Antitumor Action of Dichloro(4,5-dimethyl-*o*-phenylenediamine-*N*,*N*′) platinum(II)[1] (37239)

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The observations by Rosenberg and coworkers (1–3) of the effects of certain platinum complexes on growth of *Escherichia coli* led to the subsequent demonstration of the particular effectiveness of *cis*-dichlorodiammineplatinum(II) (*cis*-PtII, NSC-119875) against a number of rodent tumors (4–10); clinical trials of this compound have been reported from several institutions (11–14).

We report now the antitumor action of a bidentate arylamine congener of *cis*-PtII, along with studies of certain of its effects on tumor cells *in vitro*.

Materials and Methods. The previously undescribed dichloro(4,5-dimethyl-*o*-phenylenediamine-*N*,*N*′)platinum(II) (DDPP) was synthesized from 4,5-dimethyl-*o*-phenylenediamine (Aldrich Chemical Co.) and potassium tetrachloroplatinate (a generous gift from Matthey Bishop, Inc.) by a straightforward adaptation of a method used for the synthesis of other *cis*-dichloro-platinum compounds containing amine ligands. The ligand (0.33 g) was dissolved in 100 ml of distilled water and acidified slightly with a few drops of 1.0 *N* HCl. To this was added a filtered solution of 1.0 g of K_2PtCl_4 in 10 ml of water, and the mixture was heated in a water bath at 70° for 30 min. The yellow product was filtered, washed with water and ether, and dried overnight at 45°. The yield was 76%. Elemental analyses (Galbraith Laboratories) showed the following percentage values (theoretical values are in parentheses): C, 23.87 (23.89); H, 2.95 (3.01); N, 6.87 (6.97); Cl, 17.75 (17.63); Pt, 48.48 (48.51). Its infrared absorption spectrum, recorded as a KBr pellet with a Perkin-Elmer model 467 spectrometer, is shown in Fig. 1, and its structural relationship to *cis*-PtII is shown in Fig. 2.

The course of the hydrolytic aquation reaction of DDPP, in which the neutral dichloro complex is converted to the di-aquated, doubly positively charged species upon the loss of two chloride ions, was measured with a Buchler-Cotlove chloridometer.

The *E. coli* strain B (ATCC no. 11303) was grown on a synthetic agar medium (15). Cells from the periphery of the inhibitory zone surrounding a few crystals of DDPP placed directly on the seeded agar were stained with gentian violet and photographed.

The Ehrlich ascites tumor was maintained in BALB/c mice (Flow Laboratory Animals) and the L1210 leukemia in BDF_1 mice (Sprague-Dawley). DDPP for ip injection was prepared by sonication in 0.9% NaCl to obtain a suspension sufficiently disperse to pass readily through a No. 24 hypodermic needle.

The rates of synthesis of DNA, RNA, and protein in Ehrlich ascites tumor cells *in vitro* were assessed by the respective rates of incorporation of thymidine-methyl-^{3}H, uridine-5-^{3}H, and L-leucine-^{3}H (New England Nuclear Corp.) into the acid-insoluble fraction of the cells. The cell suspensions were prepared at 1.0% (v/v) in Eagle's minimum essential medium with Hanks' balanced salt solution (MEM). Each isotopically labeled precursor was added to a final activity of 0.5 μCi/ml. DDPP was added in dimethylsulf-

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oxide (DMSO); the final DMSO concentration was 1.0% in all vessels including controls. The reactions were terminated with an equal volume of cold 10% trichloroacetic acid (TCA), and the acid-insoluble material was washed thrice with cold 5% TCA. After solubilization in 2.0 ml of 1.0 *M* hydroxide of Hyamine and addition of a phosphor solution composed of PPO (5 g/liter) and PO POP (0.3 g/liter) in toluene, radioactivity was measured with a liquid scintillation spectrometer (Nuclear Chicago Corp., Mark I).

For studies of the effects of DDPP on cell viability *in vitro,* tumor cells were prepared at 0.4% (v/v) in a suspending medium of MEM:ascitic fluid:0.067 *M* phosphate buffer, pH 7.4, in proportions of 8:1:1, and a 2×10^{-4} *M* solution of DDPP or *cis*-PtII was prepared in the same medium. One part of this solution was added to 1 part of the cell suspension, yielding a 0.2% cell suspension and a DDPP concentration of 10^{-4} *M*. At intervals during incubation at 37°, a drop was removed from each vessel, mixed with a small volume of 1% trypan blue on a slide, and viable cells were detected as those which totally excluded the dye.

Results and Discussion. The course of the aquation reaction of DDPP, as well as of *cis*-PtII, is shown in Fig. 3. Even though the initial rate of loss of chloride from DDPP was somewhat less than that of the corresponding reaction of *cis*-PtII, equilibrium values attained with the two compounds were virtually identical. The relative insolubility of DDPP in aqueous media necessitated performing these measurements on a solution of each compound in a DMSO:water mixture of 1:1. The equilibrium value attained with *cis*-PtII in water without DMSO corresponded to a value of only 31% hydrolysis, but meaningful measurements could not be made with DDPP in water without the solubilizing action of DMSO.

The morphologic aberration incurred by cells of *E. coli* grown in the presence of DD PP is shown in Fig. 4. This filamentous type of growth is typical of the so-called "unbalanced" type, and is generally considered to represent maintenance of cell synthetic processes in spite of failure of some step in the replicative mechanism.

The increased survival times of BALB/c

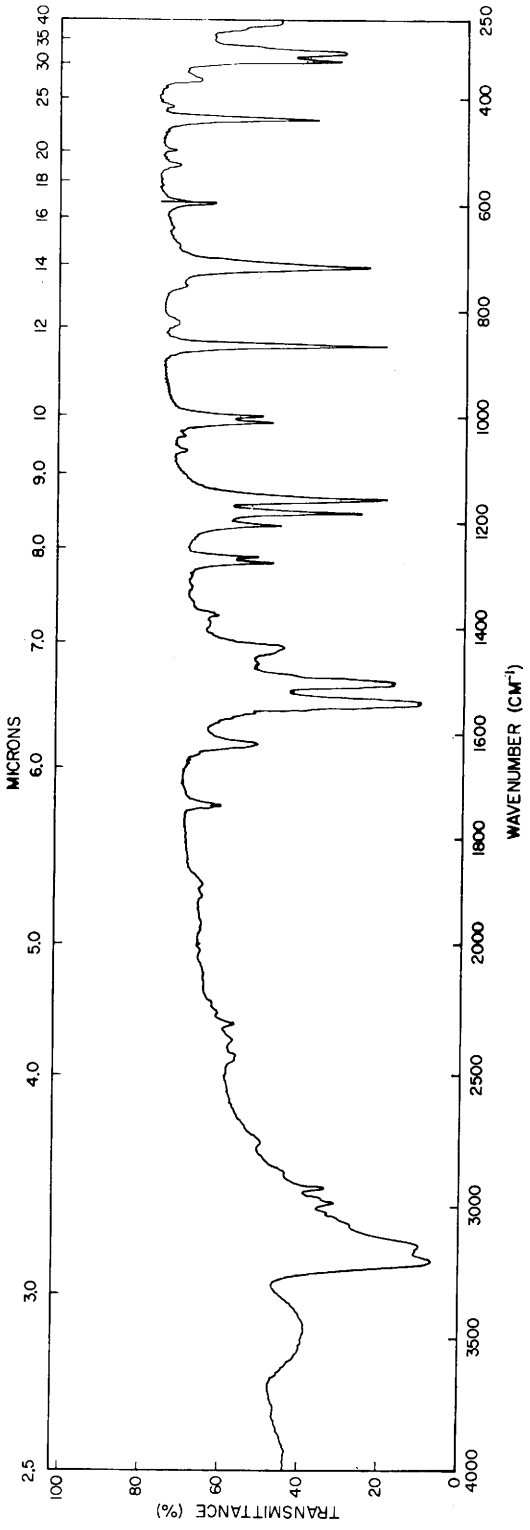
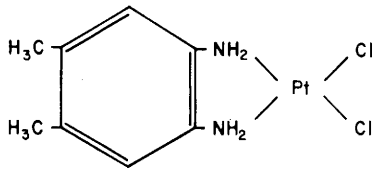
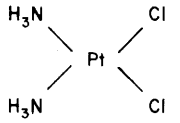


FIG. 1. Infrared absorption spectrum of DDPP, measured as a KBr pellet in a N_2 atmosphere.



DICHLORO(4,5-DIMETHYL-O-PHENYLENEDIAMINE-N,N')PLATINUM (II)
(DDPP)



cis-DICHLORODIAMMINEPLATINUM (II)
(cis-Pt II, NSC-119875)

FIG. 2. Structural relationships of DDPP and *cis*-PtII.

mice bearing the Ehrlich tumor and which were treated with DDPP are shown in Table I. Even though a substantial weight loss occurred at the highest doses used, there were no deaths resulting from toxicity; each mouse which succumbed had clear evidence of gross tumor development. Only 1 of 6 dosage regimens yielded a mean increased survival time of less than 100%.

In 3 experiments in which BDF_1 mice bearing the L1210 leukemia were treated with various regimens of DDPP, each dose schedule tested increased the mean survival time over 40%, and 10 of 12 regimens yielded a mean increased survival of over 50% (Table II). A single injection of 200 mg/kg caused no fatalities; however, a total dose of 300 mg/kg given as 2 equal injections on Days 1 and 5 was quite lethal.

To assess any similarity of action of DDPP to that of *cis*-PtII (5), Ehrlich tumor cells were incubated with DDPP *in vitro* for various intervals prior to addition of radioactively labeled precursors to monitor the rates of synthesis of DNA, RNA, and protein. Figure 5 shows that each synthetic process was impeded following a 1.5-hr incubation with DDPP; the synthesis of RNA and protein proceeded at about one-fifth the control rate while synthesis of DNA ceased vir-

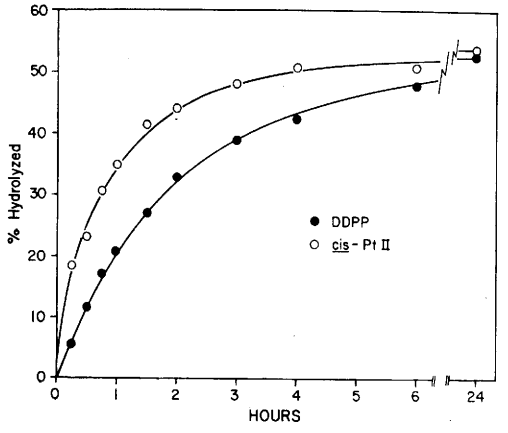


FIG. 3. Course of the aquation reaction of DDPP and of *cis*-PtII in H_2O:DMSO (1:1) at 25° as measured by loss of Cl^-. Each neutral complex was at an initial concentration of 6×10^{-3} *M*.

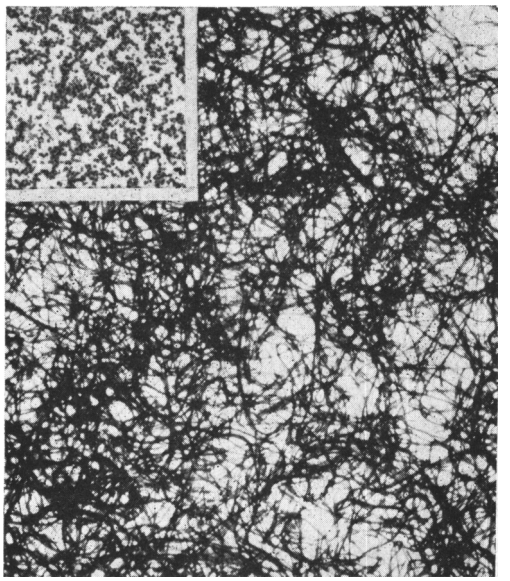


FIG. 4. Filamentous growth of *E. coli* strain B induced by DDPP. Inset, control cells. Gentian violet stain, ×300.

TABLE I. Effect of DDPP on Survival Times of BALB/c Mice Bearing the Ehrlich Ascites Tumor.[a]

Expt	Dose/injection (mg/kg)	Treated on day(s)	Mean survival time (days ± SD)	% Increase of mean survival time	p	Mean wt change[b] (g)	No. of toxic deaths
A	0		16.9 ± 2.5			+2.3	
	25	1	29.1 ± 16.8	72	<.10	+0.1	0
	50	1	43.6 ± 11.5	158	<.001	−2.6	0
	100	1	37.0 ± 9.3	119	<.001	−5.0	0
B	0		14.6 ± 1.1			+3.3	
	10	1–7	31.5 ± 5.1	116	<.001	−3.4	0
	25	1,4,7,10	35.0 ± 3.2	140	<.001	−4.3	0
	40	1,7	40.3 ± 8.7	176	<.001	−3.8	0

[a] Each group contained 8 mice, and each mouse was given 10^7 tumor cells on Day 0.
[b] Between Days 1 and 8.

tually completely. These data differ from those obtained with *cis*-PtII (5) in only a temporal fashion. With the latter drug, the action was more gradual in onset, and substantially complete inhibition was attained only after about 4-hr incubation with the drug. With graded concentrations of DDPP and using a standard preincubation time of 1.5 hr, the 50% inhibitory concentrations of DDPP on synthesis of DNA, RNA, and protein were 3×10^{-5}, 5×10^{-5}, and 5×10^{-5} M, respectively.

To detect the extent of reversibility of the inhibitory action of DDPP Ehrlich tumor cells were incubated for 1.5 hr with the compound at concentrations which would yield 80–100% inhibition of synthesis of each macromolecular class. Aliquots were sedimented,

TABLE II. Effect of DDPP on Survival Times of BDF_1 Mice Bearing the L1210 Leukemia.[a]

Expt	Dose/injection (mg/kg)	Treated on day(s)	Mean survival time (hr ± SD)	% Increase of mean survival time	p	Mean wt change[b] (g)	No. of toxic deaths
A[c]	0		164 ± 8			+2.8	
	7.5	1–5	245 ± 20	49	<.001	−0.5	0
	15	1–5	270 ± 29	65	<.001	−0.7	0
	25	1,3,5	262 ± 37	60	<.001	−2.2	0
	100	1	248 ± 15	51	<.001	−0.5	0
B	0		168 ± 12			+2.8	
	100	1	276 ± 30	64	<.001	−1.0	0
	125	1	258 ± 18	54	<.001	−1.0	0
	150	1	283 ± 32	69	<.001	−2.1	0
C	0		163 ± 6			+2.6	0
	100	1,5	284 ± 29	74	<.001	−2.0	0
	150	1,5	(Toxic)			−1.7	7
	150	1	257 ± 16	58	<.001	−0.9	0
	175	1	268 ± 13	64	<.001	−1.1	0
	200	1	265 ± 21	62	<.001	−1.7	0

[a] Each group contained 8 mice, and each mouse was given 10^6 leukemia cells ip on Day 0.
[b] Between Days 1 and 6.
[c] Two groups of 8 mice were treated with *cis*-PtII in Expt A, yielding the following: at 10 mg/kg on Day 1 only, 91% increased survival; at 2.5 mg/kg on Days 1–5, 62% increased survival. The respective weight changes in these 2 groups between Days 1 and 6 were −3.2 and −2.8 g.

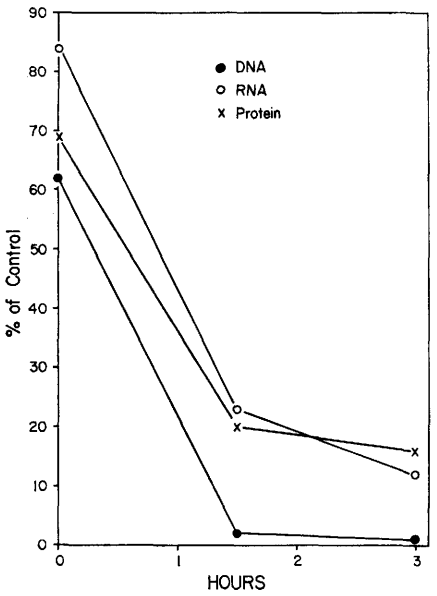


FIG. 5. Inhibition by DDPP (10^{-4} *M*) of synthesis of DNA, RNA, and protein in Ehrlich ascites tumor cells *in vitro*.

TABLE III. Persistence of the Inhibitory Action of DDPP on Synthesis of DNA, RNA, and Protein in Ehrlich Ascites Tumor Cells *in Vitro*.[a]

Synthesis of	DDPP (10^{-4} *M*) in Incubation medium	DDPP (10^{-4} *M*) in Washing medium	Inhibition (%)
DNA	—	—	
	+	—	93
	+	+	99
RNA	—	—	
	+	—	83
	+	+	92
Protein	—	—	
	+	—	80
	+	+	89

[a] A 1% (v/v) suspension of tumor cells in MEM was incubated with (+) or without (—) DDPP for 1.5 hr at 37°. The cells were then washed thrice with fresh MEM with (+) or without (—) DDPP, and finally suspended in the original volume in the appropriate medium. Aliquots of 3.0 ml were incubated for 20 min with thymidine-methyl-^{3}H, uridine-5-^{3}H, or L-leucine-^{3}H, each at 0.5 μCi/ml, prior to termination of the reaction with 3.0 ml of cold 10% TCA. Values given in the last column are the averages of 3 separate experiments, each of which was done in duplicate.

washed either with MEM devoid of DDPP or with MEM containing the compound at the same concentration as was present during the 1.5-hr period of preincubation, and finally resuspended to the original volume in the appropriate medium. Subsequent measurement of the rate of incorporation of each labeled precursor showed that inhibition, once established, was irreversible within the limits of the experimental design (Table III). In addition, cells which were labeled with thymidine-methyl-^{3}H, uridine-5-^{3}H or L-leucine-^{3}H, washed free of unincorporated radioactivity, and then incubated with DDPP at a concentration of 10^{-4} *M* showed a considerable time-dependent loss of the incorporated label from the acid-insoluble fraction of the cells (Fig 6).

To obtain a direct measure of the loss of cell viability upon incubation with DDPP, cells were incubated in the supplemented MEM with the compound at 10^{-4} *M* and an aliquot was examined at each 30-min interval to determine the percentage of cells which excluded trypan blue. Figure 7 shows a more rapid rate of loss of viability in the presence of DDPP (50% nonviable cells after 2.8 hr) than occurred in the presence of an equivalent concentration of *cis*-PtII (50% nonviable cells after 6.2 hr). Since DDPP had a slower rate than *cis*-PtII as regards aquation with the loss of Cl^- (Fig. 3), presumably an event tantamount to pharmacological "activation," the more rapid rate of loss of cell viability conferred by DDPP may be a reflection of a more rapid rate of passage of this organoplatinum congener through the cell membrane.

Summary. Dichloro(4,5-dimethyl-*o*-phenylenediamine-*N*,*N*')platinum(II) (DDPP) was synthesized by the reaction of 4,5-dimethyl-*o*-phenylenediamine with potassium tetrachloroplatinate. The resulting compound extended up to 176% the survival times of BALB/c mice bearing the Ehrlich ascites tumor and up to 74% the survival times of BDF_1 mice bearing the L1210 leukemia. In studies using Ehrlich ascites tumor cells *in vitro*, DDPP was found to be a potent inhibitor of synthesis of DNA, RNA, and protein; the concentrations which conferred 50% inhibition following 1.5 hr of incubation with the

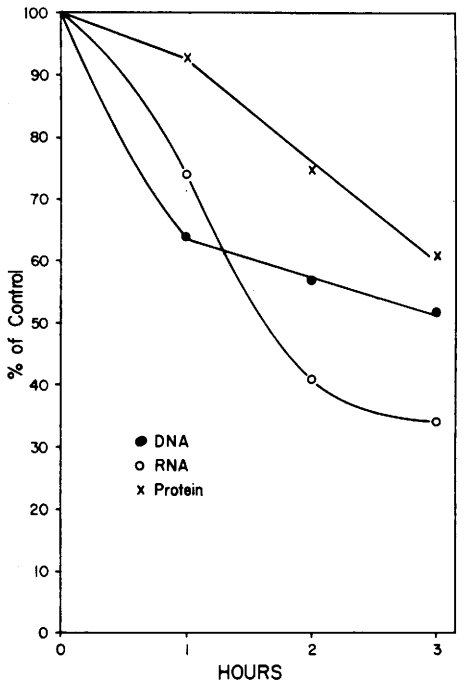


FIG. 6. Decrease in acid-insoluble radioactivity in cells containing thymidine-methyl-^{3}H-labeled DNA, uridine-5-^{3}H-labeled RNA, and L-leucine-^{3}H-labeled protein upon subsequent incubation with DDPP (10^{-4} *M*).

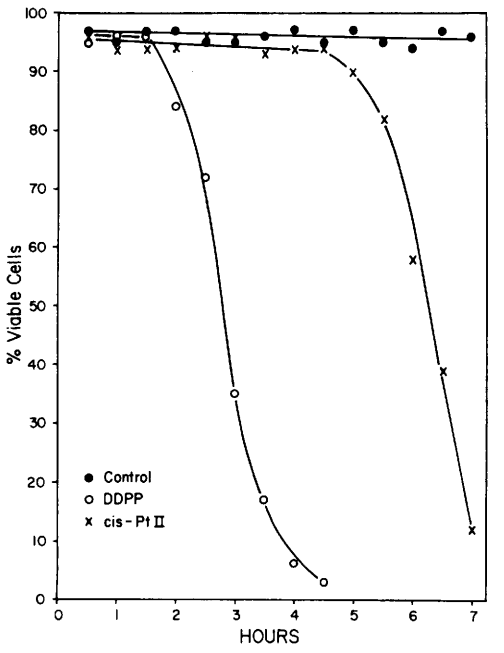


FIG. 7. Reduction of viability of Ehrlich ascites tumor cells *in vitro* as a function of time of incubation with DDPP or *cis*-PtII (each at 10^{-4} *M*). A total of 200 cells was counted to obtain each point on the graph, and the values are the means of 3 experiments.

compound were 3×10^{-5} to 5×10^{-5} *M*. Inhibition of nucleic acid and protein synthesis *in vitro*, once established, was not ameliorated by washing the cells with fresh medium devoid of DDPP. Cellular DNA, RNA, and protein, each appropriately labeled with a radioisotopically labeled precursor, became more acid-soluble upon incubation of the cells *in vitro* with DDPP. The rate of loss of viability of cells incubated *in vitro* with DDPP, as assessed by the trypan blue exclusion method, was found to be considerably greater than the rate obtained upon incubation of cells with an equivalent concentration of *cis*-dichlorodiammineplatinum(II).

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