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Circadian Variation of 5-Fluorouracil and *Cis*-Platinum Toxicity in Mice

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ABSTRACT

Circadian variations of organ specific and lethal toxicities were investigated in female C_3H mice following intraperitoneal injection(s) of 5-fluorouracil (5-FU) and/or *cis*-platinum (CDDP) with different doses at one of the four equidistant time points, viz., 3, 9, 15 and 21 HALO (Hours After Light On). Lethality, peripheral WBC count, spleen size, femoral bone marrow cell population and changes in body weight were analyzed. A single injection of 5-FU (300 mg/kg) showed 45% mortality with 3 HALO and 15% with 9 HALO treatment ($p<0.05$); that with CDDP (16 mg/kg) was 80% in 3 HALO and 10% in 9 HALO group ($p<0.01$). Peripheral WBC count on the 4th post-treatment day showed that drug induced leukopenia was less severe with 15 HALO dosing compared to the other HALO points ($p<0.01$). The reduction in spleen size following treatment was maximum in 3 HALO treated group with both drugs ($p<0.01$). The combination therapy of these two drugs also showed circadian variation of toxicity. The results suggest that the toxic effects of chemotherapy are dosing time dependent.

Key words: *Circadian rhythm, Chemotherapy, Anticancer agents, Toxicity, Dosing time*

Time dependent drug delivery systems have been a modern trend in cancer chemotherapy. Since most of biological functions exhibit circadian rhythmic patterns, the response to a given stimulus is also expected to be time dependent[20], which has been documented for a variety of stimuli including anticancer agents[10,23,24].

Since cancer chemotherapy has its limitations due to the toxic effects it produces, several attempts have been made to schedule a suitable time for drug administration so as to achieve an optimum drug tolerance and to obtain the maximum therapeutic effect. Daily variations in drug pharmacokinetic and pharmacodynamic parameters[30], as well as the rhythm of tissue susceptibility to drug toxicity, are of particular importance in this respect. Both animal experiments and human studies have shown that host tissues are affected by anticancer agents according to a time dependent pattern[15]. For example, 5-FU is better tolerated in the mid-rest phase in mice[2], while the greatest toxicity of adriamycin is late in the activity cycle of the rodents[27].

In the present study, the chronotoxic effects of two commonly used anticancer agents, 5-fluorouracil (5-FU) and *cis*-platinum (CDDP), were investigated. The purpose of this study is to establish a safer dosing time for the administration of these agents considering the pattern of variation in toxicity according to dosing time.

MATERIALS AND METHODS

Mice

Studies were carried out in the animal facility of this institute with female C_3H mice of mean age 16 weeks, purchased from Nihon Kurea (Osaka, Japan). The mice were randomized and housed five per cage in a compartment with a automatized lighting regimen. A light-dark (L-D) cycle of 12 hours of light alternating with 12 hours of darkness was maintained with the light on at 6:00 a.m. and off at 6:00 p.m.. The mice, prior to use in the experiments, were synchronized to this L-D cycle for a period of at least four weeks, while pellet food and tap water were supplied freely.

Anticancer agents

5-FU and CDDP were obtained from Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan) and Bristol-Myers Squibb K.K. (Tokyo, Japan), respectively. The drugs were diluted in RPMI 1640 medium just before use, at the required concentration.

Experimental protocol

The mice were grouped for different drugs and different time points. Four time points, viz., 3, 9, 15 and 21 HALO were arbitrarily chosen for treatment and evaluation of toxicities. Where HALO stands for Hours After Light On, 3 HALO corresponds with 9:00 a.m., 9 HALO with 3:00

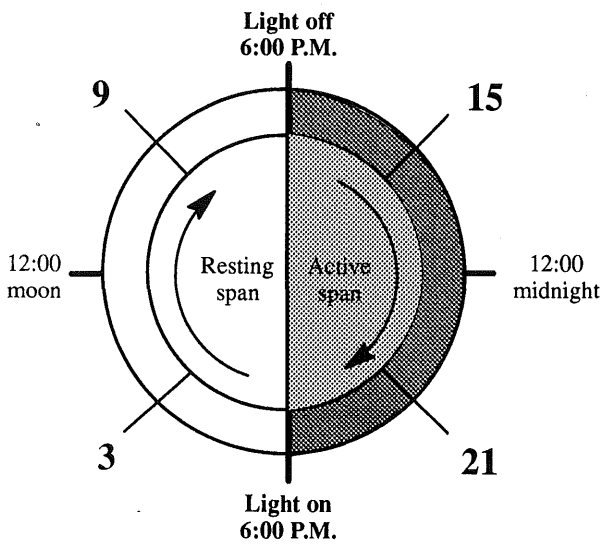

Fig. 1. Clock hours and HALO (Hours After Light On) Time. 3, 9, 15 and 21 HALO correspond with 9:00, 15:00, 21:00 and 03:00 hours local time, respectively.

p.m., 15 HALO with 9:00 p.m. and 21 HALO with 3:00 a.m. local time (Fig. 1). Drugs were injected via the intraperitoneal route at the doses shown in Table 1. The parameters observed for evaluation of toxicities are shown in the same table. The mice were treated and sampled at the corresponding HALO times (Fig. 2). For the individual drug group, the indicated dose (Table 1) was injected as a single dose at each of the four HALO points and parameters were checked on day 4. For combined sequential treatment, the indicated doses were injected for five consecutive days at the respective HALO times and were administered for a second cycle of five days similarly after an interval of 10 days.

The body weights of all groups were measured daily between 9:00 to 10:00 a.m.. The WBC count was carried out on day 0 (prior to treatment) and day 4 (post-treatment) with blood collected in a WBC pipette by sectioning the tail. It was immediately diluted with Turk solution and counted under a microscope. The mice were sacrificed by cervical dislocation on the 4th post-treatment day and their spleens and femurs were dissected out. The spleens were weighed immediately. The femurs were sectioned at each end and bone marrow cells were flashed out of the cavities with 3.0 ml of RPMI 1640 medium using a 26G needle. The cell count was carried out under a microscope. For the survival rate, each group of mice was observed twice daily at 12 hour intervals to record the incidence of death. Three sets of experiments, each having a minimum of eight mice per HALO point group, were carried out for each parameter and presented as results. A statistical analysis of the data has been done by χ^2 and students t test.

Each study was provided with a corresponding control group in which the mice received RPMI 1640 medium only.

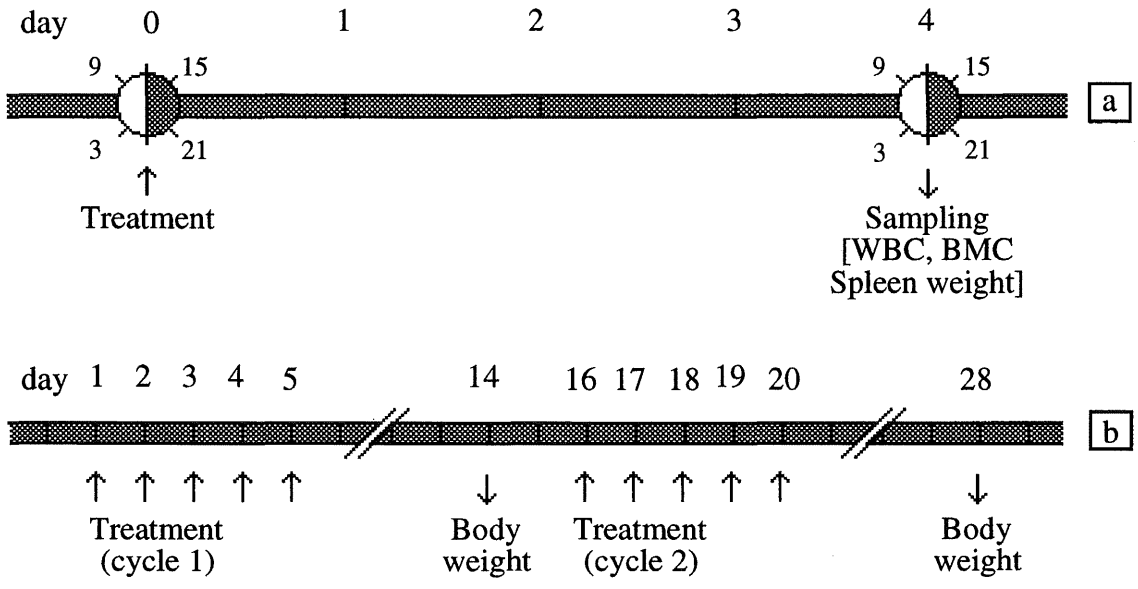

Fig. 2. Treatment and sampling schedule. Mice were treated and sampled at the corresponding HALO times. a, single dose treatment for individual drug group; b, combined sequential treatment for two cycles of five days each, at an interval of 10 days.

Table 1. Experimental Protocol

Study[1]	Mice[2]/Study	Drugs and Doses	Toxicity Parameters
1†	140	5-FU[3] 300 mg/kg	Survival Rate
2†	120	CDDP[4] 16 mg/kg	Survival Rate
3†	40	5-FU 300 mg/kg	WBC count, spleen size
4†	40	CDDP 16 mg/kg	WBC count, spleen size
5†	48	5-FU 50 mg/kg	BMC[5] population
6†	48	CDDP 5 mg/kg	BMC population
7*	80	5-FU 40 mg/kg and CDDP 2 mg/kg	Survival Rate, changes in body weight

[1]Each study was carried out for four HALO points; [2]Total number of mice in each study; [3]5-fluorouracil; [4]Cisplatinum; [5]Bone marrow cells; †Single dose treatment; *Combined sequential treatment.

RESULTS

Survival rate

A single injection of an LD_{50} dose of 5-FU (Fig. 3) or CDDP (Fig. 4) showed that both the overall survival and the mortality rate were dependent on the time of treatment ($p<0.05$, 5-FU; $p<0.01$, CDDP). With both drugs the overall survival rate was highest with 9 HALO treatment (mortality 15% with 5-FU and 10% with CDDP) and lowest with 3 HALO (mortality 45% with 5-FU and 80% with CDDP). With either drug death was initiated earlier in the 3 HALO treated group, while in the 9 HALO group the onset of mortality was later. In the case of 5-FU, maximum mortality was occurred during the 9th to 14th days in all groups except 9 HALO. In the case of CDDP, the onset of death was earlier than that with 5-FU and maximum mortality was recorded during the 3rd to 9th days in 3, 15 and 21 HALO groups. The death pattern and overall survival rate of the 15 and 21 HALO groups appeared to be similar with each drug.

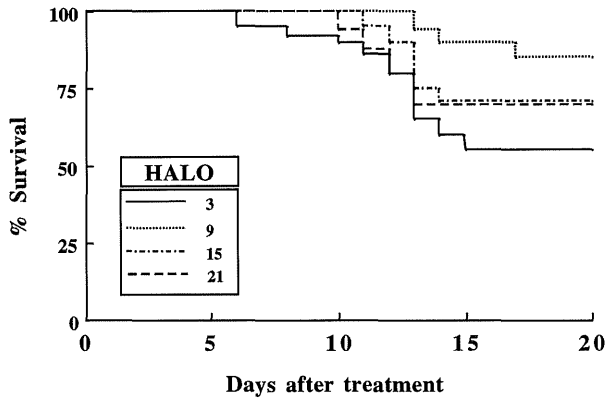

Fig. 3. Survival rate of different HALO groups following treatment with a single dose of 5-FU (300 mg/kg), i,p., at respective HALO times. 3 HALO point treatment showed the maximum mortality and 9 HALO point the minimum.

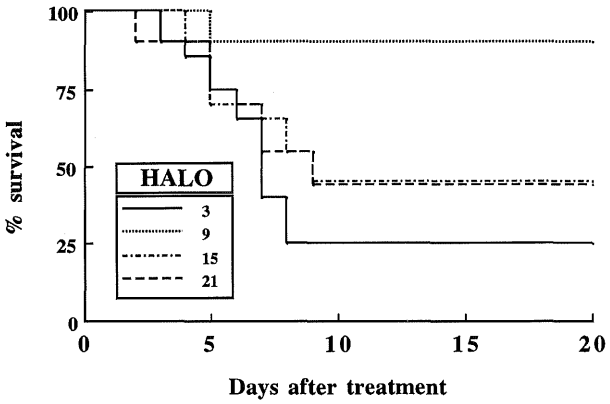

Fig. 4. Survival rate of different HALO groups following treatment with a single dose of CDDP (16 mg/kg), i.p., at respective HALO times. Maximum mortality was observed with 3 HALO treatment and minimum with 9 HALO.

The doses used in combined sequential treatment did not cause any mortality when the mice were treated according to the same schedule with each of those drugs alone (5-FU 40 mg/kg or CDDP 2 mg/kg) (data not shown). Following combined treatment mortality ensued and maximum death was recorded during the 10th to 15th days in all HALO groups (Fig. 5). After this period mortality continued only in the 3 HALO treated group, resulting in the highest mortality (60%). The minimum mortality was recorded in the 9 and 15 HALO groups (20%).

In all three drug groups the studies were terminated on the 30th post-treatment day after which no death occurred during an additional observation period of 30 days. No death was recorded in the control groups throughout this period.

Peripheral WBC count

On post-treatment day 4 the peripheral WBC count (Fig. 6) showed that the extent of drug induced leukopenia was also related to the dosing

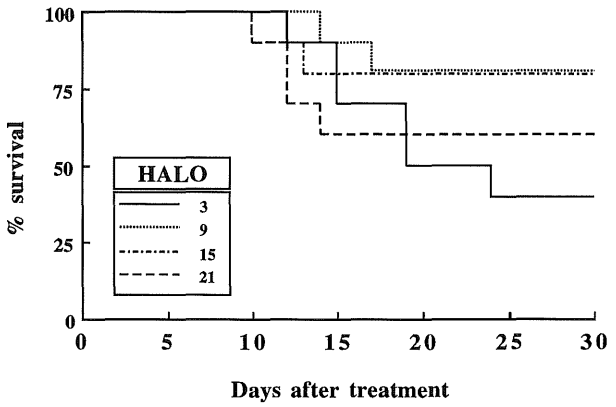

Fig. 5. Survival rate of different HALO groups following combined sequential treatment (see Table 1) with 5-FU (40 mg/kg) and CDDP (16 mg/kg), i.p., at respective HALO times. Maximum mortality was recorded with 3 HALO treatment. 9 and 15 HALO treatment caused minimum mortality.

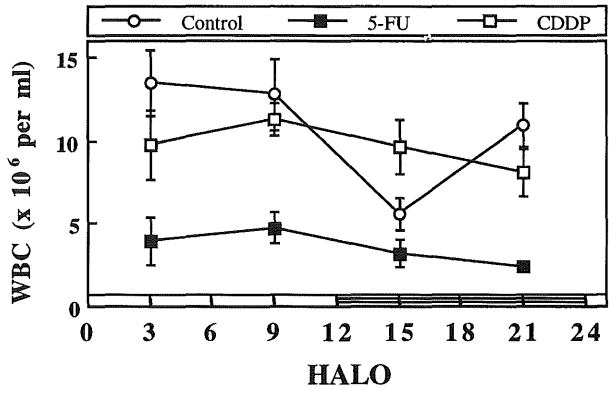

Fig. 6. Peripheral WBC counts in control mice of different HALO groups at respective HALO times and those of 5-FU (300 mg/kg) and CDDP (16 mg/kg) treated mice on 4th post-treatment day; (mean ± SD). Higher leukopenia, as calculated by T/C (%), occurred with both drugs in the 3 HALO treated group. The 15 HALO treated group suffered less leukopenic stress in the case of 5-FU and showed no leukopenia in the case of CDDP.

time. The mice were treated and blood from the tail vein was sampled at the corresponding HALO time. Compared to the normal circadian variation of WBC count of the control group, 15 HALO treatment appeared to produce a lesser extent of drug induced leukopenia with either drug ($p<0.01$). In the case of 5-FU, drug induced leukopenia was less severe by 30% in the 15 HALO group compared to the other HALO point treatments. Treatment with CDDP showed no leukopenia in the 15 HALO group as compared to its control. Maximum leukopenia was recorded with 3 and 21 HALO treatment in both drug groups.

Spleen weight

As shown in Table 2, compared to their controls, in both drug groups, reduction in spleen weight was found to be dependent on dosing time. This effect was found to be maximum with 3 HALO treatment (T/C 30% in 5-FU and 51% in CDDP) ($p<0.01$). The minimum reduction was recorded in the 9 HALO group in the case of CDDP (T/C 75%) ($p<0.01$), while in the case of 5-FU no statistically significant difference was found among the groups of 9, 15 and 21 HALO time treatments.

Bone marrow cell population

Following the LD_{50} dose, the number of bone marrow cells on day 4 was extremely low and did not show any difference among the treated points (data not shown) in either drug group. However, treatment with a lower dose (about one fourth of LD_{50} dose) showed a statistically significant difference ($p<0.01$) between the 21 HALO treated group and other three HALO points (Table 3). With both drugs, 21 HALO groups suffered a higher bone marrow suppression — 48% and 54% of the control in case of 5-FU and CDDP, respectively.

Table 2. Changes in spleen weight (4th post-treatment day) following circadian stage dependent treatment (mean ± SD)

Time	Spleen Weight (g)			T/C (%)	
(HALO)	Control[1]	5-FU[2]	CDDP[3]	5-FU	CDDP
3	0.1176 ± .0095	0.0432 ± .0024	0.0600 ± .0071	37*	51*
9	0.1180 ± .0141	0.0486 ± .0029	0.0877 ± .0071	41	75*
15	0.1109 ± .0571	0.0502 ± .0031	0.0677 ± .0101	45	61
21	0.1126 ± .0042	0.0496 ± .0039	0.0597 ± .0055	44	54

[1]Treated with RPMI 1640 ; [2]300 mg/kg, i.p., single dose; [3]16 mg/kg, i.p., single dose;
*Statistically significant difference from the other HALO points ($p<0.01$).

Table 3. Changes in femoral bone marrow cell population after 24 hours following circadian stage dependent treatment (mean ± SD)

Time	No. of cells per femur ($\times 10^6$)			T/C (%)	
(HALO)	Control[1]	5-FU[2]	CDDP[3]	5-FU	CDDP
3	3.88 ± 0.99	2.74 ± 0.07	2.29 ± 0.10	71	75
9	3.83 ± 0.88	2.85 ± 0.06	3.1 ± 0.07	74	81
15	4.04 ± 0.12	2.97 ± 0.06	3.3 ± 0.09	73	81
21	3.97 ± 0.66	1.92 ± 0.04	2.16 ± 0.05	48*	54*

[1]Treated with RPMI 1640; [2]50 mg/kg, i.p., single dose; [3]5 mg/kg, i.p., single dose;
*Statistically significant difference from the other HALO points ($p<0.01$).

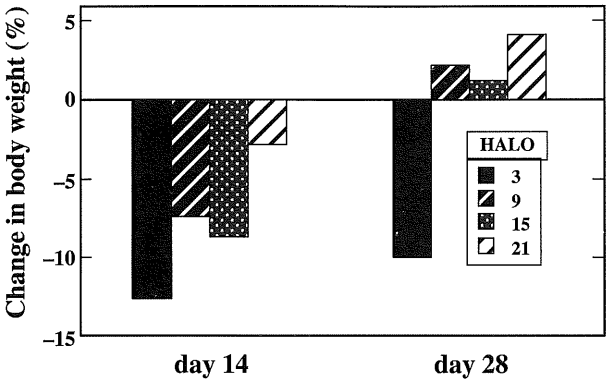

Fig. 7. Changes in the body weight of different HALO groups calculated as a percentage of initial weight following combined sequential treatment (see Fig. 2) with 5-FU (40 mg/kg) and CDDP (2 mg/kg), i.p., at respective HALO times. The 3 HALO treated group suffered maximum and persistent loss in body weight (day 14 and day 28) while the other HALO groups recovered and increased their initial weights (day 28).

Changes in body weight

Following the combined sequential treatment (see Table 1 and Fig. 2) with 5-FU and CDDP, body weights were measured daily for 30 days from the day of initial treatment. Changes in body weight (BW) were calculated by the following formula:

$$\text{Changes in BW (\%)} = \frac{BW_{n\,day} - BW_{0\,day}}{BW_{0\,day}} \times 100$$

The overall kinetic pattern of the body weight changes following treatment at different HALO times were similar in all groups (data not shown) except that the severity of loss of weight was more pronounced with 3 HALO treatment. Fig. 7 shows the changes in body weight on day 14 and day 28, i.e., two weeks after the initial treatment of each cycle. The 3 HALO group suffered the maximum body weight loss (13% of initial weight) among the four HALO points investigated. This group remained in a state of negative balance throughout the follow-up period. All other groups, as seen in the day 28 plot, regained and exceeded initial weight. The 21 HALO point was found to be the least toxic treatment time if loss of body weight and rapidity of recovery from that state is considered. The control group, receiving only RPMI 1640 medium, steadily gained weight during the experimental period.

DISCUSSION

A drug, from the time of its administration, passes through numerous complex pathways up to excretion. To accomplish its intended pharmacological effects, or to exert untoward side effects, the drug is dependent on the functions of different organ systems or on the availability of the necessary enzyme systems, both of which could be biologically rhythmic[6,7,13,16,18,33]. Each drug affects numerous target tissues, each of which may differ in its rhythm and susceptibility. In addition, each drug may affect a different site (cellular or molecular) in the same tissue and each site may exhibit a different rhythm of susceptibility.

In our experiment, we observed that the toxicity of 5-FU and CDDP is dependent on the time of administration of these drugs. With both drugs, we found a higher lethality, higher bone marrow suppression, a higher degree of leukopenia, greater reduction of spleen weight with the 3 HALO treatment. These findings give rise to speculation of a higher target organ susceptibility to these drugs during that time.

As for lethality following 5-FU treatment, Hrushesky et al[11] reported 100% lethality in mice following 2 HALO treatment. This is in accordance with our findings. This is further supported by Gonzales et al[5]. On the other hand, Burns et al[2] and Popovic et al[22] reported higher lethality following treatment with 5-FU at 17 and 20-22 HALO, respectively. However, both demonstrated that 5-FU is better tolerated during the mid to late rest span in mice which is consistent with our data.

For 5-FU, the variation of the level of 5-FU deg-

radation enzyme dihydro-pryrimidine dehydrogenase (DPD) which catabolizes more than 80% of the administered dose of 5-FU[10], has been reported to be circadian stage dependent. In rodents, Harris et al[7] showed the peak level of DPD is at around 10 HALO and that there is a trough at 22 HALO. In addition, an inverse relationship between the level of DPD and the plasma level of 5-FU in humans has been reported[6]. Very recently, the level of thymidine kinase (TK), the first enzyme in the thymidine phosphorylation pathway for fluoropyrimidine anabolism, has been reported to be circadian stage dependent and has been suggested responsible for the circadian variation of 5-FU toxicity[33]. The TK level in different tissues has remarkable diurnal variations and is inversely related to DPD level. These reports may support the speculation of low toxicity with 9 HALO treatment since only a small amount of active drug of the total administered dose is available at that time (high DPD and low TK level). The converse is true when the same dose is administered at 3 HALO.

Regarding CDDP, Levi et al[16] demonstrated a better tolerance of the drug during the mid to late activity (dark) span in mice. But this is not consistent with our study when lethality is considered. Hurshesky et al[12] showed highest lethality following CDDP treatment at 01 HALO, which, in our experiment, was found to be in 3 HALO (01 HALO not investigated). They found the lowest mortality, however, with 17 HALO treatment which was with 9 HALO in our experiment. The lowest toxicity following 9 HALO treatment in our study has been further supported by other parameters evaluated, like bone marrow cell population and spleen size.

The mechanisms for circadian changes in CDDP toxicity include alteration in drug pharmacokinetics with significant variations in plasma binding and urinary excretion[8,13,18]. The early onset of mortality in this drug group, beside the low hematopoietic toxicity compared to 5-FU, may be due to the more pronounced nephrotoxic effect of the drug. Though CDDP was expected to be tolerated better during the mid to late activity (dark) span[26] as the glomerular filtration rate is largely increased at this time[3,32], in our study, the discrepancy may be due either to differencs in dosage, route of administration and treatment schedule, or to different biological rhythms in the strains of mice used in the experiments.

We have reported the circadian variation of tissue DNA synthesis with a peak at 3 HALO and a higher suppression of the synthesis activity at the same HALO point following circadian stage dependent chemotherapy with 5-FU and CDDP[28]. This may also contribute to a reason for higher toxicity following treatment with these agents at the 3 HALO point.

In the combined treatment, 3 HALO dosing showed maximum toxicity both in mortality and in loss of body weight. Interestingly, the kinetic pattern of changes in body weight following the combined treatment was almost similar to that of 5-FU alone (data not shown) except that the former showed greater severity in toxicity. This finding suggests that CDDP, by some means, potentiates the action of 5-FU. Petit et al[21] have reported that CDDP helps to maintain a higher blood level of 5-FU when given in combination. As has been seen, both 5-FU and CDDP showed greater toxicity with 3 HALO dosing, but their net toxic effect is much higher following combination therapy than when either drug is used alone. However, minimum toxicity considering the loss of body weight was with 21 HALO treatment. But the mortality of this group was higher compared to the 9 and 15 HALO groups. Similar works on combined therapy with 5-FU and CDDP have not been reported so far. Based on our data, 9 to 15 HALO (late resting to early active phase) appeared to be a safer dosing time for combination therapy in mice.

As for hematological toxicity, 15 HALO treatment appeared to be less toxic considering the drugs' inducement of leukopenia. The rhythm characteristics of the changes of WBC count following treatment with anticancer agents were similar to those already reported by Boughattas et al[1]. The circadian pattern of bone marrow suppression in our study also supports the variation of drug induced leukopenia following time dependent treatment. The possible reason for time related variations of hematological toxicity is that circadian rhythm characterizes the cellular proliferation in the bone marrow. DNA synthesis is highest in the light (rest) span of mice[25] and bone marrow granulocytic precursors exhibit an increased proliferation ability in the dark (activity) span[17]. Circadian variation of DNA synthesis of bone marrow cells showed peak synthesis activity during the early resting span (3 HALO) and a trough during the early activity span (15 HALO); and following circadian stage treatment, a higher suppression of synthesis activity was found with 3 HALO treatment and a lower suppression with 15 HALO[28]. Therefore, the lowest hematological toxicity corresponds to the administration of drugs near the middle of the dark (activity) span. The demonstration of circadian rhythm in the proliferative capacity of murine totipotent and committed stem cells[19,29] further supports this hypothesis.

Apart from these mechanisms, a number of other organs may be more susceptible to drug toxicity at a particular time that may be common to a number of drugs. For example, bone marrow toxicity was found to be greater with 3 HALO

treatment with both drugs in our experiment. Also, intestinal toxicity such as jejunal lesion has been found to be dependent on the treatment[1]. The circadian pattern of DNA synthesis in different tissues has also been reported to a play role in the susceptibility to drugs[2]. Large inter- and intra-individual variations in the time of peak blood level (and hence the effect) of 5-FU in humans have been reported[21,31] which may be the case for other anticancer agents as well. A circannual variation of circadian rhythm is also reported[19]. These all may well contribute to the higher lethality related to dosing time.

Discrepancies in the circadian rhythmicity of any single parameter can be noted in the works of a number of investigators, but the existence of circadian rhythm is considered to be a fact[19]. Though the susceptibility of normal tissue is rhythmically variable during the circadian cycle, that of malignant tissue may be less so[14]. Studies have been carried out to investigate the dosing time related efficacy. Further studies need to be carried out to understand the mechanisms of time dependent toxicity, as well as, of factors like enzyme level[4,6,7] and tumor blood flow[9], which are responsible for maximum drug efficacy.

In the present study, it is noteworthy that a number of target organs may be more susceptible to chemotherapy at a particular time point, a fact which we are the first to report. A number of similar works have been reported but data on the combination of 5-FU and CDDP is still lacking. Evaluating the data of all the parameters, the late rest to early active span appeared to be the better tolerance time for the two anticancer agents in mice, either singly or in combination. Our preliminary study suggests that (1) more anticancer agents need to be tested for their time dependent toxicity and/or efficacy, (2) the mechanisms of drug induced toxicity in relation to dosing time need to be investigated and (3) more time points in a 24-hours span need to be evaluated for toxicities and efficacies to work out an optimum dosing time.

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