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Antitumor Activity against Human Tumor Samples of *cis*-Diamminedichloroplatinum(II) and Analogues at Equivalent *in Vitro* Myelotoxic Concentrations[1]

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ABSTRACT

We compared the antitumor activity of *cis*-diamminedichloroplatinum(II) (cisplatin; CDDP) with three CDDP analogues: *cis*-diammine-1,1-cyclobutanedicarboxylateplatinum(II) (CBDCA), *N*-methyliminodiacetato-1,2-diamino(cyclohexane)platinum(II) (MIDP), and *N*-(2-hydroxyethyl)-iminodiacetato-1,2-diamino(cyclohexane)platinum(II) (HIDP). Fresh human tumor samples in the adhesive tumor culture system were utilized for this comparison. The equitoxic concentrations of all four drugs were derived based on their inhibitory activity against human bone marrow samples. For these normalized concentrations, CDDP proved to have a higher cytotoxic activity than its analogues. CBDCA's *in vitro* activity had a significant correlation with CDDP activity ($r = 0.67$) *in vitro*. However, the structurally similar substances MIDP and HIDP demonstrated a much greater degree of association ($r = 0.90$). Our data suggest that CBDCA, HIDP, and MIDP have overall less activity than CDDP when tested at equitoxic *in vitro* concentrations. Close association between CDDP and CBDCA also reflects known clinical experience with these two drugs, suggesting the method of comparison used here is probably appropriate. These conclusions, however, must be validated by clinical trials.

INTRODUCTION

Since its introduction into clinical trials in 1972, cisplatin (CDDP)[6] has been found effective for the treatment of many types of cancer (1–8). However, the side effects of CDDP therapy are considerable, including nephrotoxicity, gastrointestinal toxicity, neurotoxicity, ototoxicity, and visual disturbances (1, 8–11). Of these, renal impairment and neurotoxicity are the major dose-limiting toxicities. Intravenous hydration with mannitol diuresis and hypertonic saline (12) and administration of sulfur-containing platinum-binding agents, such as penicillamine (13, 14), thiourea (15), and sodium thiosulfate (16, 17), have been used to reduce the severity of nephrotoxicity. Although not dose-limiting, CDDP-induced myelosuppression has also been noted in several clinical studies (10, 11, 18).

In order to circumvent or reduce major dose-limiting toxicity, and to perhaps improve antitumor efficacy, second generation CDDP analogues have been synthesized (19–26). Many of these analogues have resulted in less nephrotoxicity in preclinical models, and several have been selected for clinical evaluation (27–30). Among them is carboplatin (CBDCA), a nonnephrotoxic analogue that has shown greater potential in various Phase I and II clinical trials (5, 16, 31–33). Two recently developed CDDP analogues, MIDP and HIDP (34), are active and less nephrotoxic in animal models (35).

To help define a role for these analogues relative to CDDP and CBDCA in the treatment of human tumors, the present study explores the concept of evaluating antitumor activity of CDDP, CBDCA, MIDP, and HIDP (Fig. 1) in the adhesive human tumor-cell culture system (ATCCS) (36, 37) at equitoxic concentrations to human bone marrow granulocyte-macrophage colony-forming cells *in vitro*.

MATERIALS AND METHODS

Drug Preparation. All drugs were dissolved in 0.9% sodium chloride, stored in 0.2 ml (20X) aliquots at −70°C, and used within one month. CDDP and CBDCA were obtained from Bristol-Myers, Inc. (Syracuse, NY). MIDP and HIDP were synthesized in the Pharmacology Department at The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston (Houston, TX).

Human Bone Marrow GM-CFC Cultures. The concentrations of the drugs that produced 50% (IC_{50}) and 90% (IC_{90}) inhibition of human bone marrow GM-CFC *in vitro* were determined as follows. Normal human bone marrow was obtained from the posterior iliac crest by aspiration. Donor consent and institutional approval were obtained. The marrows were collected in 1 ml of calcium-free Dulbecco's PBS containing 300 units of preservative-free heparin. Mononuclear cells were concentrated by Ficoll-Hypaque density separation (density, 1.077 g/ml). GM-CFC was measured by a modified bilayer soft-agar culture procedure (38). Unless specified, tissue culture reagents were purchased from K. C. Biological (Kansas City, KS). A 1-ml nutrient underlayer consisting of Eagle's alpha modification of minimal essential medium (α-MEM), 15% FBS, 10% colony-stimulating factor (human placental-conditioned medium) (40), and 0.5% Bacto-agar (DIFCO, Detroit, MI) was placed into 35-mm plastic culture dishes. After solidification of the underlayer, 1 ml of α-MEM upper layer containing 1.5×10^5 mononuclear cells, 0.32% agar, 15% FBS, and appropriate concentrations of drugs (or diluent as controls) was added. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 12% O_2 for 8 to 10 days. Five experiments, using cells from five different donors, were done for each compound to determine GM-CFC toxicity over a several-log range of concentrations. Experiments yielding at least 30 GM-CFC colonies of 40 or more cells in the control plates were considered evaluable and scored using an Olympus 200M stereo microscope.

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[6] The abbreviations used are: CDDP, *cis*-diamminedichloroplatinum(II); CBDCA, *cis*-diammine-1,1-cyclobutanedicarboxylateplatinum(II); MIDP, *N*-methyliminodiacetato-1,2-diamino(cyclohexane)platinum(II); HIDP, *N*-(2-hydroxyethyl)-iminodiacetato-1,2-diamino(cyclohexane)platinum(II); FBS, fetal bovine serum; GM-CFC, granulocyte-macrophage-colony-forming cells; PBS, phosphate buffered saline; IC, inhibition concentration; ATCCS, adhesive tumor-cell culture system.

Survival curves were constructed on semilog scale and the IC_{50} and IC_{90} values for each drug were calculated by interpolation.

Primary Human Tumor Cell Culture. *In vitro* antitumor activity of the drugs was tested at GM-CFC equitoxic concentrations using primary human tumor cells in the ATCCS (36, 37). Human tumor specimens were collected from the Department of Pathology and transported to our laboratory in tissue culture medium. Preservative-free heparin (10 unit/ml) was added to fluid samples immediately after aspiration. Both solid specimens (minced into 1-mm cubes) and fluid specimens were incubated overnight at 37°C with continuous stirring in a mixture containing 0.75% collagenase (Type III; Cooper Biomedical, Malvern, PA), 0.005% DNase (Sigma Chemical, St. Louis, MO), and 10% FBS. The disaggregated cells were then washed with Hanks' balanced salt solution (calcium and magnesium free). Cell count and viability of the nucleated cells larger than 10 μm were determined using a hemocytometer and the trypan blue exclusion method.

The digested tumor cell suspensions were suspended in a 0.3% methyl-cellulose F12 attachment medium and then inoculated in 24-well culture plates in duplicate, at a seeding density of 25,000 cells/well. The plates were previously coated with an extracellular matrix material for enhancement of tumor cell attachment and growth (36). Because plating efficiency is unpredictable with primary cell culture, a cell inoculum titration was made at 25,000, 12,500, 6,250 and 3,125 cells/well to evaluate the plating efficiency of the culture at low cell density and to extrapolate the true control values in overplated cultures (40). Additional controls included a Day 1 culture fixed after 24 h of incubation to record the starting cell population, a culture treated continuously with [^{3}H]thymidine (10 μCi/ml, 56 μCi/mmol; ICN Radiochemicals, Irvine, CA) to estimate the nondividing background adherent cell population and a cell-free well that contained only culture medium to serve as the blank. The remaining wells were used for drug exposure tests.

Drug Exposure and Analysis. After a 24-h attachment period, the medium was removed, the plates were washed with PBS, and the adherent cells refed with Ham's F12 culture medium containing 10% swine serum (J. R. Scientific, Woodland, CA), 10 μg/ml transferrin, 0.5 μg/ml hydrocortisone, 5 ng/ml epidermal growth factor (41), 5 μg/ml insulin (Collaborative Research, Lexington, MA), 100 unit/ml penicillin, 100 μg/ml streptomycin (GIBCO, Grand Island, NY), and 2.7 μg/ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. Drugs, reconstituted in the same medium, were added to the cells at this time. Each drug was tested at four concentrations over a 6- to 8-fold range, at the equitoxic GM-CFC concentrations and above. After a 5-day incubation with cells, the drugs were removed by aspiration, and the cultures replenished with drug-free medium and reincubated for 7 more days. Both incubations were in a humidified atmosphere of 5% CO_2 in air. At the end of incubation, the plates were washed in PBS, and the cells fixed in 70% ethanol for 20 min and then stained with a 0.05% crystal violet solution. Tumor cell growth was quantitated by measuring the cell staining density by digital image analysis (36). The surviving fraction for each drug concentration was plotted against drug concentration on semilog scales and the IC_{50} value determined by interpolation for each drug.

Reproducibility of the Primary Cell Culture Assay. Intralaboratory variations in the primary human tumor-cell culture assays were assessed by dividing the cell yield following enzymatic digestion between two technicians, who then cultured the specimens with separately prepared drug sets and independently derived the IC values. Interlaboratory reproducibility of the assay was also evaluated similarly between two independent laboratories. Results were analyzed by the linear regression method. The correlation coefficients were 0.99 for four intralaboratory experiments (10 pairs of drug points) and 0.79 for six interlaboratory determinations (six pairs of drug points).

Ranking of Drug Activity and Comparison of Cross-Reactivity. Equitoxic GM-CFC IC values were used to normalize *in vitro* cytotoxicity. The therapeutic index (TI; GM-CFC IC_{50}/human tumor IC_{50}) was calculated, and the relative *in vitro* therapeutic efficacy of each drug was determined according to the ranking of its TI, using Friedman's two-way analysis of variance by rank (42). Cross-reactivity (resistance or sensitivity) between drugs was analyzed by the linear regression method and Spearman's rank correlation coefficient method (43).

RESULTS

***In Vitro* Toxic Dose Range of Human Bone Marrow GM-CFC.** The GM-CFC IC_{50} and IC_{90} values for CDDP were 0.29 and 0.78 μg/ml, respectively. The GM-CFC IC_{50} and IC_{90} values for the platinum analogues tested were all greater than that for CDDP on a weight basis (Fig. 2 and Table 1). Specifically, the IC_{50} and IC_{90} values for MIDP, CBDCA, and HIDP were 1.3-, 2.1-, and 4.1-fold greater than that for CDDP, respectively (Fig. 2).

Chemosensitivity of Human Tumor Cultures. Using the above

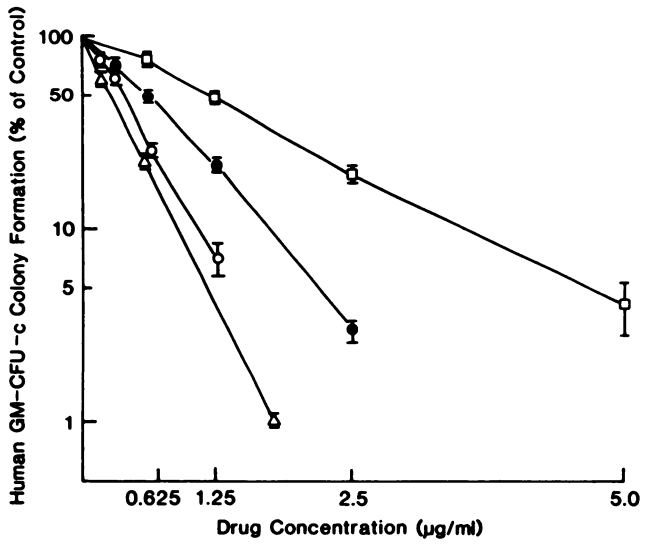

Fig. 2. Dose response of human bone marrow GM-CFC to cisplatin (△), CBDCA (●), MIDP (○), and HIDP (□). *Point*, mean ± SEM of five experiments from cells of five different donors.

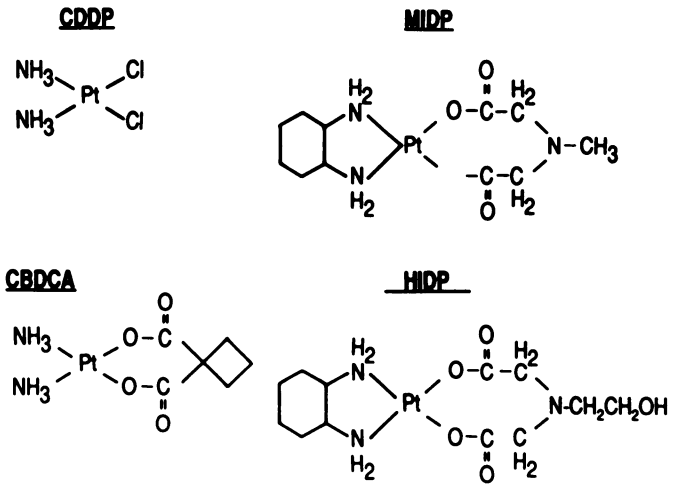

Fig. 1. Chemical structures of cisplatin (CDDP) and its analogues CBDCA, MIDP, and HIDP.

Table 1 *Inhibitory concentrations to bone marrow GM-CFC and tumor growth*

The values (μg/ml) are mean ± SEM from five GM-CFC experiments and 31 human tumor specimens.

Drug	GM-CFC (IC_{50})	GM-CFC (IC_{90})	Human tumors (IC_{50})
Cisplatin	0.29±0.09	0.78±0.04	0.14±0.03
CBDCA	0.60±0.03	1.69±0.06	0.61±0.12
MIDP	0.39±0.01	1.08±0.06	0.88±0.18
HIDP	1.19±0.06	3.60±0.29	3.50±0.62

data, a panel of human tumors was subsequently tested in the ATCCS at their GM-CFC IC_{50} to IC_{90} equitoxic drug concentrations. This meant that three analogues were tested at doses weighing 1.3 to 4.1 times more than CDDP (see Table 1 and Fig. 2).

Tumor response *in vitro* was defined as 50% inhibition of growth within the GM-CFC dose-range. The 50% and 90% inhibitory concentrations were calculated for each drug on every tumor specimen if drug sensitivity was seen within the range tested. Altogether, 31 tumor specimens (15 nonsmall cell cancer of the lung, six melanoma, three ovarian, and seven miscellaneous) were evaluated.

The response rates to CDDP and its analogue are shown in Table 2 and Fig. 3. *In vitro* tumor response rate to CDDP was higher (>90%) than any of its analogues; however, a dose response was evident with all analogues (Table 2). The two structurally similar analogues (MIDP and HIDP) exhibited a similar range of response rates (Table 2). Another way of showing the relative response rates is to normalize the *in vitro* therapeutic efficacy by dividing GM-CFC IC_{50} by tumor IC_{50} values (Table 3). CDDP was again found to have the highest therapeutic index, followed in order by CBDCA, MIDP, and HIDP.

The correlation between the therapeutic index of CDDP and its analogues on individual tumors was analyzed (Table 4). CDDP and CBDCA showed a significant overall correlation ($r = 0.67$) and highest degree of association in lung tumor ($r = 0.95$) cell cultures. The association between CDDP and the two new analogues (MIDP and HIDP) ($r = 0.49$ and 0.56, respectively) was generally less than with CBDCA, with the exception of the lung tumor cell cultures, in which MIDP appeared to correlate well with the therapeutic efficacy of CDDP ($r = 0.79$).

CBDCA was found to more closely associate with MIDP overall and in lung tumors than HIDP (Table 4). The structurally similar MIDP and HIDP had high associations overall ($r = 0.90$), in lung tumors ($r = 0.85$), in melanoma cultures ($r = 0.97$, data not shown), and in a small number of mixed tumor types ($r = 0.97$, data not shown).

Table 2 *Response rates (%) of human tumors at equitoxic GM-CFC IC_{50} and IC_{90} doses*

Drug	IC_{50}	IC_{90}
Cisplatin	94	100
CBDCA	55	94
MIDP	32	71
HIDP	29	65

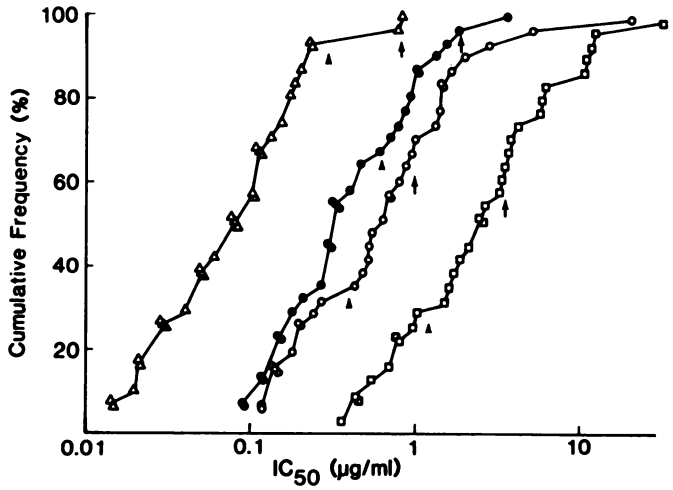

Fig. 3. The IC_{50} cumulative response rate of 31 tumors to cisplatin (△), CBDCA (●), MIDP (○), and HIDP (□). The average GM-CFC IC_{50} value for each respective drug is indicated by ▲, and the average GM-CFC IC_{50} value by ↑. The response rate of tumors within and below the GM-CFC dose range is clearly greater for cisplatin (△) and CBDCA (●), followed by MIDP (○) and HIDP (□).

Table 3 *In vitro therapeutic index of cisplatin and analogues to human tumors*

Figures are the mean ± SEM from 31 human tumors. Therepautic index = IC_{50} to GM-CFC/IC_{50} to human tumors.

Drug	Therapeutic index
Cisplatin	5.7 ± 1.0
CBDCA	2.3 ± 0.3[a]
MIDP	1.1 ± 0.2[a,b]
HIDP	0.9 ± 0.2[a,b,c]

[a] $P < 0.005$ compared to cisplatin.
[b] $P < 0.02$ compared to CBDCA.
[c] $P < 0.01$ compared to MIDP.

DISCUSSION

It is essential to develop analogues to active drugs that have limiting extramedullary toxicities such as renal, cardiac, or neurological toxicity. However, it has been more difficult to identify analogues superior to the original drug than it has been to identify drugs with totally different mechanisms of action and chemical structures. Particularly at the *in vitro* level, some methods of defining whether an analogue would be superior to the parent compound should be devised.

We have previously reported on the approach of defining the response rates of drugs in the ATCCS within the dose range defined by *in vitro* GM-CFC toxicity and comparing that response rate with standard agents (37). Using this system, active drugs are defined as drugs that caused a response rate (tumor growth inhibition of either 50% or 90%) of 30% and above up to a GM-CFC IC_{90}. In comparing analogues, the first criterion would be that all analogues achieve comparable or superior response rates and that the patterns of cross-reactivity between the analogues and the parent drugs be evaluated in case the analogue has a different activity spectrum. However, to gain a true perspective on the relative response rate of the parent and analogue drugs *in vitro*, a measure of cross-reactivity at equitoxic concentrations is fundamental.

The testing of CDDP and the three related compounds, CBDCA, MIDP, and HIDP, against 31 tumors has helped to validate our approach and improve our understanding of the relationship of these drugs to one another. Firstly, all three analogues fulfill our *in vitro* criteria for being active agents, demonstrating a response rate of 30% or above within the GM-CFC IC_{90} range. The parent compound, CDDP, achieved the highest *in vitro* response rate at normalized concentrations.

Table 4 *Correlation of cross-reactivity between cisplatin analogues*

Correlation values (r) were analyzed by the linear regression method. Significance values (p) were determined by Spearman's rank correlation coefficient for association analysis.

	CBDCA	HIDP	MIDP
All tumors ($n = 31$)			
Cisplatin	$r = 0.67$	$r = 0.49$	$r = 0.56$
	$p < 0.002$	$p < 0.05$	$p < 0.01$
CBDCA		$r = 0.6$	$r = 0.76$
		$p < 0.05$	$p < 0.002$
HIDP			$r = 0.90$
			$p < 0.002$
Lung tumors ($n = 15$)			
Cisplatin	$r = 0.95$	$r = 0.62$	$r = 0.79$
	$p < 0.001$	$p > 0.05$	$p < 0.005$
CBDCA		$r = 0.57$	$r = 0.83$
		$p < 0.05$	$p < 0.001$
HIDP			$r = 0.85$
			$p < 0.001$

However, the superior response rate of CDDP should be assessed with some reservations, because its clinical activity may be limited by extramedullary toxicities and it is only moderately marrow sparing. It could be that CDDP was tested at a higher biologically equivalent concentration than the other analogues, so therapeutic activity may be more equal than it appears. The superior response rate of CDDP over other analogues within the GM-CFC range is further exemplified by its therapeutic index (Table 3).

Prior experience[7] has demonstrated that no significant associations exist between the different classes and concentrations of many standard drugs needed to inhibit tumor growth 50% or 90% on the same tumor specimens. In these 31 tumors, some relationship of response among all drugs was observed, and a stronger relationship was evident between those drugs most related structurally (Fig. 1). The best correlation was between MIDP and HIDP (Table 4), suggesting that both are likely to have similar clinical spectrum. In cross-reactivity studies between CDDP and CBDCA, although seen in clinical trials, there was the occasional tumor which responded to a lower concentration of CDDP and a higher concentration of CBDCA (data not shown). A larger number of tumors should be tested against CDDP and CBDCA *in vitro* to more clearly delineate the cross-reactivity. The relationship of CDDP to the newer analogues, HIDP and MIDP, was less than that to CBDCA; this appeared to be due to a number of tumors being responsive to CDDP and unresponsive to HIDP or MIDP. Although the correlations for specific tumor types were not indicated due to the small numbers of some tumor types, there appears to be some tumor type specificity among the compounds. For example, the *in vitro* ranking by the two-way analysis of variance of Friedman (42) to be CDDP>CBDCA>MIDP>HIDP for all tumors ($p < 0.005$). However, the order for lung tumors (Table 4) is significantly different, with CDDP>CBDCA>HIDP>MIDP ($p < 0.005$). Therefore, a Phase II *in vitro* screening may provide valuable preclinical information.

Testing analogues at equitoxic concentrations in the ATCCS appears valid, because the *in vitro* degree of cross-reactivity with CDDP and CBDCA is already borne out by the current clinical experience.

The newer clinical analogues show less cross-reactivity with CDDP, but also have lower response rates and may not be as clinically useful as CDDP or CBDCA. This conclusion, however, awaits confirmation by clinical trials.

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