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**CURE WITH CISPLATIN (II) OF MURINE MALARIA INFECTION
AND IN VITRO INHIBITION OF A CHLOROQUINE-RESISTANT
PLASMODIUM FALCIPARUM ISOLATE**

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SUMMARY: Antiplasmodium properties of cisplatin [cis-platinum (II) diamine dichloride], a neoplastic drug, have been assessed in in vivo and in vitro model systems of malarial parasite. A well-tolerated dose of 6 mg/kg body weight of the compound cured the mice infected with *Plasmodium berghei* and the amount of cisplatin required for in vitro inhibition (IC₅₀) of a chloroquine-resistant *Plasmodium falciparum* isolate was smaller than either chloroquine or quinine. The minimum inhibitory concentration (MIC) needed to prevent the in vitro multiplication of asexual blood parasites was 30 ng/ml. Late ring and trophozoite stages of the erythrocytic cycle were the most susceptible, whereas schizont and early ring stages were the least sensitive to the toxic effect of cisplatin. Multiple smaller doses were more effective in curing malaria in mice than a single large dose. In a few of the mice treated with a single intraperitoneal large dose of 6 mg/kg body weight, there was a delay in appearance of parasitemia but most of them recovered completely but slowly. This compound exerts its toxicity mainly by randomly damaging and cross-linking DNA strands as shown by Southern hybridization with a synthetic oligonucleotide probe, which is a repeat sequence in the falciparum genome. The report clearly demonstrates the antimalarial potentials of this compound and suggests a closer evaluation of this and other related compounds, specially in combination with antimalarial drugs to probe their synergistic properties.

INTRODUCTION

Species of *Plasmodium* are the causative agents of malaria in man and some other vertebrates. *P. berghei* is responsible for murine malaria in mice and *P. falciparum* causes "malignant tertian" human malaria. Appearance and speedy spread of resistance in *P. falciparum* to common synthetic antimalarial drugs like chloroquine (1,2), pyrimethamine (3,4), and proguanil (4-6) used for treatment are showing an increasing rate of curative failures (7,8). The current grim situation is thus a cause of concern of malaria epidemiologists, indicating urgent need to identify and develop alternate compounds capable of curing resistant malaria. Malaria pathogenicity can be diminished by administration of drugs which inhibit active multiplication of asexual erythrocytic parasites and cure can be achieved by effectively eliminating the blood forms from the host. The parasite clearance may be achieved either by lethal action of the compound used for treatment and/or by its ability to evoke a stronger immune response against the parasite. Cisplatin has twin properties, being employed for neoplastic treatment (9,10) and immunomodulation (11,12). To our knowledge, no work has been reported on the effect of this compound on malaria parasites. These considerations prompted us to assess in vitro and in vivo antiplasmodium properties of cisplatin. Further to understand the mode of action of this compound, studies on stage-specific in vitro susceptibilities of erythrocytic parasites and on interaction of cisplatin with intact and restriction enzyme-digested *P. falciparum* DNA have been carried out. The later experiments were performed with a view to look for changes, if any, in the number and size of restriction fragments generated, following incubation of *P. falciparum* DNA with cisplatin, on a Southern blot with a *P. falciparum*-specific oligonucleotide probe.

MATERIALS AND METHODS

Parasites: The rodent malaria parasite, *P. berghei*, was obtained from National Institute of Communicable Diseases, Delhi, India and maintained in Swiss albino mice of 30-40 g by weekly mechanical passage of infected erythrocytes drawn from a donor mouse by cardiac prick into a sterile, heparinized syringe. The biology of these parasites has been reviewed by Killick-Kendrick and Peters (13). A chloroquine-resistant *P. falciparum* isolate, FCD-4, was collected locally

from a malaria patient with his consent (14) and established for in vitro continuous cultivation of erythrocytic stages by the method of Trager and Jensen (15).

Stock solution of cisplatin: A stock solution of cis-platinum (II) diammine dichloride, obtained from Sigma Chemicals, St. Louis, MO., USA, was prepared by dissolving 1 mg of the compound in 60% dimethylsulfoxide (DMSO). Further dilutions were made with buffered RPMI-1640 culture medium.

Erythrocytic schizontocidal activity

In vitro susceptibility of *P. falciparum*: The erythrocytic schizontocidal activity of cisplatin on *P. falciparum* was determined by exposing parasites in triplicates to graded concentrations of cisplatin by the modified 48-hr test method (16). The experiments were conducted in 24-well tissue-culture plates by setting up microcultures with eight different concentrations, ranging from 1 to 1,000 ng/ml. The parasite material for experiments was obtained from stock cultures and subjected to sorbitol lysis (17), to get synchronized ring stages. The parasitemia was adjusted to less than 1% with uninfected fresh erythrocytes and this material was made to 50% cell suspension with complete RPMI medium. Aliquots of 20 μ l of the suspension were added into series of wells of the test plate, each holding 480 μ l of complete medium with or without the drug, yielding a final cell suspension of 2%. The loaded test plates were incubated at 37 C in a candle-jar for 96 hr with daily changes of medium. The drug was included in experimental wells for the first two days only. Blood smears were made at the end of 48 and 96 hr, stained with Giemsa. A minimum of 5,000 erythrocytes were enumerated to determine parasitemia. The percent reduction of parasitemia in relation to control was calculated from the percent inhibition of growth. Fifty percent and 90% inhibitory concentrations (IC₅₀ and IC₉₀, respectively) were extrapolated from semilog plot of various concentrations against percent inhibition.

In vitro stage-specific sensitivity: To investigate the preferential in vitro toxicity to any specific stage of *P. falciparum*, asynchronous culture material was subjected to triple sorbitol treatment at 0, 40 and 47-hr intervals, to obtain synchronized parasites of 0-7 hr old (17). These synchronized parasites were in early ring stage for another 12 hr, for next 12-24 hr of development in the late ring or early trophozoite stage, 24-36 hr old in late trophozoite stage and during subsequent 36-48 hr of growth in the schizont stage of the erythrocytic schizogonic cycle. The synchronized stages were subjected to drug pressure for 12 hr each, the respective period of their residence in the specific stage. The experiments were conducted in triplicates in multi-well plates and slides made at the end of schizogonic cycle. The toxicities of cisplatin to ring-, trophozoite- and schizont-infected stages were determined by calculating the percent inhibition of parasitemia in drug-treated parasites to that in drug-free controls.

In vivo susceptibility of *P. berghei*: The curative property of cisplatin was assessed by comparing the survival times of mice untreated or treated with cis-

platin receiving the same amount of parasite inoculum in the form of infected erythrocytes obtained from a donor mouse. The test mice were taken in groups of five. Preliminary experiments were conducted in batches each consisting of three mice to arrive at the optimum tolerable dose of cisplatin effective in curing the mice. These preliminary doses ranged from 2-12 mg/kg body weight. Each member of the test group was injected intraperitoneally with 10^8 parasitized erythrocytes. Cisplatin was given intraperitoneally but 8 hr later either as a single dose or in four equally divided doses each in 1-ml saline at 4-hr intervals. Appropriate sets of control mice were maintained. The development of parasitemia was monitored by making Giemsa-stained blood films from the cut tail vein of the experimental and control animals every 24 hr for a period of 60 days.

Platination of DNA: The binding of cisplatin to *P. falciparum* DNA extracted from erythrocytic stages (18) was studied by incubating 4 μ g of DNA at 37 C for 24 hr with 50 or 100 ng of cisplatin in a total volume of 10 μ l. The incubated material was then digested with restriction enzyme *Hind* III at 37 C for 2 hr (19). In another set of experiment, the *Hind* III-digested DNA material was incubated with cisplatin. These digested samples were size-fractionated on a 0.7% agarose gel along with control samples of restriction enzyme-uncleaved or -cleaved DNA not incubated with cisplatin. The gel was stained with ethidium bromide and visualized under UV before the material was transferred onto a nylon membrane by Southern blotting. The membrane was hybridized with γ - 32 P end-labeled 21-mer synthetic oligonucleotide, which is known to be repetitive sequence in *P. falciparum* genome (20), at 42 C under stringent washing conditions. The sequence of the oligomer used to probe the membrane was as follows:

5'-AGGTCTTAAGTTGACTAACAT-3'

RESULTS

In vitro inhibitory effects of cisplatin on multiplication of the synchronized cultures of *P. falciparum* are presented in Fig. 1. Complete inhibition of the multiplication was achieved with a minimum amount of 30 ng/ml of cisplatin present in culture medium for a duration of 48 hr. From the dose response curve, IC_{50} and IC_{90} values determined after 48 hr were found to be 6.2 and 24 ng/ml, respectively and 3.8 and 26 ng/ml, respectively after 96 hr. Abnormal parasites were observed from the drug-treated wells at the end of 48 hr, showing different degrees of vacuolization or pale staining, whereas normal healthy parasites multiplied to about 6-fold in control wells in a schizogonic cycle. Most of the abnormal parasites in treated wells degenerated or lysed within 96 hr.

Fig. 1. In vitro growth inhibition response of asexual erythrocytic stages of *P. falciparum* to different concentrations of cisplatin in a 48-hr test. Slides were made at the end of 48 and 96 hr. Starting synchronized parasitemia was less than 1%.

Fig. 2. In vitro growth inhibition of different asexual erythrocytic stages of *P. falciparum* to various concentrations of cisplatin in a 48-hr test. Slides were made at the end of 48 hr. Starting parasitemia in each case was less than 1%.

Table I. Effects of cisplatin on *P. berghei*-infected mice

Administration of cisplatin	Mice showing parasitemia and deaths / five infected mice	
	Parasitemia within 2 weeks	Death within 60 days
Untreated	5	5
6 mg/kg in a single dose	3	1
6 mg/kg in four doses at 4-hr interval	0	0

Each mouse received intraperitoneally 1×10^8 parasites. Cisplatin was injected intraperitoneally 8 hr later.

The stage-specific toxicity of cisplatin is given in Fig. 2. Schizont stages were the least susceptible to the toxic effect of the compound followed by young rings whereas trophozoites and old rings were the most susceptible. The schizont stages exposed to 30 ng/ml of cisplatin in the medium resulted in only 36% growth inhibition.

Ability of cisplatin to clear *P. berghei* infection from mice is presented in Table I. A total dose of 6 mg/kg body weight given in four equally divided ip injections at 4-hr intervals effectively cured the infected mice and no parasitemia could be observed for 60 days in any of the treated mice. The same dose when given as a single ip injection finally cured 80% of the infected mice. Initially, however, in three out of five of these test animals, parasite appearance was delayed by 11 days compared to controls and parasite population increased subsequently leading to death of one of the mice, whereas in the other two mice, appearance of high parasitemia was followed by slow decline with final disappearance of the parasites after 14 days. Four weeks onwards from the start of the experiment, no parasite could be observed in these two surviving mice till day 60. In all the control animals, not receiving treatment with cisplatin, healthy parasites appeared

Fig. 3. An autoradiogram showing hybridization bands of *P. falciparum* DNA with species-specific synthetic γ - ^{32}P -labeled 21-mer-oligonucleotide probe. The DNA samples (4 μg in each lane except 3, which was blank) were size fractionated on 0.7% agarose gel and Southern transferred onto nylon membrane for hybridization.

Lanes 1: Undigested *P. falciparum* DNA, 2: *P. falciparum* DNA digested with *Hind* III, 3: Kept blank, 4: *P. falciparum* DNA incubated with DMSO prior to digestion with *Hind* III, 5: *P. falciparum* DNA digested with *Hind* III and incubated with DMSO, 6: *P. falciparum* DNA incubated with 50 ng of cisplatin, 7: *P. falciparum* DNA digested with *Hind* III and incubated with 50 ng of cisplatin, 8: *P. falciparum* DNA incubated with 100 ng of cisplatin and later digested with *Hind* III, and 9: *P. falciparum* DNA digested with *Hind* III and incubated with 100 ng of cisplatin.

in circulation within 24 hr of receiving the infective inoculum and they died in less than 10 days due to high parasitemia.

Autoradiograph of the restriction enzyme-digested falciparum DNA incubated with or without cisplatin and probed with a repetitive synthetic oligonucleotide is shown in Fig. 3. No hybridization band was observed in the lanes containing DNA or digested DNA incubated with cisplatin, whereas control lanes showed clearly the presence of one or several hybridization bands.

DISCUSSION

One of the most inexpensive ways of searching new lead antimalarial compounds for drug development could be to screen such drugs that have been developed for other diseases and evaluate their antimalarial potentials, either alone or in combination with other known antimalarial drugs. Cisplatin is a drug with a high potency in treatment of a few human malignancies (9,21,22) and is broad-spectrum anticancer drug in animals (9,23). *P. falciparum* also possesses genes with affinities to multiple drug-resistance (mdr) genes of human cancer cells (24,25) and this particular factor provided a strong clue indicating that some anticancer drugs may possess antiplasmodium properties. The results presented in this report amply demonstrate the antiplasmodium potential of cisplatin. Apparently non-pathogenic dose of this compound cured the malaria-infected mice and inhibited in vitro multiplication of a chloroquine-resistant falciparum isolate at a low concentration included in the medium. The amount of cisplatin required to achieve in vitro 50% inhibition of parasitemia in isolate FCD-4 was found to be less than either chloroquine ($IC_{50}=17.5$ ng/ml, unpublished data) or quinine ($IC_{50}=11$ ng/ml) (14). Not much difference in IC_{50} or IC_{90} value calculated from the slides made at the end of 48- or 96-hr experiments suggests that there was an insignificantly delayed mortality of the parasites due to cisplatin toxicity. Most of its lethal effect is exhibited within an erythrocytic schizogonic cycle.

Each of the synchronized schizogonic stages was subjected to in vitro uniform drug pressure for 12 hr with the differential outcome with regard to parasite survival and multiplication. Intraerythrocytic merozoites in the schizont and the early rings are the least susceptible to toxic effects of cisplatin, perhaps in these stages, no active DNA synthesis takes place (26) and the parasite plasma membrane is the most intact, thus retarding free entry of the compound into the parasite milieu. Cisplatin is known to prevent the multiplication of cancer cells by random inter- and intralinking of DNA strands of these dividing cells (27). The most damaging outcome, therefore, is seen in the early and late trophozoite stages of malaria parasite, known to be actively involved in DNA synthesis (26).

Toxicity to the host is an important consideration of any candidate compound with a potential of being employed as a drug. The amount of cisplatin used in this study to cure malaria infection in mice is well tolerated by the host. The dose of 6 mg/kg body weight given either as a single ip injection or in four equally divided doses at 4-hr intervals with saline did not cause any mortality in control mice. Other cancer workers have also reported that a therapeutic dose of 7 mg/kg

body weight of cisplatin causes less than LD₁₀ in mice and, as a single ip injection, the administered LD₅₀ dose is 13 to 14 mg of cisplatin/kg body weight (9). Slow infusion of cisplatin gives better results in cancer treatments (9) and also kidney toxicity is ameliorated with saline hydration (28). The present report shows also that a 1.5 mg/kg body-weight dose of cisplatin four times at 4-hr intervals with saline is far more superior than a single dose of 6 mg/kg in treatment of mouse malaria. This is perhaps due to longer retention of a higher level of cisplatin in the body for an extended period of time, as the half life of cisplatin in mice has been reported to be only 20 hr and an erythrocytic schizogonic cycle of mouse malaria is of 24-hr duration. In the two mice, which received a single 6 mg/kg body-weight dose of cisplatin, appearance of parasitemia was followed by slow but complete recovery; this could be due to the immunostimulation property of cisplatin which has been clearly demonstrated in several reports (11,29,30). Cisplatin has a trypanocidal activity in mice (31). The preliminary result in this study clearly demonstrates the antiplasmodium properties of cisplatin in both the in vitro and in vivo systems. Therefore, it would be worthwhile to investigate the antimalarial properties of this and other related platinum complexes with regard to their ability to cure polyresistant *Plasmodium* species. Probing the synergism properties in combination with other antimalarial drugs having different modes of actions should also be important, but all this needs further serious investigations.

Molecular basis of cisplatin toxicity has been attributed to lesions in DNA molecules of cancer cells leading to inter- and intrastrand DNA cross-links, finally resulting in cell death (27,32). We explored the interaction of cisplatin with *P. falciparum* DNA, by incubating it with intact DNA and digesting it later with restriction enzymes and by incubating predigested DNA with cisplatin. When these samples were run on agarose gel and stained with ethidium bromide, no fluorescence was observed in lanes containing samples incubated with cisplatin, showing that cisplatin prevents intercalation of ethidium boromide with DNA. Following blotting of these samples on to the nylon membrane, probed with radio-labeled 21-mer repeat sequence, no hybridization in these lanes are visible. This may be due to inter- and intra-strand cross linking of DNA by cisplatin, thus preventing the specific binding of the probe to complementary sequences on the DNA which is clearly seen in the form of distinct bands in control lanes of the autoradiograph. Thus, the mode of action of this compound on *Plasmodium* may be similar to the reported mechanism of action on neoplastic cells.

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