CANCER CHEMOTHERAPEUTIC PROPERTIES AND TOXICOLOGIC EFFECTS OF CIS-PLATINUM(II) DIAMMINO DICHLORIDE

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This is to certify that the

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CANCER CHEMOTHERAPEUTIC PROPERTIES AND TOXICOLOGIC EFFECTS OF CIS-PLATINUM(II) DIAMMINO DICHLORIDE

presented by

Richard J. Kociba

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Stuart D. Sleight
Major professor

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ABSTRACT

CANCER CHEMOTHERAPEUTIC PROPERTIES AND TOXICOLOGIC EFFECTS OF CIS-PLATINUM(II) DIAMMINO DICHLORIDE

by Richard J. Kociba

The cancer chemotherapeutic properties of the compound Cis-Platinum(II) Diammino Dichloride [Cis-Pt.(II)] were evaluated against the Dunning Ascitic Leukemia (DAL) and the intramuscular Walker 256 Carcinosarcoma. A single intraperitoneal (IP) treatment with 2 mg./kg. or 4 mg./kg. Cis-Pt.(II) on Day 1 after tumor implantation inhibited the development of DAL in Fischer 344 rats. Recovered rats were refractory to a challenge dose of DAL tumor cells on Day 30. Necropsy examination at time of termination on Day 60 failed to reveal any evidence of DAL. A single treatment with 4 mg./kg. Cis-Pt.(II) delayed until Day 4 or Day 7 after implantation of DAL was sufficient to arrest tumor development and thus led to an extended survival time. Adhesions present between adjacent abdominal viscera at time of necropsy indicated the DAL had undergone regression subsequent to delayed treatment with Cis-Pt.(II).

Treatment of rats bearing the intramuscular Walker tumor with 2 mg./kg. Cis-Pt.(II) administered IP on Day 3 after implantation inhibited tumor development to less than 20% by Day 7. Delayed treatment on Day 7 after implantation of the Walker tumor with a single IP injection of 2 mg./kg. or 4 mg./kg. Cis-Pt.(II) also caused a marked regression of tumor development and extended the survival time.

The LD_{50} of Cis-Pt.(II) was determined to be 12.2 mg./kg. in the male rat. The LD_{90} and LD_{10} were determined to be 26.5 mg./kg. and 5.5 mg./kg., respectively. A minimal therapeutic index of 2.75 was calculated from the limited data available.

Histologic alterations subsequent to the IP injection of 12.2 mg./kg. Cis-Pt.(II) were most pronounced in those tissues having cellular constituents which have rapid turnover times. Thymic atrophy (due to lymphocytic depletion), splenic depletion of lymphoid elements, intestinal epithelial denudation and bone marrow depression were most severe at 2-4 days after intoxication. Renal tubular sloughing and elevated blood urea nitrogen levels also occurred at this time. Rats surviving the intoxication showed regeneration of the tellular elements in those tissues which were affected.

Panleukocytopenia, reticulocytopenia and blood platelet depression was also most severe at 2-4 days after injection of 12.2 mg./kg. Cis-Pt.(II). This was followed by regenerative increases in rats surviving the intoxication. Erythrocyte counts, packed cell volumes and hemoglobin levels were not depressed by the toxic dosage of Cis-Pt.(II), and serum bilirubin levels were not elevated.

Serum albumin levels were essentially unchanged, whereas depression of total serum protein levels occurred at the time coincident with lymphoid depression. Serum levels of uric acid, calcium, inorganic phosphorus and cholesterol were not altered, whereas lactic dehydrogenase, alkaline phosphatase and glutamic-oxaloacetate transaminase levels were depressed subsequent to injection of 12.2 mg./kg. Cis-Pt.(II). A slight hyperglycemia occurred 2-4 days postinjection, and may have reflected the dehydration due to enteritis.

The data presented in this study indicated the oncostatic properties and toxicologic effects of Cis-Pt.(II) were similar to those described for the compounds which have been referred to as radiomimetic agents.

CANCER CHEMOTHERAPEUTIC PROPERTIES AND TOXICOLOGIC EFFECTS OF CIS-PLATINUM(II) DIAMMINO DICHLORIDE

By

Richard J. Kociba

A THESIS

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Dedicated to my wife and family

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I wish to express my appreciation to Dr. S. D. Sleight, my major professor, for his advice and assistance during the course of this study. I am also indebted to the members of the Michigan State University Biophysics Department, because without the help of Dr. B. Rosenberg and co-workers this study would not have been possible.

A special note of thanks is due to the staff members of the Department of Pathology who assisted in the preparation of tissues for microscopic examination. The invaluable training in histopathology received from Dr. R. F. Langham was of utmost importance during the interpretation of tissue alterations in this study.

The cooperation of Dr. R. B. Foy of the Laboratory of Clinical Medicine during this study is also greatly appreciated.

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INTRODUCTION

The testing of about a quarter million chemical compounds and preparations against model tumor systems, mainly by the Cancer Chemotherapy National Service Center (CCNSC), has revealed the relative effectiveness of certain classes of drugs which have been utilized in the chemotherapeutic treatment of cancer.

As a result, the present day cancer chemotherapist has available an impressive array of chemical agents, of diverse biochemical action, that possess the potential to retard the progression of some malignant diseases. While the ability of these agents to maximally damage tumors with minimal harm to the host may well be increased by manipulation of the dosage and schedules of drug administration, the relative refractoriness of various tumor types to therapeutic management dictates that basic research provide new and potent therapeutic regimens for the treatment of cancer.

A recent symposium, sponsored by the American Cancer Society and the International Union Against Cancer, critically evaluated the general area of cancer chemotherapy and concluded one of the more important approaches in the future must involve the preparation and evaluation of new drugs that are more sufficiently selective against the tumor cell (Mandel and Rall, 1969).

It is in harmony with this general idea that this study of the oncostatic and toxicologic properties of the compound Cis-Platinum(II) diammino dichloride [Cis-Pt.(II)] was undertaken.

LITERATURE REVIEW

Prevailing Course of Cancer Chemotherapy Studies

Cancer chemotherapy has been defined as the use of any chemical substance administered systemically which, while relatively nontoxic to the host, will interfere with, favorably modify, or destroy a neoplastic growth or alleviate its deleterious effects on the host (Karnofsky, 1948). In the past 20 years there has been a great expansion in cancer research, particularly in the field of cancer chemotherapy. The decreasing importance of infectious disease as therapeutic problems in contrast to the increasing prevalence of cancer, and the shift in medical research toward biochemical and physiologic studies, have served to stimulate study of this disease. The intensification of work on the problems presented by cancer in all its manifestations, and the influence of various agents on the growth of normal and neoplastic tissues, will no doubt also result in information not only applicable to cancer therapy, but with important implications for many other medical and biological problems.

Studies in cancer chemotherapy can be divided into 3 major categories:

1. Elucidation of the neoplastic component of the cancer cell. If the basic abnormality of the cancer cell were understood, it would be possible to search for specific methods of controlling the pathologic process. Such knowledge would lead to indisputably reliable methods available for testing compounds for chemotherapeutic activity against cancer. Despite the suggestions that cancer is caused by viruses, is due to somatic mutations, or has a common biochemical abnormality, no single view has

received general support (Oberling, 1952). As a result there is no satisfactory basis on which to initiate a direct chemotherapeutic attack on cancer. As the fundamental studies on the nature of cancer would ultimately lead to a rational approach to cancer chemotherapy, they are of utmost importance.

2. Study of substances inhibiting or modifying the growth of normal or neoplastic tissues. As cancers can arise from practically every tissue of the body, neoplastic cells retain many of the properties of normal cells. The study of the properties of normal tissues and their susceptibilities to specific agents has led to the development of useful chemotherapeutic agents. Most of these agents, although injurious to certain normal cells, offer temporary remission or prolonged partial control of some neoplastic processes.

3. Empirical trials of chemical and biological preparations for detection of tumor-inhibiting activity. Studies of this type have utilized various types of *in vitro* tests such as antimicrobial tests, tests in tissue culture involving destruction of normal or tumor cells, or inhibition of embryonic development (Gellhorn and Hirschberg, 1955). In these types of systems, the influence of drugs is measured on only one parameter of response. For this reason *in vivo* pharmacologic testing has been recommended in which transplantable tumors in rodents are used to detect any antitumor activity. In these systems tumor cells are implanted into rodents, treatment is administered and the influence of therapy on tumor growth and survival time of the animals can be measured.

Function of the Cancer Chemotherapy National Service Center in Coordination of Research Efforts

The Cancer Chemotherapy National Service Center (CCNSC) has been established as a branch of the National Cancer Institute to act as an

organizational framework in which cooperative research could develop. One of the principal roles of the CCNSC has been to coordinate the screening of candidate compounds for antitumor activity. The majority of materials are screened in several laboratories where the candidate compounds are supplied by the CCNSC and the screening methods used are those specified by the CCNSC.

The CCNSC has published protocols (CCNSC, 1962) for the specific test systems, and has listed a series of transplantable rodent tumors to be used in the screening studies. The first phase of the operation involves the use of 3 transplantable mouse tumors, namely the Sarcoma 180, Adenocarcinoma 755 and Leukemia L1210 to screen for antitumor activity. Response is determined by tumor inhibition and prolongation of survival time.

Those compounds which show evidence of antitumor activity are then subjected to more intensive studies to firmly evaluate their oncostatic properties. Additional tumors may be utilized at this point to determine the range of antitumor activity. Compounds that have demonstrated sufficient antitumor activity at this stage are now subjected to pharmacologic studies in animals to determine the mode of absorption, tissue distribution, metabolism, and excretion. Studies to characterize any toxicologic properties of the compound must be also conducted prior to human pharmacologic trials. The CCNSC (1959) has established uniform protocols to be followed by the medical centers affiliated with the CCNSC program. The effects of the candidate anticancer compound are first explored in patients with advanced neoplastic diseases which are no longer responding to any known therapy. If the new agent looks promising in this preliminary trial and it is not excessively toxic, it then will undergo definitive evaluation in a large scale inter-institutional cooperative clinical study. Pooling of these data plus statistical analysis and collaborative

reporting will then allow comparison of the anticancer properties of this compound with those of other active drugs, and with surgical intervention or radiation therapy.

Utilization of Dunning Ascitic Leukemia in Cancer Chemotherapy Studies

The Dunning Leukemia arose as a spontaneous tumor in the laboratory of W. F. Dunning and has been described as a monocytic type of leukemia (Dunning and Curtis, 1957). The tumor cells were described as round to oval cells having considerable size variation, which were observed to possess ameboid movement when examined by darkfield techniques. The use of this transplantable tumor in cancer chemotherapy studies was first reported by Dunning (1958), who propagated the tumor by subcutaneous implantation into Fischer 344 rats. Jones *et al.* (1958) described the usefulness of the Dunning Leukemia in quantitative pharmacological studies of cancer chemotherapy agents. These workers cited the fact that this tumor was readily transplanted with 100% success, with no spontaneous regressions. The most useful index of therapeutic activity against the Dunning Leukemia was considered to be the prolongation of life as measured by increased survival time.

The Dunning Leukemia has since been converted to an ascitic form (Dunning Ascitic Leukemia or DAL). The conversion of neoplasms into ascitic tumors has been discussed by Klein and Klein (1956). Koprowska (1956) and Siegler and Koprowska (1962a) studied the mechanism of ascitic tumor formation, which proceeded from an initial penetration of tumor cells into the mesentery within the first day. This was followed by their proliferation within the mesentery and the subsequent exfoliation into the fluid accumulating in the peritoneal cavity.

In another communication Siegler and Koprowska (1962b) described the host responses to a transplantable ascitic tumor. The ultrastructural aspects of the invasion of ascitic tumor cells into the abdominal wall has been reported by Birbeck and Wheatley (1965). Their findings suggested the mesothelial cells became modified in their contacts with neighboring cells, and subsequently exfoliated. Tumor cells rapidly filled the resultant spaces left by the exfoliated cells and eventually formed a continuous layer on the abdominal wall.

Baserga (1963) used autoradiography to determine the growth rate of ascitic cells derived from the Ehrlich ascitic tumor. His work indicated a replicative cycle of approximately 18 hours.

The CCNSC (1962) has chosen Dunning Ascitic Leukemia as one of the principal tumor systems to be used in screening studies. The tumor is propagated by the transplantation of 5×10^6 neoplastic cells from a donor Fischer 344 rat bearing a Day 7 development of the DAL into the peritoneal cavity of recipient Fischer 344 rats. Any compound which prolongs the survival time at least 25% past the expected 8-11 days is considered significant and will warrant further testing.

Sugiura and Creech (1956) concluded the ascitic form of a transplantable tumor was a more sensitive system for the detection of antitumor activity. However, the intraperitoneal (IP) injection of chemicals into ascitic tumor-bearing animals represents a form of intratumoral treatment; as such, it possibly has less significance than treatment of solid tumors growing at a distance from the injection site.

Utilization of the Walker 256 Carcinosarcoma in Cancer Chemotherapy Studies

The Walker Carcinosarcoma 256 (W256) tumor developed spontaneously in the region of the mammary gland of an albino rat in 1928 in the

laboratory of George Walker at the Johns Hopkins University School of Medicine. At necropsy the subcutaneous tumor was the size of an egg and was incompletely encapsulated. On microscopic examination the tumor was diagnosed as adenocarcinoma.

Initial transplantation attempts were only partially successful, but later transplants grew in nearly 100% of the recipient rats. Over the years of continuous propagation of this tumor, a carcinomatous variant and a sarcomatous variant have developed (Stewart *et al.*, 1956).

An early description of the Walker tumor was presented by Earle (1935), who studied the cells both *in vitro* and *in vivo*. Fisher and Fisher (1961) reported on the ultrastructural and histochemical features of the Walker tumor. They proposed the neoplasm was essentially a carcinoma, based on the effacement of adjacent cellular borders, which was in keeping with the fundamental concepts concerning the arrangement of epithelial cells.

The fine cytology of the Walker tumor was also studied by Mercer and Easty (1961). These workers reported that dense cytoplasmic particles usually associated with protein synthesis were mainly free in the cytoplasm, and not connected to the intercellular reticulum. No virus particles were observed in the neoplastic cells during this study.

The CCNSC has recommended the use of both the subcutaneous and the intramuscular forms of the Walker tumor in cancer chemotherapy screening studies (CCNSC, 1962).

Protocols for the use of the intramuscular Walker Tumor (IM W256) specify the implantation of tumor brei material obtained from a donor rat into the pelvic muscle mass of recipient standard randombred rats. Treatment with the candidate compound is begun on Day 3 postimplantation, and normally is continued through Day 6. On Day 7 both treated and

control rats are killed and the tumor weights are determined. Any candidate compound which can inhibit development of the intramuscular tumor to 60% or less when compared to nontreated tumor-bearing rats has been considered significant by the CCNSC.

The IM W256 tumor metastasizes to regional lymph nodes as well as various visceral organs by Day 7-10 postimplantation.

Initial Description of Oncostatic Properties of Platinum Compounds

Rosenberg, VanCamp and Krigas (1965) noted an unexpected response of *Escherichia coli* when under the influence of a current delivered between platinum electrodes. Individual organisms attained lengths up to 300 times normal under these conditions. Exhaustive studies disclosed that several new chemical species were created by the electrical current. Several platinum salts were subsequently shown to induce cell elongation when added to the growth medium.

Further studies by Rosenberg *et al.* (1969) reported one of the more active compounds, Cis-Pt.(II), was capable of retarding the growth of the Sarcoma 180 tumor in mice. Additional data showed the compound markedly increased the survival times of mice bearing the L1210 Leukemia. The most recent report (Rosenberg and VanCamp, 1970) described the successful regression of large Sarcoma 180 tumors following delayed treatment with Cis-Pt.(II).

Howle and Gale (1970) have also reported on the chemotherapeutic action of Cis-Pt.(II) against the Ehrlich Ascitic neoplasm. They concluded the Ehrlich Ascitic neoplasm was approximately as sensitive to Cis-Pt.(II) as the Sarcoma 180 and Leukemia L1210 tumors.

Current Research Regarding the Metabolism and Basic Mechanism of Action of Cis-Pt.(II)

Knowledge in regard to the fate of the compound Cis-Pt.(II) following injection or the mechanism of action against the tumor cells has been meager. Studies by Renshaw and Thomson (1967) in regard to the distribution of platinum ions within *Escherichia coli* showed the metal was associated with metabolic intermediates, nucleic acids and cytoplasmic proteins.

Howle and Gale (1970) reported that Cis-Pt.(II) inhibited the incorporation of isotopic precursors into deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins.

Studies in progress by Harder (1970) are attempting to further define the basic mechanism of action while the studies of Allen (1970) have been directed towards defining the metabolic fate of the Cis-Pt.(II) in the mammalian system.

No known data in regard to the toxicologic parameters of Cis-Pt.(II) have been published prior to this time.

MATERIALS AND METHODS

General Plan

A 3-phase study was undertaken to investigate the cancer chemotherapeutic properties and toxicologic effects of Cis-Pt.(II) in the male albino rat. Phase 1 utilized rats bearing the Dunning Ascitic Leukemia (DAL) in evaluating the chemotherapeutic properties of Cis-Pt.(II) and Phase 2 utilized rats bearing the intramuscular form of the Walker 256 Carcinosarcoma (W256). Phase 3 was an attempt to define the toxicologic parameters of the Cis-Pt.(II) compound in the male rat.

Source of Animals

Permission to obtain tumor bearing rats was granted by Dr. John Venditti, of the National Cancer Institute, and donor rats bearing the Dunning Ascitic Leukemia were obtained from a frozen tumor bank maintained by the National Cancer Institute.* Male rats of the Fischer 344 strain were obtained from a commercial supplier,** and were used in all phases of the work with the DAL.

Additional donor rats bearing the Walker 256 Carcinosarcoma were obtained from the same source* previously mentioned. Standard random bred rats obtained from a second commercial supplier*** were used for all experiments with the Walker tumor.

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*Isidore Wodinsky, Arthur D. Little Co., Cambridge, Mass.

**Charles River Company, Cambridge, Mass.

***Spartan Research Animals, Haslett, Mich.

All toxicologic studies were conducted on male standard random bred albino rats weighing 100-150 grams.

Maintenance of Animals

All rats were maintained in quarters in the Michigan State University Center for Laboratory Animal Resources (CLAR). All animal care was performed by CLAR personnel in accordance with acceptable procedures designated by the CLAR Director.

Hematologic Determinations

Hemoglobin (Hb) values were determined by the cyanmethemoglobin method. Packed cell volumes (PCV) were estimated by the microhematocrit method. Reticulocytes were stained with new methylene blue prior to the counting procedure. Procedures as outlined by Coles (1967) were followed in determination of the above parameters.

An electronic counter* was used for all erythrocyte and leukocyte determinations. Phase microscopy was used to facilitate the manual determination of the circulating platelet counts.

Serum Component Determinations

An automated multi-channel analyzer** operated by personnel of a private laboratory*** was utilized for determination of serum components.

Histologic Preparations

A buffered solution of 10% formalin was used as the fixative for all tissue specimens. Tissue preparation and staining was performed according

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*Coulter Counter, Coulter Electronics, Hialeah, Fla.

**Auto-Analyzer, Technicon Corp., Ardsley, N.Y.

***Laboratory of Clinical Medicine, Lansing, Mich.

to commonly accepted methods as outlined in the Armed Forces Institute of Pathology Manual (1967). Hematoxylin and eosin were routinely used in the staining process.

Bone Marrow Preparation

The femur of the rat was the source for all bone marrow collected. A combination Wright-Giemsa stain was used on all bone marrow smears. The dried smear was immersed for 5 minutes in Wright's stain, followed by 1 minute in phosphate buffer and 30 minutes in dilute Giemsa solution.

Source of Cis-Pt.(II) Compound

The compound Cis-Pt.(II) was made available by Dr. B. Rosenberg and co-workers. Sterile saline (0.85% NaCl) was used in the preparation of all aqueous solutions of Cis-Pt.(II), and all solutions were prepared within 1 hour of time of injection.

Preparation of Thioglycollate Agar

A commercial source of thioglycollate media* was used in the preparation of sterile tubes of agar which were used to check for bacterial contamination during transplantation procedures.

Phase 1. Treatment of Rats Bearing Dunning Ascitic Leukemia

Part A. Day 1 Treatment of DAL with Cis-Pt.(II). Thirty-six rats of the Fischer 344 strain were randomly divided into 6 equal groups. A donor Fischer 344 rat bearing DAL in the 7th day of development was killed by cervical dislocation and was immediately immersed in a solution of 50% ethanol. The integument of the ventral abdominal area was aseptically reflected, and needle and syringe were used to aseptically aspirate a

*Difco Laboratories, Detroit, Mich.

small quantity of ascitic fluid. Four tubes containing thioglycollate agar were inoculated with several drops of ascitic fluid. Two tubes were incubated at 25 C. and 2 at 37 C. The cellular content of the ascitic fluid was quantitated with the aid of an electronic counter, and smears of the ascitic fluid were stained with Wright's stain.

Following quantitation of the cellular content of the ascitic fluid, 30 of the 36 recipient rats were each implanted with 5×10^6 neoplastic cells into the peritoneal cavity.

On Day 1, each rat received a single intraperitoneal injection according to the schedule outlined in Table 1. Solution concentrations of Cis-Pt.(II) were adjusted so that each rat received a comparable quantity of diluent. All rats were observed daily and complete necropsies were conducted as soon as possible after death.

All rats surviving on Day 30 were examined for the presence of ascitic fluid by paracentesis of the peritoneal cavity.

Part B. Challenge of Day 30 Survivors with DAL Cells. On Day 1' (Day 31 postimplantation) all surviving rats from Part A were implanted with 5×10^6 neoplastic cells (Table 1). All rats were again observed daily, with necropsies performed on any dead rats.

On Day 30' all surviving rats were killed with an overdose of ether and representative specimens from all organs of the body were fixed in 10% buffered formalin for histologic examination.

Part C. Delayed Treatment of DAL with Cis-Pt.(II). Recipient male Fischer 344 rats were implanted with 5×10^6 neoplastic DAL cells on Day 0. A single intraperitoneal injection of 4 mg./kg. Cis-Pt.(II) was given according to the schedule outlined in Table 2. The tumor-bearing control group of rats received only sterile diluent on Day 1.

Rats surviving on Day 30 were killed and tissue specimens collected in the manner previously outlined.

An additional group of 6 recipient Fischer 344 rats was implanted intraperitoneally with 10^7 DAL cells on Day 0, and treatment with 4 mg./kg. Cis-Pt.(II) was delayed until Day 7 (Table 2). Day 30 survivors were terminated in the manner outlined in Part B.

Phase 2. Treatment of Rats Bearing Intramuscular Walker 256 Carcinosarcoma

Part A. Day 3 Treatment with Cis-Pt.(II). A standard random bred rat bearing an intramuscular Walker 256 tumor in the 7th day of development was killed by cervical dislocation. The site of tumor development was swabbed with 50% ethanol and the integument was reflected to expose the tumor mass. A portion of the tumor mass was aseptically removed and sterile saline was used to prepare a 1:6 dilution which was homogenized in a sterile tissue grinder. Additional portions of the tumor mass were used to inoculate tubes of thioglycollate agar for incubation at both 37 C. and 25 C.

Within 20 minutes of the preparation of the tumor brei, 0.2 ml. aliquots of the brei were implanted into the muscle mass of the right pelvic limb of recipient rats. All rats were observed daily, and on Day 3 all rats not bearing a palpable intramuscular tumor were discarded. A single IP injection of Cis-Pt.(II) was given on Day 3 according to the schedule outlined in Table 3.

On Day 7, all rats were killed with an overdose of ether and the pelvic limbs were removed at the coxofemoral articulation. Weight of the IM tumor was obtained from the difference in weights of the tumored and nontumored right and left pelvic limbs. Tissue specimens were preserved in the manner previously outlined for histologic examination.

Part B. Day 7 Treatment with Cis-Pt.(II). Recipient rats were implanted with the IM Walker tumor and a single IP injection of Cis-Pt.(II) was given on Day 7 according to the schedule outlined in Table 4. All rats surviving at Day 30 were terminated and processed in the manner previously outlined.

Phase 3. Evaluation of Toxicologic Effects of Cis-Pt.(II)

Part A. Determination of LD_{50} in Male Rats. Groups of 8-10 normal male albino rats (100-130 grams body weight) were used in determination of the LD_{50} according to the method outlined by the CCNSC (1964). The compound Cis-Pt.(II) was prepared in sterile saline medium within 30 minutes of administration of a single IP injection. The data were fitted to a straight line by the method of least squares (Lewis, 1966), and the LD_{50} was estimated (Figure 13).

Part B. Serial Toxicity Study of Cis-Pt.(II) in Male Rats. The normal values of circulating cellular and serum components were established by means of a minimum of 6 normal male rats for all determinations.

On Day 0, a group of rats were given a single IP injection of 12.2 mg./kg. Cis-Pt.(II). On Days 1, 2, 3, 4, 5, 7, 9, 20 and 28 a minimum of 3 rats were killed, and various parameters of toxicity were determined. A terminal blood sample was collected for measurement of the circulating cellular and serum components. Representative tissues were prepared for histologic examination. Bone marrow was removed within 5 minutes of killing and stained according to the manner previously outlined.

RESULTS

Phase 1. Treatment of Rats Bearing Dunning Ascitic Leukemia

Part A. Day 1 Treatment of DAL with Cis-Pt.(II). The earliest deaths occurred on Days 4 through 6 in the Groups 1 and 2 rats which had received 16 mg./kg. or 8 mg./kg. Cis-Pt.(II) (Table 1). Necropsy of these rats revealed no evidence of DAL, and these deaths were tentatively presumed to be associated with the toxicologic properties of Cis-Pt.(II). From Day 11 through 13 additional deaths occurred in Groups 1, 2, 4 and 5. In all cases necropsy examination revealed terminal stages of DAL (Figure 1). Histologic examination confirmed the presence of large masses of neoplastic cells (Figure 2), and examination of the ascitic fluid revealed the presence of neoplastic cells and erythrocytes (Figure 3).

On Day 30, the groups of rats which had received 4 mg./kg. or 2 mg./kg. Cis-Pt.(II) had 6/6 and 5/6 survivors, respectively. Paracentesis of the peritoneal cavities of these rats failed to reveal any ascitic fluid or neoplastic cells.

The only other rats surviving on Day 30 were those of Group 6 which had not been implanted with DAL and had received only saline on Day 1. Clinically, the rats of Groups 3 and 4 were indistinguishable from Group 6 rats on Day 30. Thus a single treatment with 4 mg./kg. or 2 mg./kg. Cis-Pt.(II) on Day 1 after implantation of DAL was sufficient to arrest tumor development.

Table 1. Treatment of DAL with Cis-Pt.(II) followed by reimplantation of DAL cells into Day 30 survivors

Gp. No.	No. Rats	DAY 0 DAL Cells Implanted	DAY 1 Cis-Pt.(II) (mg./kg.)	DAY 30 Survivors	DAY 1' DAL Cells Implanted	DAYS 9'-15' Deaths Due to DAL	DAY 30' Survivors
1	6	5×10^6	16	0/6*	-	-	-
2	6	5×10^6	8	0/6*	-	-	-
3	6	5×10^6	4	6/6	5×10^6	0/6	6/6
4	6	5×10^6	2	5/6	5×10^6	0/5	5/5
5	6	5×10^6	0	0/6	-	-	-
6	6	0	0	6/6	5×10^6	6/6	0/6

*Some deaths due to drug toxicity (Days 4-5), remainder due to DAL (Days 11-13).

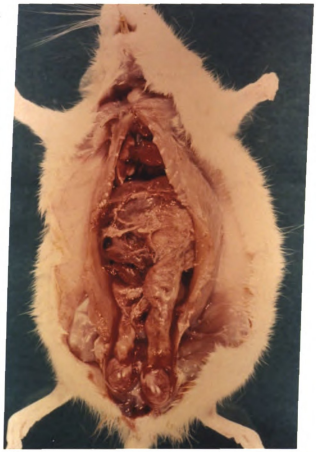

Figure 1. Gross appearance of Fischer 344 rat bearing the terminal stages of DAL. The ascitic fluid has been removed to demonstrate the extensive infiltration of the neoplastic process into all organs of the abdominal and inguinal regions.

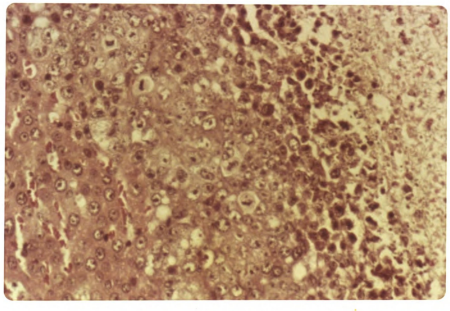

Figure 2. Histologic appearance of DAL cells infiltrating the subcapsular region of the liver. H & E stain. x 125.

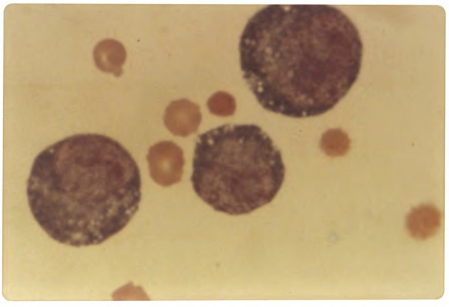

Figure 3. Histologic appearance of neoplastic cells intermixed with erythrocytes in the ascitic fluid collected from the peritoneal cavity of a Fischer 344 rat bearing DAL. Concentration of DAL cells ranged from 75 million to 150 million/cubic centimeter of ascitic fluid. Note large size and prominent nucleoli of DAL cells. Wright's stain. x 625.

Part B. Challenge of Day 30 Survivors with DAL Cells. Following implantation of the challenge dose of DAL cells, paracentesis of the peritoneal cavities of Group 6 rats revealed abundant neoplastic cells (Figure 3). No ascitic fluid or neoplastic cells were demonstrable in rats of Groups 3 and 4. From Day 9' through Day 15' all rats of Group 6 died (Table 1) and postmortem examination confirmed terminal DAL in all 6 rats (Figure 1). Rats of Groups 3 and 4 had no gross or histologic evidence of DAL when terminated on Day 30'.

Part C. Delayed Treatment of DAL with Cis-Pt.(II). All rats treated with a single IP injection of Cis-Pt.(II) on Days 1, 4 or 7 had 100% survivors on Day 30, whereas the tumor-bearing control group of rats had 1/6 survivors on Day 30 (Table 2). Gross and histologic examination disclosed no evidence of DAL. Rats treated on Day 7 had fibrous adhesions between adjacent abdominal viscera (Figure 4).

The group of rats implanted with 10^7 neoplastic cells and treated on Day 7 had 3/6 succumb to terminal DAL. Examination of Day 30 survivors revealed extensive adhesions between adjacent visceral structures (Figure 4).

Phase 2. Treatment of Rats Bearing Intramuscular Walker 256 Carcinosarcoma

Part A. Day 3 Treatment with Cis-Pt.(II). The gross appearance of the untreated rats revealed a consistent development of neoplasia by Day 7 (Figure 5). The average tumor weight in these untreated rats was 5.5 grams on Day 7, whereas rats treated on Day 3 with 4 mg./kg. or 2 mg./kg. Cis-Pt.(II) had average tumor weights not exceeding 1.1 gram and 1 gram, respectively (Table 3). Figure 6 shows the gross appearance of those

Table 2. Delayed treatment of DAL with Cis-Pt.(II)

	DAY 0	DAY 1	DAY 4	DAY 7	DAY 30
No. Rats	DAL Cells Implanted	Cis-Pt.(II) (mg./kg.)			Survivors
6	5×10^6	4			6/6
6	5×10^6		4		6/6
6	5×10^6			4	6/6
6	5×10^6	0			1/6
6	10^7			4	3/6

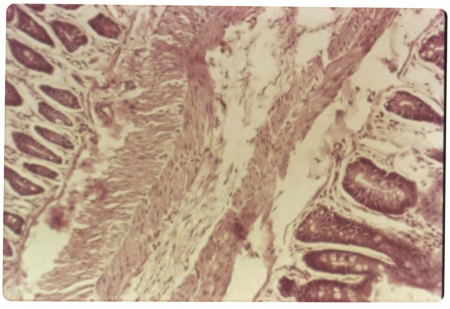

Figure 4. Histologic appearance of fibrous adhesions between abdominal viscera following the regression of DAL as a result of delayed treatment with Cis-Pt.(II). H & E stain. x 63.

Figure 5. Gross appearance of rats bearing Day 7 development of the IM Walker tumor. Average tumor weight 5.5 gm. on Day 7.

Table 3. Day 3 treatment of IM W256 Carcinosarcoma with Cis-Pt.(II)

No. Rats	DAY 0 Tumor Brei Implanted	DAY 3 Cis-Pt.(II) (mg./kg.)	DAY 7 Average Tumor Weight (gm.)
6	0.2 ml.	4	1.1
6	0.2 ml.	2	< 1
6	0.2 ml.	0	5.5

Figure 6. Day 7 gross appearance of IM Walker tumor-bearing rats following treatment with 2 mg./kg. Cis-Pt.(II) on Day 3. Maximal tumor weight 1.0 gm. on Day 7.

rats treated with 2 mg./kg. Cis-Pt.(II) on Day 3 and killed on Day 7. Histologic examination of the intramuscular tumor site following treatment revealed that the neoplastic process had been inhibited to the point where only focal areas of neoplastic cells remained on Day 7 (Figure 7). Histologic appearance of the intramuscular tumor mass from untreated rats on Day 7 is shown in Figure 8.

Part B. Day 7 Treatment with Cis-Pt.(II). Treatment on Day 7 extended survival time of all rats to at least 30 days, at which time all rats were terminated. Figure 9 shows the gross appearance of rats treated with 4 mg./kg. Cis-Pt.(II) on Day 7 and killed on Day 30. Gross evidence of neoplasia was limited to 3 metastatic nodules in the subcutaneous region of 1 rat. Histologic examination of the initial intramuscular site of implantation revealed the neoplastic process had been reduced to a focal area of tumor cells intermixed with cellular debris (Figure 10).

Figure 11 shows the gross appearance of rats treated with 2 mg./kg. Cis-Pt.(II) on Day 7 and killed on Day 30. Gross evidence of neoplasia was limited to 1 rat with local tumor development at the site of initial implantation. Histologic examination of the regressed tumor site disclosed similar findings previously described for the other group of treated rats.

The average survival time of rats bearing the untreated Walker 256 tumor was 16 days (Table 4). The gross appearance of rats succumbing to the terminal Walker 256 tumor is shown in Figure 12. Histologic examination revealed occasional metastatic tumor cells located in the liver, lung, lymph nodes, and brain.

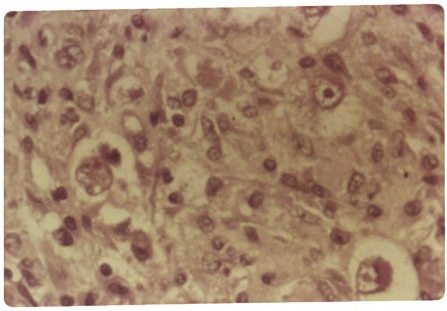

Figure 7. Histologic appearance of IM Walker tumor cells following treatment with 2 mg./kg. Cis-Pt.(II). H & E stain. x 250.

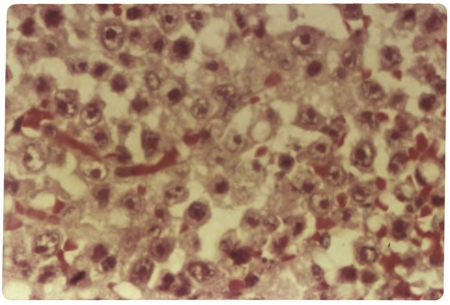

Figure 8. Histologic appearance of IM Walker tumor cells from untreated rats. Note high mitotic index and prominent nucleoli present in tumor cells. H & E stain. x 250.

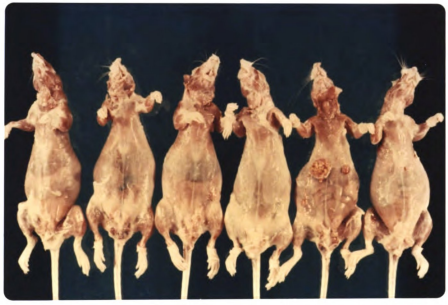

Figure 9. Day 30 appearance of IM Walker tumor-bearing rats treated with 4 mg./kg. Cis-Pt.(II) on Day 7. Gross evidence of neoplasia limited to metastatic nodules in subcutaneous abdominal region of 1 rat.

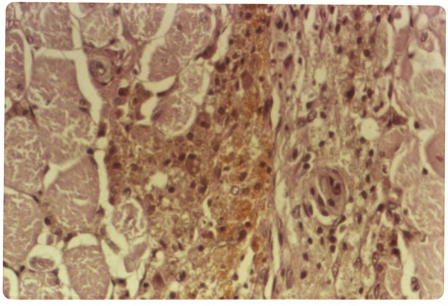

Figure 10. Day 30 histologic appearance of initial IM site of Walker tumor implantation following treatment with 4 mg./kg. Cis-Pt.(II) on Day 7. The neoplastic process has been reduced to focal areas of tumor cells intermixed with cellular debris. H & E stain. x 125.

Figure 11. Day 30 gross appearance of IM Walker tumor-bearing rats treated with 2 mg./kg. Cis-Pt.(II) on Day 7. Gross evidence of neoplasia limited to 1 rat showing tumor development at site of initial implantation.

Table 4. Day 7 treatment of IM W256 Carcinosarcoma with Cis-Pt.(II)

	DAY 0	DAY 7	DAYS 14-17	DAY 30
No. Rats	Tumor Brei Implanted	Cis-Pt.(II) (mg./kg.)	Deaths	Survivors
6	0.2 ml.	4	0/6	6/6
6	0.2 ml.	2	0/6	6/6
6	0.2 ml.	0	6/6	0/6

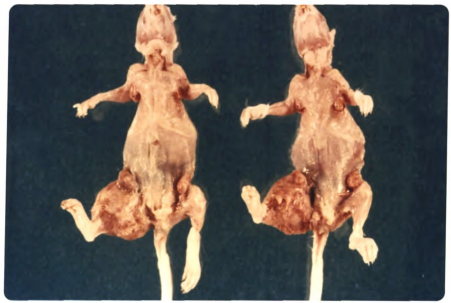

Figure 12. Gross appearance of rats succumbing to terminal effects of IM Walker tumor. Average survival time 16 days. Metastatic nodules located in lymph nodes, liver, lung and other organs.

Phase 3. Evaluation of Toxicologic Effects of Cis-Pt.(II)

Part A. Determination of LD_{50} in Male Rats. Figure 13 shows the data fitted to a straight line by the method of least squares. The LD_{50} was estimated at 12.2 mg./kg. for the male albino rat.

Part B. Serial Toxicity Study of Cis-Pt.(II) in Male Rats

1. Hematologic and Serum Chemistry Alterations. Following a single IP injection of 12.2 mg./kg. Cis-Pt.(II) a marked leukopenia occurred. Figure 14 illustrates this decrease in total leukocytes which was most severe at 3 days postinjection. This was followed by a regenerative increase in which the counts returned to the normal range by Day 5 through Day 7. By Day 9 the circulating leukocyte counts were elevated above the normal range. Determinations made on Day 20 and Day 28 showed a gradual return of the total leukocyte count to the normal range. Examination of the neutrophil and lymphocyte counts also revealed maximal depression at 3 days after injection, followed by a regenerative increase and finally a return to the normal range.

The number of circulating platelets was also depressed most severely on Day 3 postinjection. A maximal depression of reticulocytes occurred by Day 4 postinjection (Figure 15). This was followed by a regenerative increase, in which the reticulocyte counts returned to the normal range. Determination of circulating erythrocytes, packed cell volumes and hemoglobin values revealed no marked alterations following injection of Cis-Pt.(II).

Examination of blood urea nitrogen (BUN) values (Figure 16) revealed a sharp increase by Day 3-Day 4, followed by a return to the normal range.

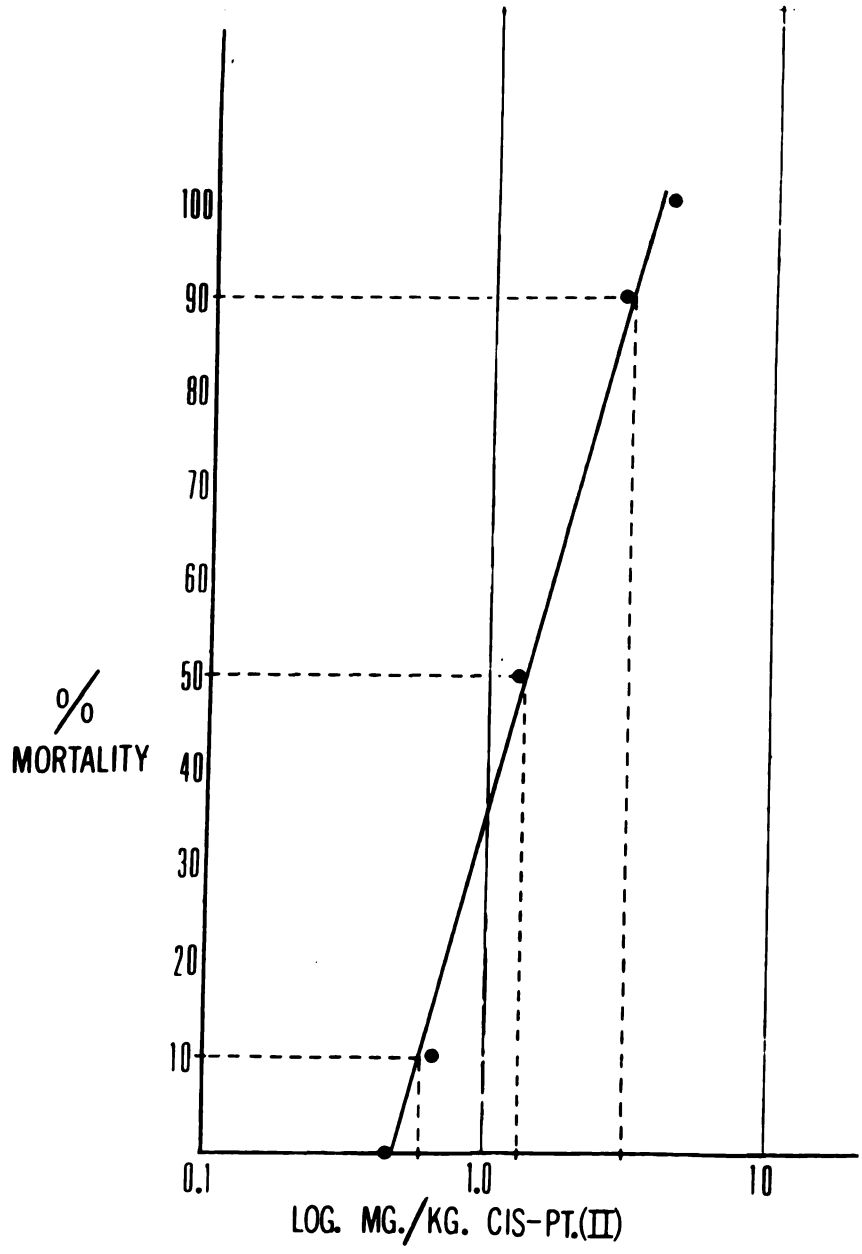

Figure 13. Determination of LD_{50} following the fitting of the data to a straight line by the method of least squares. LD_{50} estimated at 12.2 mg./kg. Cis-Pt.(II) for the male rat. LD_{90} and LD_{10} can also be estimated at 26.5 mg./kg. and 5.5 mg./kg., respectively.

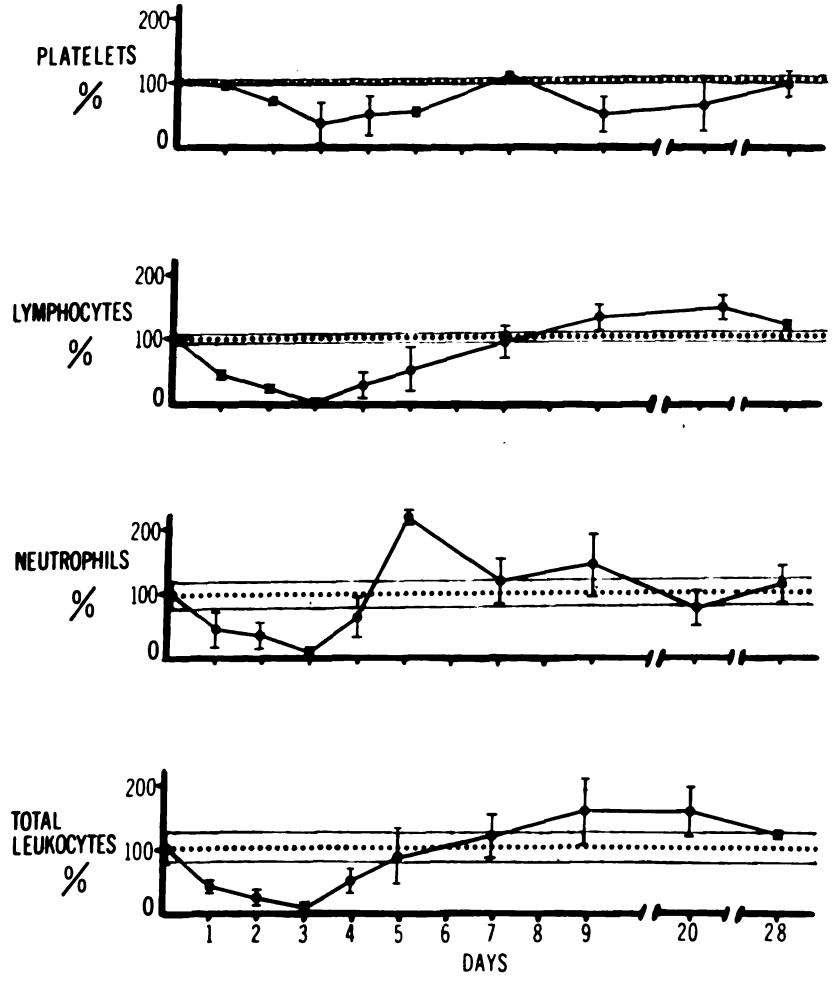

Figure 14. Serial alterations observed in blood leukocytes and platelets following a single IP injection of 12.2 mg./kg. Cis-Pt.(II) on Day 0. All values converted to relative percentage of the normal values (100% $\pm$ 1 std. dev.) established for the control rats used in this study. All plotted points signify mean $\pm$ 1 std. dev.

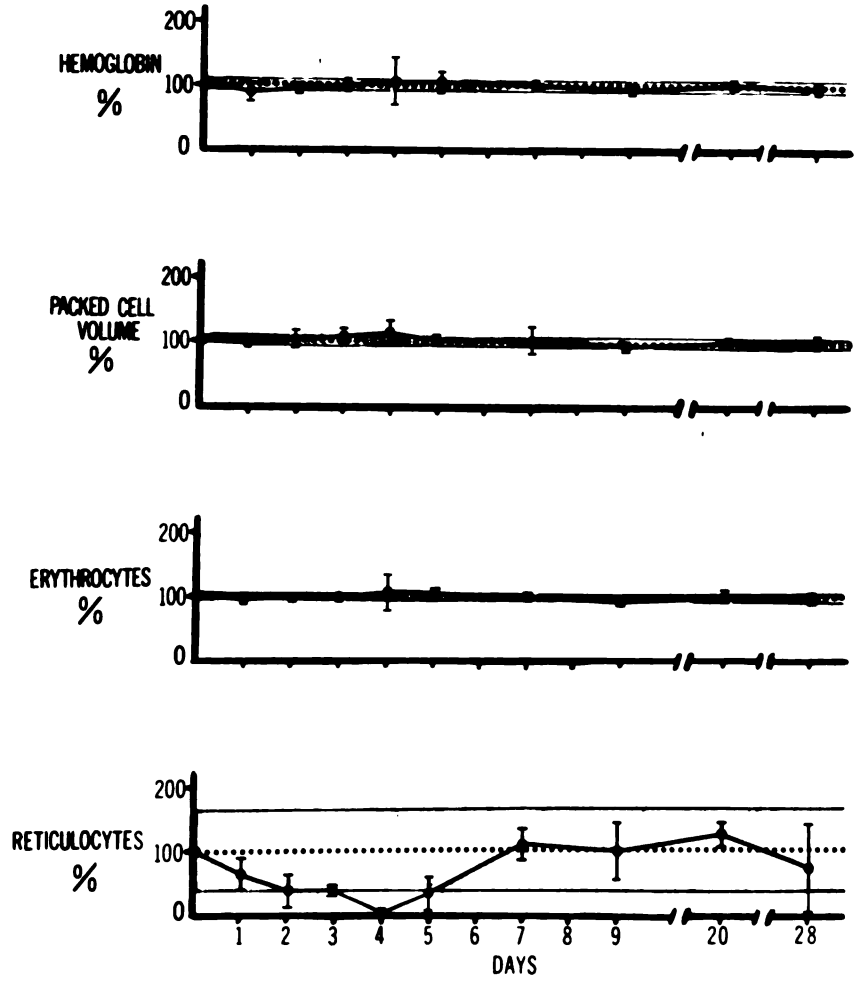

Figure 15. Serial alterations in blood reticulocytes, erythrocytes, packed cell volumes and hemoglobin values following a single IP injection of 12.2 mg./kg. Cis-Pt.(II) on Day 0. All values converted to relative percentage of normal values (100% $\pm$ 1 std. dev.) established for the control rats used in this study. All plotted points signify mean $\pm$ 1 std. dev.

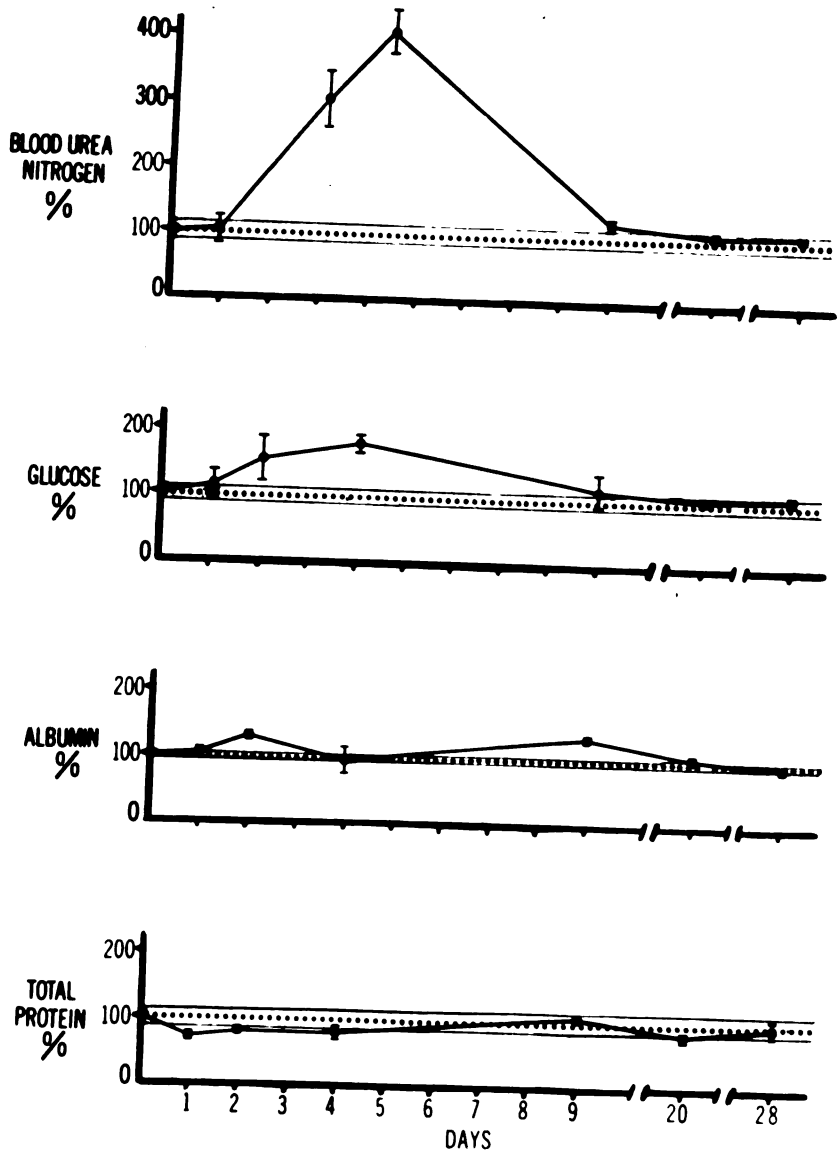

Figure 16. Serial alterations in blood urea nitrogen, glucose, albumin and total protein values following a single IP injection of 12.2 mg./kg. Cis-Pt.(II) on Day 0. All values converted to relative percentage of normal values (100% $\pm$ 1 std. dev.) established for the control rats used in this study. All plotted points signify mean $\pm$ 1 std. dev.

Serum glucose levels were elevated 2-4 days postinjection, followed by a return to normal (Figure 16).

Serum albumin levels were inconsistently elevated following injection of Cis-Pt.(II), whereas total protein levels were depressed from Day 1-Day 4 postinjection. Later determinations showed both albumin and total protein values near the normal range of values (Figure 16).

Total bilirubin values and uric acid values were in the lower portion of the normal range of values (Figure 17). Calcium and inorganic phosphorus values remained in the normal ranges.

Figure 18 shows changes in 3 serum enzymes and cholesterol values following injection of 12.2 mg./kg. Cis-Pt.(II). Cholesterol values fluctuated only to a small degree, and in most cases stayed within normal limits.

Lactic dehydrogenase values, alkaline phosphatase values and glutamic oxaloacetate transaminase values were irregularly decreased following intoxication with Cis-Pt.(II), and in no case were any values increased above normal levels (Figure 18).

2. Bone Marrow. Injection of 12.2 mg./kg. Cis-Pt.(II) caused a general shift to the right, with a relative decrease of the immature pronormoblasts and myeloblasts in the bone marrow. By Day 2, swelling and dissolution of hematopoietic cells, vacuolation of cytoplasm, fragmentation of cytoplasm and arrested mitotic activity were noted in bone marrow elements.

Maximal depletion of the bone marrow occurred on Day 3, with the general hypocellularity of the marrow accompanied by engorgement of dilated sinusoids with erythrocytes.

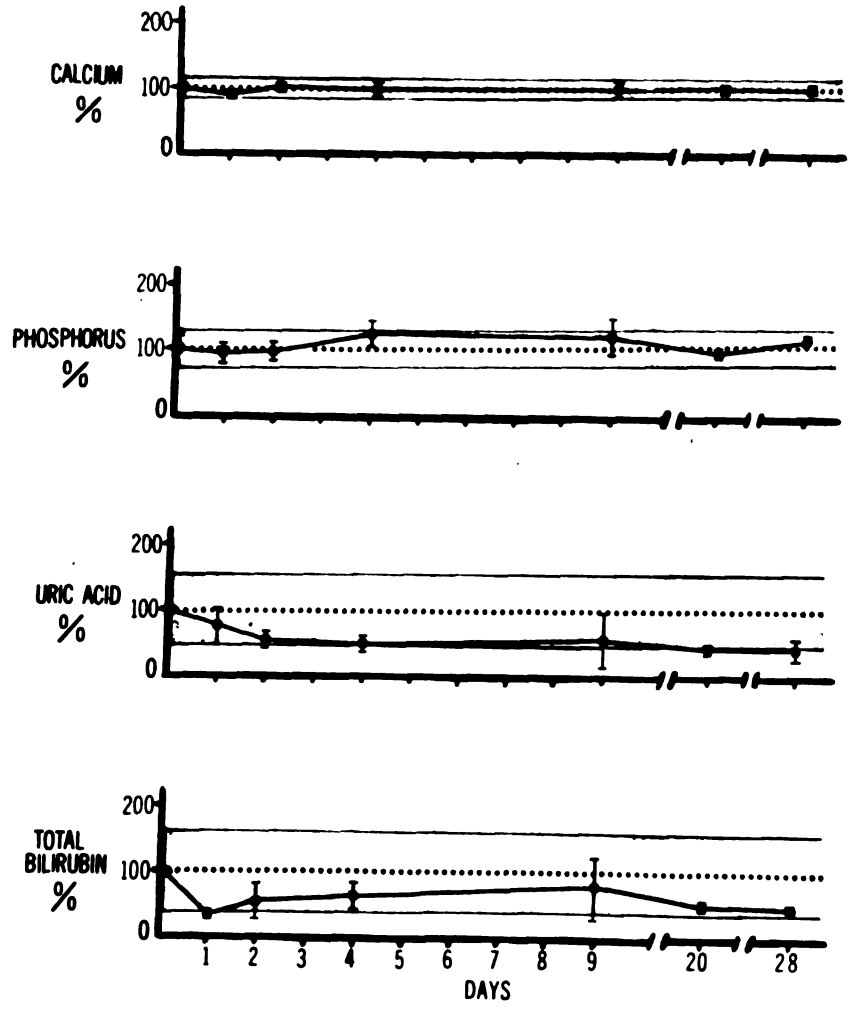

Figure 17. Serial alterations in total bilirubin, uric acid, calcium and inorganic phosphorus following the IP injection of 12.2 mg./kg. Cis-Pt.(II) on Day 0. All values converted to relative percentage of the normal values (100% $\pm$ 1 std. dev.) established for the control rats used in this study. All plotted points signify mean $\pm$ 1 std. dev.

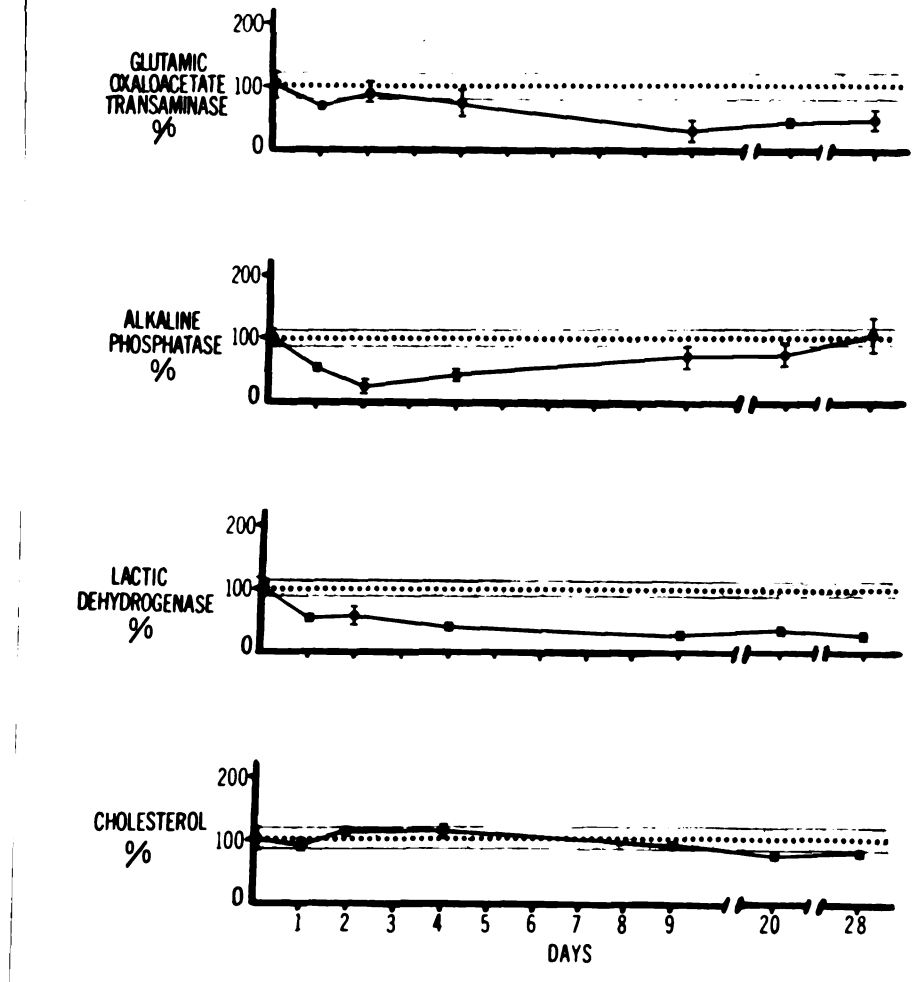

Figure 18. Serial alterations in serum enzymes and cholesterol values following a single IP injection of 12.2 mg./kg. Cis-Pt.(II) on Day 0. All values converted to relative percentage of normal values (100% $\pm$ 1 std. dev.) established for the control rats used in this study. All plotted points signify mean $\pm$ 1 std. dev.

Rats surviving the LD_{50} dose began to show regenerative activity throughout the depleted bone marrow by 4-5 days after injection. There was a marked shift to the left as the new stem cells appeared in the marrow. From Day 9 through Day 20 the bone marrow continued to show evidence of increased regenerative activity.

3. Thymus and Lymphoid Tissue. The involution of lymphoid tissue in the body was most easily followed in the thymus (Figure 19). Moderate atrophy was noted by Day 2, with further involution through Day 4. As the thymus underwent this involution, the cortical portion shrank somewhat more rapidly than the medulla, and during the period of cortical contraction the lymphocytes appeared more numerous in the medulla than in the cortex, thus tending to reverse the normal histologic pattern. Maximal involution of lymphoid elements caused the thymus to be small and fibrous on Day 3-Day 4. Rats surviving the LD_{50} showed thymuses which were increased in size, and histologically exhibited hyperplastic changes. As a result of this lymphoid hyperplasia the cortex reassumed its normal lymphocytic appearance, while the medulla became more conspicuous for its reticular cells and epithelial elements.

4. Spleen. A progressive reduction in the size of the spleen (Figure 20) resulted from the disappearance of lymphocytes with atrophy of the Malpighian corpuscles. The ultimate stage in the regression of the spleen was noted at Day 3-Day 4 after injection, at which time the disappearance of lymphocytes from the Malpighian corpuscles and the disappearance of myeloid and erythroid precursors from the red pulp gave the organ a somewhat fibrous appearance.

The spleens of rats surviving the LD_{50} dosage showed excessive lymphoid regeneration, with the enlargement of the Malpighian corpuscles.

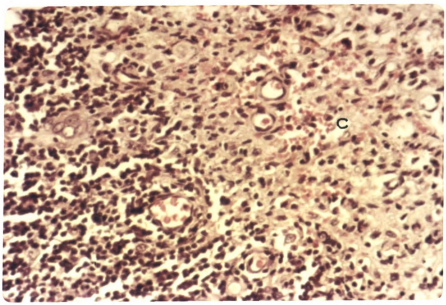

Figure 19. Histologic appearance of thymus on Day 4 following IP injection of 12.2 mg./kg. Cis-Pt.(II) on Day 0. Note extreme involution of the cortical area (C) due to lymphoid depletion. H & E stain. x 125.

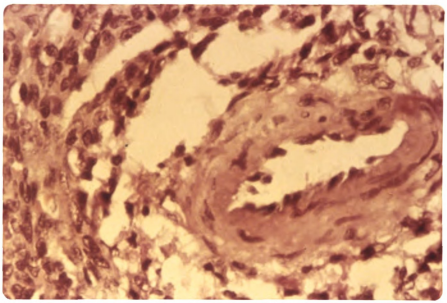

Figure 20. Histologic appearance of spleen on Day 4 following IP injection of 12.2 mg./kg. Cis-Pt.(II) on Day 0. Note depletion of lymphocytes from Malpighian corpuscle. H & E stain. x 250.

In the red pulp, clusters of primitive hematopoietic cells reappeared, with megakaryocytes, granulocytes and erythrocytes found around these areas. These regenerative changes were most pronounced from Day 9 through Day 20.

5. Intestinal Tract. Clinical evidence of diarrhea and alterations of the intestinal tract were consistently indicative of intestinal injury following the LD_{50} dosage of Cis-Pt.(II). The lesions were most severe in the small intestine. By Day 1 after injection the villi of the intestinal mucosa were edematous and the lining cells were swollen and distended with clear vacuoles (Figure 21). At Day 3-Day 4 when the lesions were most severe, the crypts of Lieberkuhn appeared as cyst-like spaces, lined by flat, elongated cells. The mucosal surface was irregularly denuded, or showed patchy areas of hypoplasia (Figure 22). In later stages a slight fibrous reaction sometimes led to deposits of connective tissue in the villi which tended to become shorter, broader and stubby. The maximal epithelial alterations and sloughing coincided in time with the development of fluid distention and mucous diarrhea.

The intestinal epithelium of rats surviving the LD_{50} dosage showed patchy areas of epithelial hyperplasia, with the crypts lined by hyperchromatic cells with increased mitotic activity.

6. Urinary System. Kidneys of rats killed on Day 2 after injection had minimal evidence of tubular vacuolation and dilatation. Irregularities in staining characteristics were also noted in the tubular epithelium, suggesting early tubular necrosis. By Day 3 there was further tubular necrosis, with hyaline casts present in the tubular lumina. Extensive loss of tubular elements was noted by Day 4, especially in the deeper aspects of the renal cortex (Figure 23).

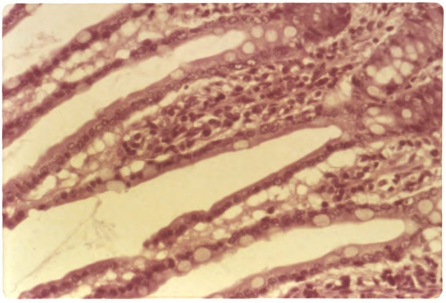

Figure 21. Histologic appearance of intestinal epithelium on Day 1 following IP injection of 12.2 mg./kg. Cis-Pt.(II) on Day 0. Note vacuolation of cytoplasm of some cells and the dilatation of lymphatic channels. Mitotic activity has been inhibited in the crypts of Lieberkuhn. H & E stain. x 125.

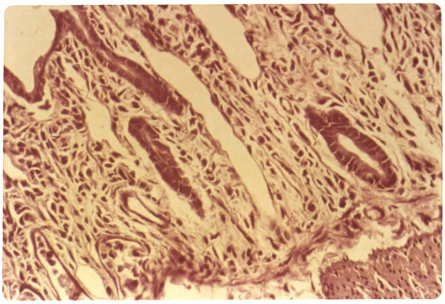

Figure 22. Histologic appearance of intestinal epithelium on Day 4 following IP injection of 12.2 mg./kg. Cis-Pt.(II) on Day 0. Note hypoplasia of epithelium and cyst-like appearance of crypts as a result of inhibition of normal cell replication. H & E stain. x 63.

Rats surviving the LD_{50} dosage showed variable degrees of tubular epithelial regeneration. The tubular alterations, which were most severe at Day 4, closely paralleled the elevation in the BUN values at this time.

7. Respiratory System. No consistent alterations were observed in the upper or lower respiratory tract following the LD_{50} dosage of Cis-Pt.(II).

8. Circulatory System. Some evidence of congestion was noted in various organs, such as the thymus, following administration of the LD_{50} dosage. No histologic evidence of cardiac injury was observed.

9. Male Reproductive System. Some sections of the testes possibly suggested a temporary arrestment of normal spermatogenesis, but to fully evaluate any injury to the testes, additional studies are indicated. Other factors, such as marked weight loss, have been shown to diminish pituitary activity (Mulinos and Pomerantz, 1940) and must be considered.

10. Central Nervous System. The brain showed no histologic abnormalities following the LD_{50} dosage of Cis-Pt.(II). Work by Jean Allen (1970) has indicated this compound does not cross the blood-brain barrier to any great extent.

11. Liver. No consistent changes were observed in the liver which could have been attributed to treatment with Cis-Pt.(II). Variable degrees of glycogen depletion were observed.

12. Musculoskeletal System. No abnormalities were observed in sections of skeletal muscle, cartilage or osseous tissue which were examined.

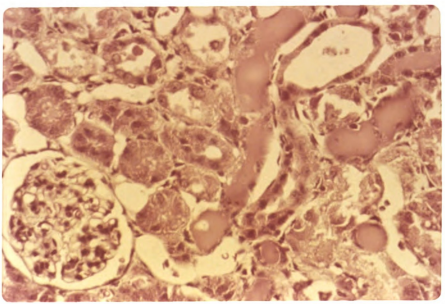

Figure 23. Histologic appearance of renal tubules on Day 4 following IP injection of 12.2 mg./kg. Cis-Pt.(II) on Day 0. Note the extensive tubular sloughing and presence of hyaline casts in lumina of tubules. H & E stain. x 125.

13. Endocrine System. No consistent histologic changes were noted in thyroid, parathyroid, pancreas or adrenal glands. The pituitary gland was not studied.

DISCUSSION

The initial experiments utilized the Dunning Ascitic Leukemia to evaluate the cancer chemotherapeutic properties of Cis-Pt.(II). This transplantable neoplasm, first described by Dunning and Curtis (1957), has proven very useful both as a screening test for potential anticancer agents (Jones *et al.*, 1958) as well as for quantitative pharmacological studies of cancer chemotherapeutic agents. The remarkable uniformity of neoplastic development, survival time and extent of organ infiltration all serve as critical parameters for evaluating the effects of candidate compounds on this neoplasm.

The CCNSC has chosen the DAL as one of a select few transplantable neoplasms for use in cancer chemotherapy studies. The CCNSC has published recommended protocols (CCNSC, 1962) to be followed during the transplantation of this neoplasm, as well as during the evaluation of any cancer chemotherapeutic effects which a candidate compound may exhibit against the development of this neoplasm. Any candidate compound which extends the survival time of the DAL bearing rats by a minimum of 25% following treatment from Day 1 through Day 5 postimplantation is considered significant by the CCNSC protocols. Any candidate compound giving these minimal results is then considered to warrant additional study in regard to its cancer chemotherapeutic properties.

The results of this study, in which the compound Cis-Pt.(II) was administered in the form of a single IP injection on Day 1 gave results which far surpassed the criteria established by the CCNSC.

Thus a single injection of Cis-Pt.(II) on Day 1 gave results comparable to repeated treatments from Day 1 through Day 5 with various alkylating agents, such as the nitrogen mustards or cyclophosphamide which have been very effective against Dunning Leukemia (Table 5). Amethopterin (an antifolate), 6-mercaptopurine ribonucleoside (purine analog) and mitomycin C have been somewhat effective against Dunning Leukemia. It has been reported as being refractory to treatment with 6-mercaptopurine (purine analog), urethane (ethylcarbamate), 5-fluorouracil (pyrimidine analog), actinomycin D, colchicine or hydrocortisone (Skipper and Schmidt, 1962).

The challenge dose of 5×10^6 DAL cells implanted IP into all Day 30 survivors failed to reestablish the neoplastic process in any of the rats previously treated with Cis-Pt.(II) subsequent to implantation of DAL. This indicated these rats had been exposed to DAL, had successfully overcome the neoplasia, and had a protective immunity to the challenge dose of DAL cells. In contrast, all rats which had not been initially implanted with DAL cells and then treated with Cis-Pt.(II) died approximately 2 weeks following the challenge dose of DAL cells. Postmortem examination disclosed the presence of terminal DAL in all these rats. Gross and microscopic examinations of the treated rats which were refractory to DAL cell reimplantation were conducted on Day 30' (60 days from initial DAL implantation) and failed to disclose any evidence of neoplastic cells.

The successful delayed treatment of DAL with a single injection of Cis-Pt.(II) on Day 4 or Day 7 offered additional evidence supporting the potential cancer chemotherapeutic properties of this compound. Comparable data in reference to the activity of other cancer chemotherapy agents used in the delayed treatment of DAL are scant. However, Skipper and

Table 5. Therapeutic indices of various compounds against Dunning Leukemia and Walker tumor systems*

Tumor	Walker	Walker	Dunning	Dunning	Dunning
Form	Subcut.	Subcut.	Subcut.	Subcut.	Subcut.
Days Treated	1-5	7-11	1-5	7-11	1-5
Route	IP	IP	IP	IP	IP
Days Assessed	18-21	23-28	28	1-30	28
Criteria	Tumor Weight	Tumor Weight	Survival	Life Span	Survival
Compound					
Nitrogen mustard	2	1	0	2	3
Nitromin	9	2	3	7	7
Chlorambucil	10	9	2	8	2
dl-Phenylalanine mustard	14	22	3	6	19
l-Phenylalanine mustard	14	40	3	7	18
Mannitol mustard	2	1	0	3	2
Benzimidazole mustard	7	6	2	8	8
Chloroquine mustard	0	0	0	0	0
Triethylene-melamine	7	5	2	8	1
Triethylenephos-phoramide	8	5	2	7	3
Cyclophosphamide	19	28	4	26	9
Busulfan	0	0	0	0	0
Amethopterin	0	0	2	0	-
6-Mercaptopurine	1	4	0	0	-
6-Mercaptopurine ribonucleoside	1	12	4	0	-
Azoserine	0	0	0	0	-
Urethane	0	0	0	0	-
5-Fluorouracil	0	0	0	0	-
Hydrocortisone	0	0	0	0	-
Actinomycin D	0	0	0	0	-
Mitomycin C	9	16	4	4	-
Colchicine	0	0	0	0	-

*Zero (0) indicates those agents which failed to inhibit tumor growth to 40% of controls (or increase life span by 40%) at the LD_{10} dosage. In comparing therapeutic indices it is essential that consideration be given to differences in protocols employed. The reader is referred to the original source of these data (Skipper and Schmidt, 1962) for additional information.

Schmidt (1962) have compiled quantitative data indicating the delayed treatments (Day 7 through Day 11) with the various alkylating agents were most effective against the subcutaneous form of Dunning Leukemia (Table 5). Mitomycin C was somewhat effective, while 6-mercaptopurine, urethane, 5-fluorouracil, actinomycin D, colchicine and hydrocortisone were ineffective.

Postmortem examination of the DAL implanted rats which were treated during the more advanced stages of development of the neoplasm (Day 4 or Day 7 treatment) revealed the presence of adhesions between adjacent abdominal viscera. This indicated the DAL had undergone a regression following treatment with Cis-Pt.(II).

The Walker 256 Carcinosarcoma has also been recommenced as a transplantable neoplasm to be used in cancer chemotherapy studies (CCNSC, 1962). This neoplasm has a high rate of cell division in that the cell population doubles every 1.7 days (Bertalanffy and Lau, 1962). The fine structure of this neoplasm has been well described (Fisher and Fisher, 1961). These factors, plus its high rate of reproducibility (100%), relative lack of spontaneous regression (2%) and its ability to metastasize to all parts of the body make the Walker tumor ideal for cancer chemotherapy studies.

The recommended CCNSC protocol is based on implantation of the neoplastic cells on Day 0 followed by treatment on a daily basis from Day 3 through Day 6. All rats are killed on Day 7, and any candidate compound which inhibits tumor development to 60% or less when compared to nontreated tumor rats is considered significant, and thus warrants additional study.

In this study a single IP injection of Cis-Pt.(II) on Day 3 caused a marked inhibition of the neoplasm, so that by Day 7 tumor development had been inhibited to less than 20% of the nontreated tumors.

Skipper and Schmidt (1962) listed the chemotherapeutic agents which were effective in inhibiting the early development of the subcutaneous Walker tumor. Included in this group were the nitrogen mustards, 6-mercaptopurine, 6-mercaptopurine ribonucleoside and mitomycin C. Ineffectual compounds included amethopterin, urethane, 5-fluorouracil, hydrocortisone, actinomycin D and colchicine (Table 5).

Delayed treatment of rats bearing the Walker tumor on Day 7 caused a marked regression of the tumor at the initial site of implantation. In addition, it increased the survival time of the rats and reduced metastasis to other areas of the body.

Comparison of the other cancer chemotherapeutic agents used in delayed treatment (Day 7 through Day 11) revealed that the same compounds which were previously listed as possessing activity against the early stages of the Walker tumor were also effective in the later stages of development. Compounds found ineffective in the early treatment of the Walker tumor were also ineffective against the later stages.

The LD_{50} of the compound Cis-Pt.(II) was calculated to be 12.2 mg./kg. for the male rat. The LD_{90} and LD_{10} could also be calculated to be 26.5 mg./kg. and 5.5 mg./kg., respectively. The CCNSC (1964) has stated the LD_{10}, as read from plotted dosage-mortality data, can be employed as a reproducible maximum tolerated dose (MTD). This MTD has been considered just as important as the tumor-response data when calculating the maximum effectiveness or therapeutic index (TI) of a drug.

When increase in life span of the tumor bearing host has been employed as the end point in drug evaluation, the TI has been defined as follows:

$$\frac{LD_{10}\ \text{(Nontumor bearing animals)}}{ILS_{40}\ \text{(Dose giving 40\% increase in life span)}}$$

In reference to this study where the DAL was used as the test tumor the minimal TI could thus be calculated as follows:

$$TI = \frac{5.5 \text{ mg./kg.}}{2.0 \text{ mg./kg.}} = 2.75$$

Since therapeutic dosages lower than 2 mg./kg. Cis-Pt.(II) were not utilized in this study the actual TI may be considerably greater than the value which was calculated.

Skipper and Schmidt (1962) have compared the TI of various agents used in the treatment of DAL and Walker tumor and have listed various alkylating agents as having therapeutic indices ranging from a high of 40 down to 0 (Table 5).

The antitumor properties and toxicologic effects of Cis-Pt.(II) are strikingly similar to those of alkylating agents and x-irradiation. The term "radiomimetic" was introduced by Dustin (1947) to describe certain chemicals which induced cytological effects similar to those observed after x-irradiation. Elson (1955) has listed a series of properties which such compounds may have in common with x-irradiation. One of the best known radiomimetic chemicals is nitrogen mustard.

The observed pathologic effects of Cis-Pt.(II) suggested its biologic activity was not limited to any one tissue of the body, but resulted in alterations in those tissues having rapid turnover times. Examination of data compiled by Leblond and Walker (1956) supplemented by data published by Bertalanffy and Lau (1962) and also Schalm (1965) has permitted the listing of the turnover times of the cells comprising the more actively dividing tissues of the rat:

Blood granulocyte - 0.04 days

Blood lymphocyte - 0.3 days

Intestinal epithelium - 0.6-1.6 days

Bone marrow myeloid series - 2.5 days

Blood reticulocytes - 1.8 days

Bone marrow erythroid series - 2.5 days

Thymic lymphocyte - 2.5 days

Gastric epithelium - 1.8-6.5 days

Seminiferous epithelium - 16-27 days

Epidermis - 20-35 days

Blood erythrocyte - 60-80 days

The turnover time for the neoplastic cells of the Walker tumor has been estimated to be 1.7 days (Bertalanffy and Lau, 1962).

As the resultant histologic and hematologic alterations following a toxic dose of Cis-Pt.(II) included panleukocytopenia, reticulocytopenia, atrophy of intestinal epithelium, atrophy of the thymus and bone marrow repression, it was apparent that the degrees of tissue susceptibility were directly proportional to the rate of regeneration of the cells comprising that tissue. As a result those tissues having turnover times comparable to that of neoplastic cells showed the most pronounced alterations.

The observed histologic alterations following intoxication with Cis-Pt.(II) were similar to those reportedly caused by the nitrogen and sulfur mustards (Graef *et al.*, 1948) or by x-irradiation (Upton, 1963).

Hematologic alterations were somewhat similar to those reported for other commonly used cancer chemotherapeutic agents such as the nitrogen and sulfur mustards, urethane (Dustin, 1947), 6-mercaptopurine (Clarke *et al.*, 1953) and mitomycin C (Philips, Schwartz and Sternberg, 1960). The number of circulating lymphocytes and neutrophils (short turnover times) was rapidly depressed to low levels by Day 3 after injection of Cis-Pt.(II). This was followed by a rapid regenerative increase which

created a temporary leukocytosis which eventually returned to the normal range.

The number of circulating platelets was not depressed as much as the leukocytes following intoxication with Cis-Pt.(II). Megakaryocytes have been known to be relatively more resistant to the toxic effects of radiomimetic agents (Kindred, 1947).

While the production of reticulocytes was temporarily inhibited, the number of circulating erythrocytes was not altered. This could be explained by the longer life span of the rat erythrocyte (60-80 days) and the lack of any evidence indicating hemolysis of erythrocytes following Cis-Pt.(II) intoxication. If the compound Cis-Pt.(II) were capable of hemolyzing erythrocytes it would be expected to cause an increase in the bilirubin level; however, this was not the case.

Packed cell volumes and hemoglobin levels were unchanged except for a slight elevation which occurred at the time coincident with dehydration and enteritis (Day 4).

Total protein levels in the blood include an extremely complex mixture of simple and conjugated proteins. Clinically, serum proteins are divided into 2 large classes, albumins and globulins. The albumin fraction is synthesized mainly in the liver, whereas the globulin fraction contains some constituent proteins which have been synthesized in lymphoid cells (gamma globulins). The observed decrease in total protein following intoxication with Cis-Pt.(II) may have been a reflection of the lymphoid depression which occurred at this time. Albumin levels were essentially unchanged, and the slight elevations may have been due to the dehydration and enteritis.

Uric acid levels in the blood normally reflect the exogenously derived sources of purines and endogenously formed purines from nucleoprotein metabolism. The serum levels of uric acid following intoxication with Cis-Pt.(II) remained in the lower part of the normal range established for the control rats.

Intoxication with Cis-Pt.(II) apparently had no effect on serum levels of calcium or inorganic phosphorus, as all values remained in the normal range.

The circulating level of cholesterol is normally the result of intestinal absorption and hepatic synthesis balanced by degradation and hepatic utilization of cholesterol as the precursor of bile acids and other sterols. In this study blood levels of cholesterol were not altered by Cis-Pt.(II).

Lactic dehydrogenase (LDH) is normally the enzyme of the Embden-Meyerhof glycolytic pathway which reversibly catalyzes the oxidation of lactate to pyruvate. The enzyme is particularly abundant in hepatic, cardiac and muscular tissues and has been shown to be elevated following myocardial infarction, hepatitis and muscular trauma. As the activity of LDH in the erythrocytes is 100 times that of serum, any hemolysis will alter the LDH value.

Alkaline phosphatase is an enzyme that is most active between pH 8 and pH 10 and can utilize a variety of phosphomonoesters as substrates. Alkaline phosphatase is formed in bone by osteoblasts, and is also present in liver, kidney and intestine. Elevated serum levels accompany bone conditions in which there is excessive osteoblastic or chondroblastic activity. In hepatic dysfunction, the interference with biliary excretion of the enzyme produces an elevation of the serum level.

Glutamic oxaloacetate transaminase (GOT) normally catalyzes the transfer of the amino group from the amino acid (glutamic acid) to oxaloacetate and thus plays an important role in the metabolism of amino acids. High levels of GOT are normally present in cardiac muscle, liver, skeletal muscle, kidney and erythrocytes. Increased serum levels of GOT have been noted following cellular injury to any of these tissues.

In this study, serum levels of LDH, alkaline phosphatase and GOT were higher in the control rats than in treated rats. This suggested the absence of any extensive injury to those tissues containing high enzymic levels, such as hepatic or myocardial tissues, and may indicate that Cis-Pt.(II) has a general inhibitory effect on enzymes.

Glucose levels in the blood are normally controlled by hormonal factors. Epinephrine increases blood glucose levels by promoting glycogenolysis. Adrenocortical hormones also increase blood glucose levels by promoting gluconeogenesis in the liver. The resultant hyperglycemia noted following intoxication with Cis-Pt.(II) may have been the result of stress-induced hormonal-mediated interactions which increased the blood glucose levels. Some degree of hyperglycemia may also have been associated with the dehydration and enteritis which occurred following the intoxication.

The blood urea nitrogen (BUN) level is quantitatively the most important nonprotein nitrogenous constituent. It is the chief end product of protein metabolism and normally is excreted entirely by the kidneys. Hence its blood concentration is directly related to the protein content of the diet and the renal excretory capacity. The elevated BUN levels occurred at a time coincident with the development of tubular necrosis (Day 3-Day 4) and probably reflected the tubular injury associated with the excretion of Cis-Pt.(II). The work of Allen (1970) has indicated

Cis-Pt.(II) is excreted via the urinary route. Eventually the BUN values returned to the normal range as the renal tubules underwent regeneration (Day 20-Day 28).

In simple terms, toxicity as a consequence of the use of antitumor agents is a reflection of the fact that most of the therapeutically useful compounds are cytotoxic agents that affect normal as well as neoplastic cell replication, and in general these agents have a low therapeutic index. The compound Cis-Pt.(II) apparently will not be different in this respect from other cytotoxic agents.

As far as is known, neoplastic cells synthesize DNA and divide in essentially the same way as their normal counterparts. In fact, cell cycle times are not too dissimilar, although the proportion of cells in active proliferation and nonproliferation would be different. Toxicity and recovery from toxicity would then be dependent upon the distribution of normal and neoplastic cells which are in these pools (of proliferating or nonproliferating cells). The tissues of the body most frequently affected by cytotoxic agents are the bone marrow, gastrointestinal tract and lymphoid organs. The cellular constituents of these tissues all have high rates of renewal so that agents exerting their cytotoxic effects on DNA synthesis or on mitosis will encounter relatively large numbers of susceptible cells. As a result panhemocytopenia and intestinal denudation are observed clinically after the use of cytotoxic agents. In this respect Cis-Pt.(II) apparently possesses the same cytotoxicity as the other antitumor agents. Fortunately, the high rate of renewal of the cells most susceptible to cytotoxic agents means that once the offending agent is removed the regenerative process is quite rapid. Here again, my work indicated this to be the case following the use of Cis-Pt.(II), as rats surviving the LD_{50} showed regeneration in the affected tissues.

Thus the biologic action of Cis-Pt.(II) is apparently more similar than dissimilar to the other cytotoxic agents which have been used as oncostatic agents.

Much effort has been expended in the elucidation of the specific action of the cytotoxic agents that are employed as cancer chemotherapeutic agents. Brookes and Lawley (1964) have studied the mechanism of action of the alkylating agents, and have shown that sulfur mustard exerts a nucleophilic attack on the N-7 of guanine residues in DNA which labilized the glycosidic bond and subsequently caused the release of guanine from the DNA. They also showed that sulfur mustard could react with 2 guanine residues to produce a cross-linking, which they postulated to be the primary cytotoxic action. In a subsequent report Lawley and Brookes (1967) noted similar cross-linking was obtained following the addition of triethylenemelamine. Ruddon and Johnson (1968) have reported that DNA treated with nitrogen mustard had a decreased template activity for RNA polymerase and DNA polymerase. In spite of the considerable data compiled thus far, the view that the primary chemotherapeutic effect of alkylating agents results from their attack on DNA is not universally accepted (Heidelberger, 1969). Wheeler and Alexander (1964) found no correlation between the extent of alkylation of the DNA and chemotherapeutic effect. Apparently not all guanine residues in DNA are equally reactive to sulfur mustard. Until the nature of this specificity is better understood, the mechanism of action of the alkylating agents will remain somewhat obscure (Heidelberger, 1969).

A metabolite can be defined as some naturally occurring compound produced during metabolism, and an antimetabolite can be defined as a compound related structurally to the metabolite which prevents its utilization by competing with it for an enzyme.

The antifolates were one of the first antimetabolites to be utilized in cancer chemotherapy. Folic acid is normally reduced to tetrahydrofolate by the enzyme dihydrate reductase, which is powerfully inhibited by antifolates such as amethopterin (Methotrexate). Tetrahydrofolate reacts with formaldehyde (or other compounds at the same oxidation level) to give isomeric formyl or methylene tetrahydrofolates, which are the coenzymes of all one-carbon metabolism. Consequently, these coenzymes are required for 2 steps of purine synthesis and for thymidylate synthetase.

The extensive efforts by Baker and Meyer (1969) have made it possible to achieve a remarkable degree of specificity in the inhibition of dihydrate reductase enzyme isolated from L1210 tumor cells, but not affecting the enzyme isolated from mouse liver, spleen or intestine.

The general group of purine antimetabolites includes a large number of analogs of the naturally occurring purine bases (adenine and guanine). Various aspects relating to these compounds have been reviewed by Heidelberger (1967). Zimmerman and Greenberg (1965) have shown 8-azoguanine will inhibit protein synthesis as a result of being incorporated into RNA to give some sort of fraudulent messenger RNA or transfer RNA. Another purine antimetabolite, 6-mercaptopurine, has been shown to competitively inactivate a pyrophosphorylase enzyme which normally catalyzes the conversion of hypoxanthine and guanine to their corresponding nucleotides (Brockman, 1965). Brockman and Chumley (1965) have also reported that 6-mercaptopurine inhibited the formation of phosphoribosylamine which is the first step in purine biosynthesis. This inhibition was of the negative feedback type which is also exhibited by the naturally occurring purines.

The pyrimidine antimetabolites include analogs of the pyrimidine bases that normally occur in the nucleic acids (uracil, thymine and cytosine). The only pyrimidine antimetabolite which is chemotherapeutically

active is 5-fluorouracil. With all other pyrimidine analogs, the nucleoside (5-iodo-2-deoxyuridine or IUdR) has been shown to be incorporated into nucleic acids (Prusoff, Bakhle and McCrea, 1963). Additional studies have also shown the inhibition of various enzymes necessary for nucleic acid synthesis.

In addition to the alkylating agents and antimetabolites, a large number of additional chemotherapeutic agents have been subjected to studies to determine the specific cytotoxic action. Mitomycin C is an antibiotic which has the potential to cross-link complementary strands of the double helix DNA (Iyer and Szybalski, 1963). Actinomycin D has been shown to block the synthesis of RNA (Sartorelli, 1964). Administration of L-asparaginase apparently depletes the serum of the amino acid asparagine, thereby causing selective starvation of tumors for which L-asparagine is an essential nutrient. Thus L-asparaginase chemotherapy utilizes a somewhat different biologic approach than the majority of oncostatic agents which have as their basic mechanism of action some cytotoxic property.

The preceding discussion has served as a prelude to the final point to be discussed; namely the question as to how the compound Cis-Pt.(II) exerts its oncostatic effects. Data presented in this study have suggested a strong parallelism between the oncostatic properties (and toxicologic effects) of Cis-Pt.(II) and those compounds, such as the alkylating agents, which can be referred to as cytotoxic agents. The oncostatic properties of these cytotoxic agents are based on the indiscriminate ability to interfere with those processes necessary for cellular replication.

The hypothesis that Cis-Pt.(II) exerts its oncostatic effect by virtue of its general cytoinhibitory properties has been amply demonstrated by the work of Howle and Gale (1970), in which they showed the inhibition

of incorporation of isotopic precursors into DNA, RNA and proteins. A further definition of the basic mechanism of action has been the subject of several studies which are still in progress.

SUMMARY AND CONCLUSIONS

A 3-phase study of the oncostatic properties and toxicologic effects of the compound Cis-Pt.(II) was conducted. Phase 1 utilized the Dunning Ascitic Leukemia as a test tumor to evaluate the oncostatic properties of Cis-Pt.(II) and yielded data which showed that Day 1 treatment of DAL-bearing rats with 2 mg./kg. or 4 mg./kg. Cis-Pt.(II) extended the survival time to at least 60 days. This was in contrast to the expected 10-14 day survival time of untreated DAL-bearing rats. Delayed treatment of DAL-bearing rats on Day 4 or Day 7 caused a regression of the neoplastic process and also extended the survival time.

Phase 2 utilized the intramuscular Walker Carcinosarcoma 256 in a further evaluation of the oncostatic properties of Cis-Pt.(II). A single IP injection of 2 mg./kg. or 4 mg./kg. Cis-Pt.(II) on Day 3 after tumor implantation caused a marked inhibition of the neoplastic process. This same therapeutic regimen administered on Day 7 after tumor implantation also inhibited the neoplastic process at the site of initial implantation and also decreased the rate of metastasis and increased the survival time of the tumor-bearing rats.

Phase 3 was an attempt to define the toxicologic parameters of Cis-Pt.(II), and the LD_{50} was determined to be 12.2 mg./kg. in the male rat. The LD_{90} and LD_{10} were calculated to be 26.5 mg./kg. and 5.5 mg./kg., respectively. A minimal therapeutic index was calculated to be 2.75 (based on limited data available from trials which utilized the DAL).

The histologic alterations subsequent to the IP injection of 12.2 mg./kg. Cis-Pt.(II) were most pronounced in those tissues having cellular constituents which have short turnover times. Thymic atrophy (due to lymphocytic depletion), splenic depletion of lymphoid elements, intestinal epithelial denudation and bone marrow repression were most severe at 2-4 days after intoxication. Renal tubular necrosis and sloughing also occurred at this time, possibly as a result of tubular damage associated with the renal excretion of Cis-Pt.(II). Rats surviving the intoxication showed regeneration of the cellular constituents in those tissues which were affected.

Panleukocytopenia, reticulocytopenia and blood platelet depression were also most severe at about Day 3 after injection of 12.2 mg./kg. Cis-Pt.(II). This was followed by a regenerative leukocytosis and reticulocytosis in rats surviving the intoxication. Erythrocyte counts, PCV and hemoglobin values were not depressed by the toxic dosage of Cis-Pt.(II), and serum bilirubin levels were not elevated.

Serum albumin levels were essentially unchanged, whereas the depression of total serum protein levels occurred at a time coincident with maximal lymphoid depletion.

Serum levels of uric acid, calcium, inorganic phosphorus, lactic dehydrogenase, alkaline phosphatase and glutamic oxaloacetate transaminase were not elevated subsequent to the injection of 12.2 mg./kg. Cis-Pt.(II).

A slight hyperglycemia occurred at a time coincident with the occurrence of enteritis and dehydration.

Levels of BUN were elevated at a time (Day 3-4) coincident with the observation of tubular necrosis in the kidney. The BUN values returned to the normal range as the tubular epithelium underwent regeneration.

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VITA

Richard J. Kociba was born in Harbor Beach, Michigan, on April 8, 1939. He graduated from Our Lady of Lake Huron High School, Harbor Beach, Michigan.

In 1959, after service in the United States Army, he enrolled as a student in Port Huron Junior College, Port Huron, Michigan. The following year he transferred to Michigan State University, where the Bachelor of Science degree was awarded with honors in 1964. In March, 1966, the Doctor of Veterinary Medicine degree was awarded with honors.

From March, 1966, to March, 1967, the author was employed in a private veterinary practice and diagnostic laboratory at Milford, Indiana. In April, 1967, he joined the Faculty of the College of Veterinary Medicine at Michigan State University.

In March, 1969, the author was awarded the Master of Science degree in Pathology. A Postdoctoral Fellowship was awarded by the National Institutes of Health for the completion of the Doctor of Philosophy degree in Pathology.