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Lethal Nephrotoxicity and Hematologic Toxicity of *cis*-Diamminedichloroplatinum Ameliorated by Optimal Circadian Timing and Hydration*

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Abstract—*Three hundred and forty-one female F344 (Fischer) rats were kept in light for* 8 hr *alternating with darkness for* 16 hr; *some were observed for survival for* 21 days, *while others were killed for blood sampling* 4.5 days *after a single intraperitoneal (i.p.) injection of* 11 mg/kg cis-*diamminedichloroplatinum* (cis-*DDP*). cis-*DDP was administered with or without concomitant i.p. saline load at one of six equispaced circadian stages. This high dose of* cis-*DDP resulted in marked lethal and renal toxicity, but in a moderate bone marrow suppression. Blood urea nitrogen (BUN), circulating total white blood cell counts (WBC) and survival times revealed statistically significant circadian rhythms of drug toxicity* ($P < 0.03$). *Optimal tolerance for* cis-*DDP gauged by these three variables resulted from drug administration in the second half of the dark span. Renal tolerance for* cis-*DDP gauged by BUN was improved two-fold by appropriate drug timing. This benefit from drug timing alone was further improved two-fold if hydration and* cis-*DDP were given at the optimal circadian stage. Hydration-induced amelioration of* cis-*DDP nephrotoxicity requires time qualification of both hydration and* cis-*DDP.*

INTRODUCTION

SUBSTANTIAL circadian rhythms characterize most, if not all, biologic processes investigated with sampling schemes of adequate length and density [1, 2]. Circadian timing of anticancer agents can determine the extent of their subsequent host and tumor toxicity [3–8].

We have demonstrated the crucial role played by the circadian timing of a single dose of *cis*-diamminedichloroplatinum (*cis*-DDP) upon the lethal toxicity and upon the nephrotoxicity of this drug [9, 10]. The present studies examine the relevance of survival data to the renal and hematologic toxicity of *cis*-DDP.

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MATERIALS AND METHODS

Study design

Female F344 (Fischer) rats were housed singly and kept for 3 weeks on a regimen consisting of 8 hr of light (L) alternating with 16 hr of darkness (D) prior to and throughout this study. Although animals were isolated from one another, a practice which has been shown to affect many physiologic endpoints, each animal was handled identically. Therefore, differences in toxic endpoints primarily due to time of treatment may still be discerned. Food was freely available. When 50 days of age and weighing 90–150 g, the rats were stratified according to their location in the rhythmometry room and randomized to one of six groups. Rats of each group received a single dose of 11 mg/kg of *cis*-DDP intraperitoneally (i.p.) at one of six different circadian stages, separated by 4 hr, on 4/1/79. Before being injected with

cis-DDP, each rat was weighed and its rectal temperature was measured by means of a thermistor probe. The temperature was recorded and analyzed to gauge the degree of circadian synchronization of these rats.

Each of the six groups was divided into two subgroups. One subgroup received *cis*-DDP alone; the other received *cis*-DDP with concurrent hydration (2 ml of saline per 100 g of body weight). Each subgroup was further subdivided into two halves. One was followed for survival; the other was killed by decapitation at a single circadian stage 4.5 days after the midpoint of drug administration. Mortality was recorded 4 times daily. Survival times were analyzed after study truncation, 12 days after *cis*-DDP administration. No further death was observed between Day 12 and Day 21 after *cis*-DDP injection. The blood urea nitrogen (BUN) and total white blood cell count (WBC) were determined in each blood specimen. The study design is shown in Table 1.

Drug preparation

cis-DDP was supplied by the Investigational Drug Branch of the National Cancer Institute (U.S.A.) in vials containing 10 mg of *cis*-DDP, mannitol and sodium chloride. The final solution used for injection contained 1 mg *cis*-DDP, 10 mg mannitol and 9 mg NaCl per ml. The drug was freshly prepared for each injection time point and used within 30 min of constitution.

Statistical methods

Classical methods including multiple-way analysis of variance with unequal cell frequency and *t*-tests were used. In addition, the single cosinor method [11–13] was employed in order to determine the characteristics of any observed circadian rhythm. If a linear trend characterized the data, it was removed before the cosinor method was applied. This rhythmometric method validates the presence of a rhythm in the data series and yields quantified rhythm characteristics. A *P*-value from an *F*-test of the zero amplitude hypothesis is obtained. The fit of a cosine function to the data yields estimates of three rhythm characteristics with their respective 95% confidence limits. These are the mesor, a rhythm-adjusted mean; the double amplitude, the total extent of *predictable* variation accounted for by the cosine curve best fitting the data; and the acrophase, timing of the maximum of this best-fitting cosine curve. A fourth index, the percentage rhythm, is a measure of the overall variability attributable to the mathematical model (in this case a 24-hr cosine function) used to approximate the rhythm. It is equal to 100 × the sum of squared deviations from the mean (ss) of values derived from the fitted cosine curve at sampling times divided by the ss of the data themselves.

RESULTS

Survival times

Table 2 shows the characteristics of the circadian rhythm in *cis*-DDP tolerance gauged by survival time with or without concurrent hydration. Hydration resulted in a slight increase in mesor but no change in double amplitude. More pertinent from the viewpoint of chronotherapy is that the timing of *cis*-DDP which resulted in optimal survival (gauged by the acrophase) was not changed by concurrent hydration. Optimal tolerance for *cis*-DDP was associated with drug administration between 17 and 18 hr after L onset. This optimal circadian timing of *cis*-DDP after the middle of the habitual activity span (D), irrespective of the hydration protocol, is depicted in Fig. 1.

Circulating leukocyte count on Day 4.5 after cis-DDP injection

A 2-way analysis of variance failed to detect

Table 1. Study design

		No. of rats/time point						
	Concurrent hydration (day of truncation)*	Time of *cis*-DDP† (hr after light onset) 01	05	09	13	17	21	Subtotals
No	(12)	17	17	16	18	16	18	102
	(4.5)	13	10	12	10	12	10	67
Yes	(12)	18	16	18	18	18	18	106
	(4.5)	10	12	10	12	10	12	66
Total								341

cis-Diamminedichloroplatinum.

†Endpoints: I. survival until Day 12, II. variables circulating in blood on Day 4.5.

Table 2. Circadian rhythm characteristics of survival time (hr) *of rats receiving* cis-*DDP**

Hydration	No. of rats	Mesor ± S.E. (hr)	Double amplitude (hr) (95% confidence limit)	Acrophase (hr^{min})
−	102	173 ± 6†	108(62, 144)	$17^{50}(16^{00}, 19^{20})$
+	106	189 ± 6†	80(34, 126)	$17^{20}(15^{10}, 19^{40})$
Pool (±)	208	181 ± 4	94(82, 106)	$17^{40}(16^{40}, 18^{40})$

*Results from cosinor analysis. *P*-value from null-amplitude test < 0.001 in each case; mesor = 24-hr rhythm-adjusted mean (midline estimating statistic of rhythm); double amplitude = extent of variation between peak and trough in fitted cosine curve; acrophase = timing of peak in fitted cosine curve (hr after light onset).

†Mesor comparison test: $F = 2.9$, d.f. (1, 206), $0.05 < P < 0.10$.

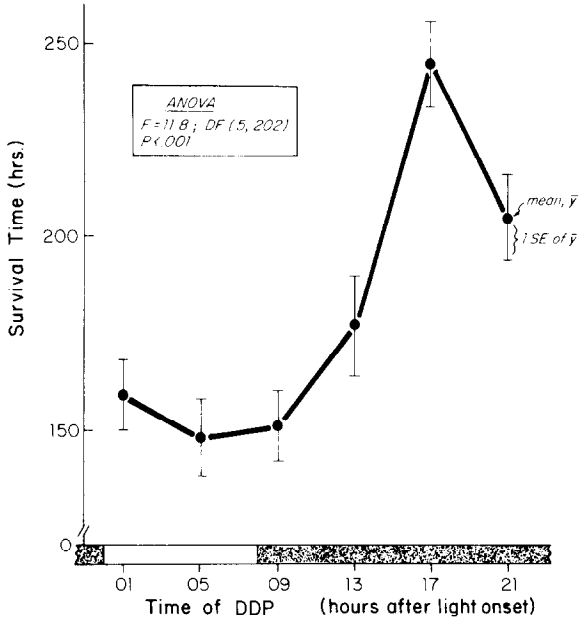

Fig. 1. Survival times of rats receiving 11 mg/kg cis-*diamminedichloroplatinum* (cis-*DDP*) *intraperitoneally at one of six different circadian stages, irrespective of any hydration regimen. Survivors on Day* 12 *after* cis-*DDP were assigned* 288 hr *as survival time. Results from analysis of variance (ANOVA).*

any effect of hydration ($F = 0.1$, $P \sim 0.75$) but revealed an interaction between circadian stage of administration and the post-injection interval (which varied by up to 20 hr since the rats given *cis*-DDP at different circadian times were all killed at the same time) ($F = 3.0$, $P \sim 0.01$). In order to further determine the relative roles of post-injection interval versus circadian stage of administration, a linear trend was fitted to the data ($P = 0.08$) and subsequently removed from them. Transformed data on leukocyte counts were then analyzed by cosinor. Results indicated a statistically significant circadian rhythm in *cis*-DDP-induced reduction of circulating leukocyte counts (Table 3).

The total predictable extent of variation of this rhythm approximates 1300 cells/mm^3. The highest total leukocyte counts in *cis*-DDP-injected rats occur when this drug is administered about 17 hr after L onset. The mean ± standard error of the circulating leukocyte count (cells/mm^3) was 6050 ± 113 in control rats, 6100 ± 290 in those rats injected with *cis*-DDP at 17 hr after light onset (HALO) and

Table 3. Circadian rhythm in cis-*DDP hematologic toxicity gauged by circulating total leukocyte count on Day* 4.5 *after* 11 mg/kg cis-*DDP. Results from cosinor analysis** *after removal of a linear trend from the data*

P	No. of samples	Double amplitude (cells/mm^3) 95% confidence limits	Acrophase (hr^{min})
0.03	119	1300(800, 1800)	$17^{10}(14^{25}, 20^{00})$

**P*-value from null-amplitude test; double amplitude = total extent of variation between maximum and minimum in fitted cosine curve; acrophase = timing of maximum in fitted cosine curve (in hr after light onset).

5070 ± 330 in those rats injected with this drug near 05 HALO.

Blood urea nitrogen

On Day 4.5 ± 0.5 after *cis*-DDP administration, irrespective of the circadian timing of this drug or of the hydration protocol, the BUN rose to 306 ± 12 mg/dl as compared to 19 ± 1 mg/dl in control rats. An effect of both *cis*-DDP timing and hydration was demonstrated by a 2-way analysis of variance ($F = 3.6$, $P < 0.005$ and $F = 6.3$, $P = 0.015$ respectively). The heavy line in Fig. 2 depicts the role of circadian timing of *cis*-DDP upon the degree of resultant azotemia.

The characteristics of the circadian rhythm in BUN as a function of treatment time are listed in Table 4. When *cis*-DDP is administered with concurrent hydration, the BUN mesor is significantly lower than the mesor of rats receiving *cis*-DDP without hydration. The timing of the rhythm in *cis*-DDP-induced BUN rise is quite similar whether or not concurrent saline hydration is employed. The extent of predictable variation in this rhythm, which gauges renal susceptibility to *cis*-DDP, appears to be increased in the hydrated group. The estimated minimum BUN resulting from 'optimal' timing of *cis*-DDP reaches 272 mg/dl if this drug is given alone and only 200 mg/dl if hydration is administered together with *cis*-DDP. The estimated maximal BUN resulting from the 'worst' timing of *cis*-DDP without concurrent hydration reaches 388 mg/dl, while an estimated BUN of 356 mg/dl results from the concurrent hydration with *cis*-DDP administration. A statistically significant inverse correlation was apparent between the mean BUN and mean survival times resulting from *cis*-DDP administration at the same circadian stage, with or without hydration (Fig. 3).

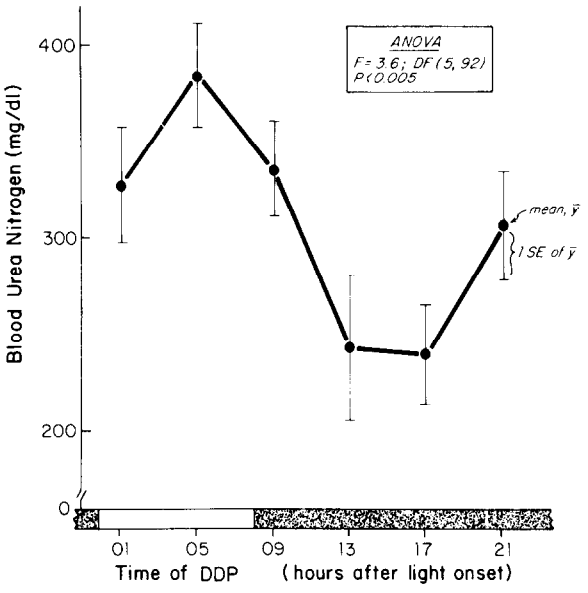

Fig. 2. Blood urea nitrogen (BUN) from peripheral blood of rats receiving 11 mg/kg cis-*diamminedichloroplatinum* (cis-*DDP) intraperitoneally at one of six different circadian stages, irrespective of any hydration regimen. BUN obtained* 4.5 ± 0.5 days *after* cis-*DDP injection. Mean ± standard error of BUN in control rats*: 19 ± 1 mg/dl. *Results from analysis of variance (ANOVA).*

DISCUSSION

Lethal, hematologic and renal toxicity of a single dose of *cis*-DDP depends largely upon drug circadian timing. Even though the effect of a lethal dose of *cis*-DDP upon the count of circulating leukocytes may be moderate [14], this effect can be further minimized by drug timing. Such an optimization of *cis*-DDP bone marrow toxicity may become an important consideration when this drug is used in combination with other anticancer agents which exert their primary toxicity on the bone marrow. The least lethal, hematologic and renal toxicity resulted from *cis*-DDP administration at the same circadian stage (17 HALO). A statistically significant inverse correlation between

Table 4. Circadian rhythms in cis-*DDP nephrotoxicity gauged by blood urea nitrogen on Day* 4.5 *after* 11 mg/kg cis-*DDP. Role of concurrent i.p. hydration. Results from cosinor analysis**

Hydration	*P*	No. of samples	Mesor ± S.E.	Double amplitude (95% confidence limits)	Acrophase (hr^{min})
−	0.05	47	330 ± 18†	116(70, 162)	$05^{20}(02^{50}, 08^{40})$
+	< 0.01	51	278 ± 17†	156(108, 204)	$01^{50}(23^{30}, 04^{10})$
±	< 0.001	98	301 ± 11	140(108, 172)	$03^{50}(02^{00}, 05^{40})$

**P*-value from null-amplitude test; mesor = 24-hr rhythm-adjusted mean; double amplitude = total extent of variation between maximum and minimum in fitted cosine curve; acrophase = timing of maximum in fitted cosine curve (in hr after light onset).
†Mesor test: $F = 4.5$, d.f. (1, 93), $P < 0.05$.

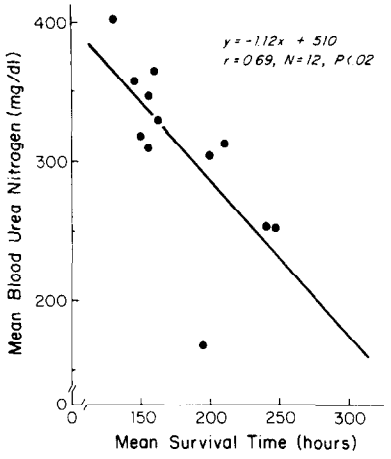

Fig. 3. Correlation between mean survival time and mean blood urea nitrogen resulting from the administration of cis-*diamminedichloroplatinum* (cis-*DDP*) *with or without hydration at the same of six circadian stages. Rats receiving the same treatment were either observed for survival for* 12 days *or killed* 4.5 ± 0.5 days *after* cis-*DDP injection.*

mean survival time and mean BUN following *cis*-DDP administration at the six times tested suggests that nephrotoxicity may contribute substantially to *cis*-DDP lethality. The discrepancy found between the effects of hydration upon survival times and BUN may be the result of a hydration regimen which was not adequate to prevent or delay death but was sufficient to demonstrate biochemical renal benefit. Other toxicities contribute to *cis*-DDP lethality. Death from a high dose of *cis*-DDP also involves gastrointestinal damage (manifested by diarrhea and anorexia), as well as bone marrow suppression and kidney failure [14–16]. While some alleviation of renal toxicity was apparently achieved by the 2% body weight hydration protocol, these other toxicities may not have been affected.

We have shown previously that nephrotoxicity of a nonlethal dose of *cis*-DDP as assessed by body weight loss, polyuria, BUN and the urinary activity of β-*N*-acetylglucosaminidase (NAG) [17], which is released into the urine after renal tubular damage, also depends markedly upon the circadian stage of drug administration. We have also shown in three previous studies that survival time is increased to a much greater extent when 3% body weight saline hydration is given concomitantly with *cis*-DDP at the circadian stage associated with optimal tolerance [10]. The present study extends this finding to BUN.

Marked circadian rhythms in renal tubular cell metabolism and division [18–24] may help to explain our results. Since *cis*-DDP is known to bind avidly to sulfhydryl groups in which lysosomal enzymes of the tubular brush border are quite rich, NAG as well as other lysosomal enzymes of the proximal tubular cells may represent the primary site of *cis*-DDP binding or be involved in the cellular entry of *cis*-DDP. We have already noted a correlation between tissue NAG and *cis*-DDP uptake. Therefore, the circadian rhythm in the activity of several lysosomal enzymes in renal proximal tubular cells [25–27] may conceivably contribute to the large amplitude of the observed circadian rhythm in *cis*-DDP nephrotoxicity.

Other kidney rescue techniques may be time-dependent as well. Although hydration does not harm renal function, even when poorly timed, this may not be the case when diuretics which act upon the renal tubular cells are used. This point is clearly illustrated by conflicting reports describing the effect of furosemide upon *cis*-DDP-induced nephrotoxicity. Some claim reduction [28, 29], while others show potentiation of *cis*-DDP nephrotoxicity [16, 30, 31]. This apparent contradiction may be understood by the recognition of the established circadian-stage dependence of the renal response to diuretics [21, 32–34], which in turn is likely to affect renal tolerance for *cis*-DDP differentially.

A chronobiologic approach to *cis*-DDP toxicity in the rat demonstrates that the optimal circadian stages of renal and hematologic tolerance for this drug are similar; *cis*-DDP is approximately half as nephrotoxic on the basis of BUN measurements when injected at this time compared to administration at a circadian stage 12 hr away from this optimal stage.

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