Antitumor and Antimitogenic Properties of the Photochemical Reaction Product of Ammonium Hexachloroiridate(IV)[1] (35457)

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Rosenberg *et al.* (1) observed that the complex hexachloroplatinate ion ($[PtCl_6]^{2-}$) in the presence of NH_4^+ ions underwent a photochemical reaction in which the chloride ions of the ligand were exchanged sequentially for 1, 2, or 3 ammonia molecules. There was a decrease of 1 negative charge on the ion at each step. The doubly negative (nonirradiated) species inhibited growth of *Escherichia coli,* the singly negative species was without effect, and the neutral species inhibited cell division without any apparent effect on other cell processes, thus inducing filamentous, unbalanced growth. The *cis* isomers of the neutral chloroammino species of platinum(II) and platinum(IV) inhibited the development of leukemia L1210 and sarcoma 180 in mice (2). The neutral form of platinum(II) also retarded markedly the rate of development of the Ehrlich ascites carcinoma; biochemical studies indicated that this may have been mediated through a pronounced and persistent inhibition of deoxyribonucleic acid synthesis (3).

The chemical proximity of iridium to platinum prompted a recent study in this laboratory of possible changes which the corresponding hexachloroiridate ion ($[IrCl_6]^{2-}$) may undergo under similar conditions (4). Spectrophotometric and electrophoretic studies were compatible with a conversion of a doubly negative species, through a less negatively charged intermediate, to a neutral species. Nonirradiated solutions were quite inhibitory to the growth of *E. coli,* but irradiated solutions were much less so. Solutions which were irradiated for 5 to 7 hr retarded bacterial cytokinesis, indicative of selective inhibition of deoxyribonucleic acid synthesis.

The present study was initiated to determine if the photochemical reaction product of ammonium hexachloroiridate(IV) exerts an antitumor action as does that of the corresponding platinum compound, as well as to assess its effect on phytohemagglutinin-induced lymphocyte mitogenesis.

Materials and Methods. Irradiation of hexachloroiridate solutions. Ammonium hexachloroiridate(IV) was purchased from Alfa Inorganics, Inc. Solutions (up to 15 mg/ml) were prepared in distilled water. Vials containing these solutions were placed into quart Mason jars containing 25 ml of 3% NH_4OH, and the jars were closed with standard lids. The solutions were irradiated approximately 2 in. from a lamp fitted with 2 General Electric 15-W fluorescent tubes. After 16 to 18 hr (except as indicated otherwise), the solutions were removed from the NH_3 atmosphere, excess NH_3 which had solubilized during irradiation was removed by bubbling a stream of air through the solution, the volume of water lost through evaporation during bubbling was restored, and crystalline NaCl was added to a final concentration of 0.9%. Since the nature of the photochemical reaction product is not known, all concentrations are expressed in terms of $(NH_4)_2IrCl_6$ which was irradiated.

Antitumor assays. The strain of Ehrlich ascites carcinoma used was one which has been maintained in this laboratory for several years, and was passaged in BALB/c mice (Flow Research Animals, Inc.). The leukemia L1210 donor and host BDF_1 mice were

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TABLE I. Effects of the Photochemical Reaction Product of Ammonium Hexachloroiridate (IV) on Survival Times of BALB/c Mice Bearing the Ehrlich Ascites Carcinoma.

Expt.	No. of mice	Dose/injection (mg/kg)[a]	Treated on day(s)	Mean survival time (days ± SD)	% Increase in mean survival time	p
A	6	0	7	13.2 ± 0.4		
	6	100	7	16.2 ± 2.5	23	<0.01
B	8	0	1	15.3 ± 1.9		
	9	200	1	22.0 ± 4.5	44	<0.0025
	9	200[b]	1	22.2 ± 5.4	45	<0.0025
C	6	0	1–5	14.5 ± 0.5		
	14	300	1–5	32.5 ± 8.5	124	<0.0005
D	8	0	1,4,7,10	14.5 ± 1.9		
	8	200	1,4,7,10	31.4 ± 4.8	117	<0.0005
	8	300	1,4,7,10	27.8 ± 9.0	92	<0.0025
E	12	0	1–4	16.1 ± 1.8		
	12	300	1–4	31.8 ± 4.1	98	<0.0005
F	10	0	1–8	10.8 ± 1.5		
	10	100	1–8	25.9 ± 5.4	140	<0.0005
	10	450	1	22.7 ± 6.6	112	<0.0005
G	10	0	1	14.2 ± 1.0		
	10	500	1	28.3 ± 6.7	99	<0.0005
H	7	0	5	13.3 ± 0.8		
	7	500	5	20.3 ± 8.4	53	<0.025

[a] Expressed as $(NH_4)_2IrCl_6$.

[b] Solution was irradiated only 7 hr.

obtained through the cooperation of Mr. N. H. Greenberg of the Cancer Chemotherapy National Service Center and Mr. L. Dudeck of Hazelton Laboratories. Mice were given an ip injection on day zero of either 2×10^7 Ehrlich ascites carcinoma cells or 10^5 leukemia L1210 cells, were housed in groups of 5 to 8, and were provided with water and Purina Laboratory Chow *ad libitum*. Iridium was injected ip in all experiments.

Lymphocyte cultures. Human blood was drawn from presumably normal donors and was defibrinated by slow swirling in a 250-ml Erlenmeyer flask containing glass beads. Nine parts of the defibrinated blood were mixed with 1 part of a 4% dextran (mol wt = 204,000) solution in capped tubes and incubated at 37° for 2.5 hr. The lymphocyte-rich serum was then withdrawn and mixed with 4 vol of Eagle's minimum essential medium with Hanks' balanced salt solution (Microbiological Associates). Phytohemagglutinin P (Difco) was added to the suspension at the ratio of 0.005 ml/ml. Two-ml aliquots were then pipetted into replicate siliconized tubes and various concentrations of irradiated solutions of $(NH_4)_2IrCl_6$ were added immediately. Final lymphocyte counts ranged from about 200,000 to 900,000/ml in various experiments, with other leukocytic elements comprising only 3 to 5% of the populations. Cultures were incubated at 37° in 5% CO_2–95% air. Penicillin and streptomycin were present at 100 units/ml and 100 μg/ml, respectively. After 2 days, 1.0 μCi of thymidine-methyl-^{3}H, uridine-5-^{3}H, or L-leucine-^{14}C (New England Nuclear Corporation) was added/ml of culture and incubation was resumed for 5.5 hr. After that interval, the cells were sedimented by centrifugation, washed once with cold 0.9% NaCl, and then suspended in cold 5% trichloroacetic acid (TCA). Following 2 washings with TCA, the acid-insoluble material was solubilized in 1 *M* hydroxide of Hyamine, transferred to a toluene-based phosphor (PPO-

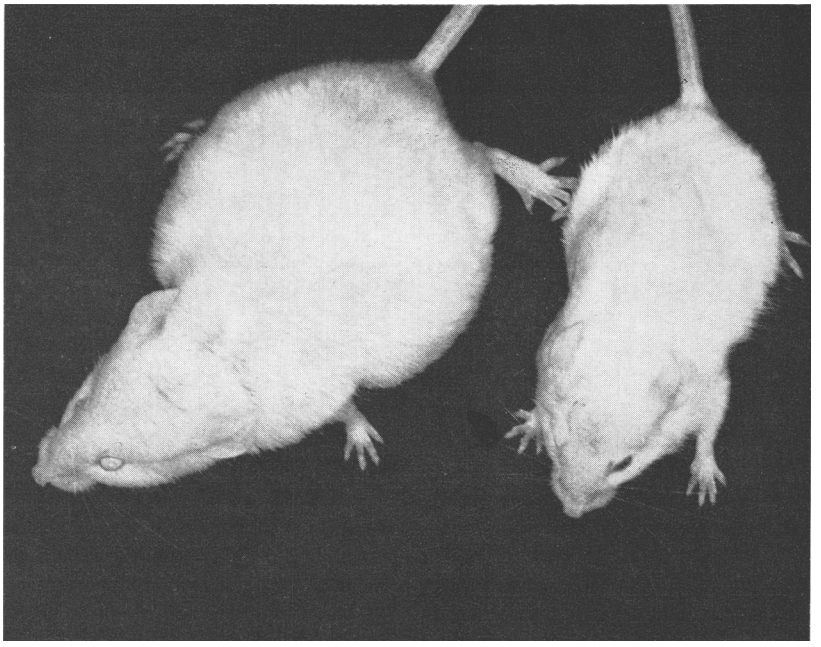

FIG. 1. Inhibition of development of the Ehrlich ascites carcinoma by the photochemical reaction product of $(NH_4)_2IrCl_6$. Both mice were inoculated with 2×10^7 tumor cells 15 days earlier. The animal on the left received 5 daily injections of 0.9% NaCl (1 ml/30 g) beginning 1 day after tumor administration; the animal on the right received the same number of injections of irradiated $(NH_4)_2IrCl_6$ at a dosage of 300 mg/kg/day.

POPOP), and radioactivity was measured with a liquid scintillation spectrometer (Nuclear-Chicago, Mark I).

Results. Table I shows the results of 8 consecutive experiments with the Ehrlich ascites carcinoma using irradiated $(NH_4)_2IrCl_6$ solutions in various treatment regimens. Of the 170 mice used in this series, all except 2 were used in calculations of percentage survival. Those excluded were 2 of the treated animals in Expt. C which died of obvious toxicity shortly after the fifth injection. In all other experiments, all animals were used in the calculations even though an occasional treated animal died with no apparent tumor prior to any of the corresponding controls. The most striking incidence of prolongation of individual survival time was one treated animal in Expt. C which finally succumbed after 58 days. Of the regimens examined, a dosage of 100 mg/kg/day on days 1 through 8 yielded the greatest extension of mean survival time coupled with only nominal evidence of toxicity as manifested by lethargy, weight loss, and unkempt coats. The gross appearance of a typical control and treated animal is shown in Fig. 1.

In regard to toxicity observed in BALB/c mice undergoing therapy, composite Fig. 2 shows the weight change patterns of treated mice at 5 different therapeutic schedules as compared with controls. A notable feature of these plots is the reversibility of the weight losses which began after some interval following cessation of therapy, the interval appearing to be related to dose per day and duration of treatment.

Survival data obtained using the L1210 leukemia in BDF_1 mice were not as impressive as that obtained with the Ehrlich carcinoma, but the moderate increases in survival times of treated animals were statistically highly significant (Table II). In this limited number of treatment regimens, the extensions of survival times in the treated groups were virtually the same irrespective of the therapeutic schedule. Much more extensive evaluation would of course be required before one could invoke the absence of a dose–response relationship.

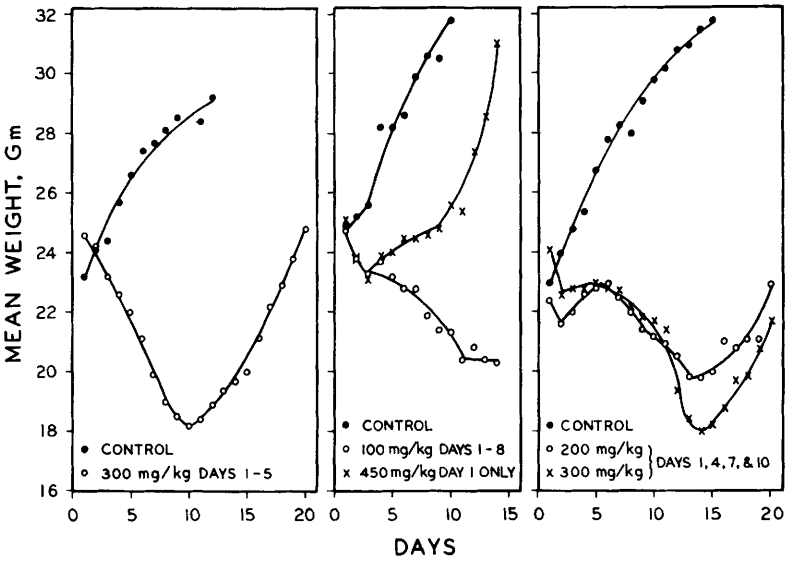

FIG. 2. Effects of 5 treatment schedules with the photochemical reaction product of $(NH_4)_2IrCl_6$ on weight changes in mice bearing the Ehrlich ascites carcinoma. The individual graphs, from left to right, correspond to Expts. C, F, and D, respectively, in Table I.

The incorporation of thymidine-methyl-^{3}H into human lymphocytes cultured *in vitro* in the presence of phytohemagglutinin was inhibited by the photochemical reaction product of $(NH_4)_2IrCl_6$. In studies to establish the dose–response relationships of the inhibition it was evident that the degree of inhibition was dependent upon the total cell population; *i.e.*, concentrations of the compound which were quite inhibitory to 2×10^5 cells/ml were only slightly active when the cell population approached 10^6. In addition, some variations of sensitivities were encountered among lymphocyte populations obtained from various donors. For example, in 8 consecutive experiments with cells from different donors and with a standard *in vitro* cell population of 2×10^5, the concentration of the product which conferred 50% inhibition ranged from 10^{-5} *M* to just over 2×10^{-4} *M*, with an average value of 6×10^{-5} *M* expressed as $(NH_4)_2IrCl_6$.

Using a concentration (10^{-4} *M*) which in most experiments conferred virtually total inhibition of lymphocyte mitogenesis, the extent of selectivity of action of the product against deoxyribonucleic acid synthesis in these cells was assessed. Using uridine-5-^{3}H and L-leucine-^{14}C to monitor ribonucleic acid and protein synthesis, respectively, no selectivity whatsoever for deoxyribonucleic acid synthesis was demonstrated (Table III).

TABLE II. Effects of the Photochemical Reaction Product of Ammonium Hexachloroiridate (IV) on Survival Times of BDF_1 Mice Bearing the L1210 Leukemia.

No. of mice	Dose/injection (mg/kg)[a]	Treated on day(s)	Mean survival time (hr ± SD)	% Increase in mean survival time	*p*
10	0	1–5	181.2 ± 5.6		
10	100	1–8	211.5 ± 8.1	17	<0.0005
10	200	1–6	216.1 ± 9.3	19	<0.0005
10	200	1,4,7	216.9 ± 6.9	20	<0.0005
10	500	1	217.5 ± 4.4	20	<0.0005

[a] Expressed as $(NH_4)_2IrCl_6$.

TABLE III. Effects of the Photochemical Reaction Product of Ammonium Hexachloroiridate (IV) on the Incorporation of Thymidine-methyl-^{3}H, Uridine-5-^{3}H, and L-Leucine-^{14}C into Phytohemagglutinin-Stimulated Human Lymphocytes *in Vitro*.

Isotopic precursor	Iridium[a]	(cpm)	% Inhibition
Thymidine	—	14,523	
	+	66	99
Uridine	—	11,286	
	+	72	99
L-Leucine	—	487	
	+	30	94

[a] Final concentration [expressed as $(NH_4)_2IrCl_6$] $= 10^{-4}$ *M*.

A closely similar lack of selectivity of action was evident in Ehrlich ascites carcinoma cells cultured *in vitro* (Table IV). Since a period of several hours of exposure of the cells to the reaction product was necessary to achieve inhibition of uptake of the isotopic precursors, as was noted also with *cis*-$[Pt(NH_3)_2Cl_2]^\circ$ (3), secondary events admittedly may have supervened to obscure such a selectivity. Thus these experiments reveal only that under conditions which are optimum for demonstrating inhibition of deoxyribonucleic acid synthesis, there was virtually equal suppression of the rates of ribonucleic acid and protein synthesis.

TABLE IV. Effects of the Photochemical Reaction Product of Ammonium Hexachloroiridate (IV) on the Incorporation of Thymidine-methyl-^{3}H, Uridine-5-^{3}H, and L-Leucine-^{14}C into Ehrlich Ascites Carcinoma Cells *in Vitro*.[a]

Isotopic precursor	Iridium[b] (*M*)	(cpm)	% Inhibition
Thymidine	0	1643	
	10^{-5}	840	49
	10^{-4}	474	71
Uridine	0	11,560	
	10^{-5}	8788	24
	10^{-4}	2321	80
L-Leucine	0	3870	
	10^{-5}	2884	25
	10^{-4}	1395	64

[a] Culture conditions were as described in the *Materials and Methods* section for lymphocytes, except (a) phytohemagglutinin was omitted, (b) 20% ascitic fluid was substituted for 20% autochthonous serum, (c) cells were incubated with iridium for 19 hr preceding a 5-hr labeling period, and (d) cell population was 7.5×10^4/ml.

[b] Concentrations are expressed in terms of $(NH_4)_2IrCl_6$.

Discussion. One of the earliest extensive surveys of antitumor actions of totally inorganic compounds was done by Collier and Krauss in 1931 (5). Of 64 such materials tested against subcutaneously implanted Ehrlich carcinoma, 40, representing compounds of copper, iron, cobalt, or rhodium, were reported to be inactive. Fourteen, containing osmium, chromium, or ruthenium, were slightly active. Of 10 lead compounds tested, $K(PbCl_3)$, $K(PbCl_3 \cdot H_2O)$, $K(Pb_2Cl_5)$, and Pb_3O_4 were reported as active. Unfortunately, those authors did not report precise quantitative data, but described their compounds only in terms of "unwirksam," "Wirkung kaum vorhanden," "deutliche Wirkung," and similar subjective ratings. No compound of iridium was tested.

Taylor and Carmichael (6) subsequently assessed 37 metallic chlorides and nitrates for effects on growth and survival of chick embryos, on tumors and embryos of eggs bearing yolk sac implants of a mouse mammary adenocarcinoma, and on development of a transplantable sarcoma in DBA mice. Of the group VIII metals investigated, $CoCl_2$, $PtCl_4$, and $RhCl_3$ were among the 10 compounds most toxic to the embryos, but some degree of preferential inhibition of growth of egg-cultivated tumors was induced by $CoCl_2$ and $RhCl_3$. Curiously, $NiCl_2$ inhibited the growth of egg-cultivated tumors 49 to 86% when injected into the yolk, but accelerated the development of the tumor 40 to 109% when introduced over the embryo membranes. $CoCl_2$ and $RhCl_3$ were only moderately active against the murine sarcoma, conferring up to 35 and 24% inhibition, respectively. No compound of iridium was tested in that study.

An extensive literature survey has revealed only 2 reports dealing with the pharmacology of iridium, excluding the earlier report from this laboratory (4) and those dealing

with radioactive iridium (^{192}Ir). In the first of these, Zinno and Cutolo (7) in 1903 reported their investigation of the actions of iridium chloride (oxidation state unspecified) *con speciale riguardo alla sua azione disinfettante*. They lauded its highly potent rapid action against *Staphylococcus aureus* and the causative organisms of typhoid, cholera, and anthrax. Spores of the anthrax bacillus were rendered nonviable following only a 10-min exposure in a 0.01 *N* solution. In the second report, Rosenberg *et al.* (8) in 1965 noted that $(NH_4)_2IrCl_6$ was bactericidal and devoid of filamentogenic activity, while $K_2Ir(NO_2)_6$ had no observable effect on either bacterial growth or cell division at the concentration tested (8.0 μg/ml). The biological properties of iridium compounds thus appear to be quite unexplored.

A recent international invitational symposium which was held at Michigan State University in September, 1970, summarized many pharmacological, toxicological, and chemotherapeutic properties of selected platinum compounds. The chemical proximity of iridium to platinum certainly would seem to warrant a survey of biological actions of representative iridium compounds, particularly the various inorganic and organic neutral complexes.

Summary. The photochemical reaction product of ammonium hexachloroiridate(IV) retards the rate of development of the Ehrlich ascites carcinoma in BALB/c mice and, to a lesser degree, the development of the L1210 leukemia in BDF_1 mice. Of the various dosage regimens examined, a maximum of 140% increase of mean survival time was obtained with mice bearing the Ehrlich carcinoma, but only a 20% increase was demonstrated with mice bearing the L1210 leukemia. Blastogenic transformation of phytohemagglutinin-stimulated human lymphocytes in culture was also inhibited by the product, as evidenced by a reduced rate of incorporation of thymidine into cultured cells. No evidence was obtained that the product was a selective inhibitor of deoxyribonucleic acid synthesis; rates of ribonucleic acid and protein synthesis were inhibited to the same extent as the rate of deoxyribonucleic acid synthesis in both cultured human lymphocytes and Ehrlich ascites carcinoma cells *in vitro*.

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