitative formation of DNA adducts was similar for the isomers. Quantum mechanical calculations, on the other hand, indicate that DNA adducts of the *R,R*- or *S,S*-DACH isomer are more stable than those of the *cis*-isomer, which may provide a partial explanation for isomeric differences in biological activity.[28] However, it needs to be recognized that these differences are tumor model-dependent, so that other mechanisms beyond DNA adduct formation need to be explored. We have examined intracellular drug uptake and platinum binding to DNA as possible explanation for the difference in potency between the *R,R*- and *cis*-isomer. Our results using L1210 cells provide a correlation between reduced uptake or DNA platination and the lower biological potency of the *cis*-isomer in the L1210/DDP cells, but this 1.5-fold difference in uptake does not account fully for the 6-fold difference in IC_{50} values between the *R,R*- and *cis*-isomers. Furthermore, DNA binding data, extrapolated to the IC_{50} concentration, suggest that the L1210/DDP line can tolerate four times as much DNA platination by the *cis*-isomer than the *R,R*-isomer at equitoxic concentrations (Table 3).

In conclusion, isomers of DACH-Cl_4-Pt(IV) demonstrate tumor model-dependent differential potencies, which appear to be only partially explained by differences in intracellular accumulation or total DNA adduct formation. Other mechanisms, such as DNA repair, are currently under considerations as providing possible explanations for the isomeric effects. These data, nevertheless, suggest that the *R,R*-DACH-Cl_4-Pt(IV) may have greater potential for clinical utility than the *S,S*- and *cis*-isomers.

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