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Circadian Time Dependence of Murine Tolerance for the Alkylating Agent Peptichemio

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Abstract—*Since the extent of host toxicity of cytostatics is considerably affected by dosing time, a chronopharmacologic approach was undertaken for optimizing the therapeutic index of the alkylating agent, peptichemio (PTC). In 4 studies involving a total of 463 male B6D2F1 mice, a highly statistically significant circadian rhythm characterized murine tolerance for PTC (8 or 10 mg/kg/day i.v. × 3 days). Six circadian stages were explored (3, 7, 11, 15, 19 and 23 Hours After Light Onset—HALO). Day-40 survival rate varied between 20% (PTC at 3 HALO) and 55% (PTC at 15 HALO) ($\chi^2 = 16.7$; $P < 0.01$). In each study, body weight loss was maximal in mice injected with PTC at 3 HALO and minimal in those treated at 15 HALO ($P < 0.01$). In a further study involving 96 male B6D2F1 mice, the toxicity of PTC on several target tissues (bone marrow, spleen, small bowel, colon, liver, kidney and lungs) was investigated by histology and leukocyte count as a function of drug dosing time. A circadian rhythm in the susceptibility of the bone marrow, the spleen and the intestinal tract was demonstrated. Optimal murine tolerance for PTC resulted from dosing it at 15 HALO, e.g. in the first half of the activity span.*

INTRODUCTION

THE CIRCADIAN time at which an anticancer drug is administered to rodents largely influences both its tolerance and its antitumor effect [1–3]. More specifically, this has been shown for the alkylating agents, cyclophosphamide [4] and melphalan [5]. Both lethal and hematologic toxicities of these drugs were minimized in rodents by injecting them at an appropriate circadian time. However, the optimal tolerance for melphalan was achieved after injection near 8 hr after light onset (e.g. rest span) and that for cyclophosphamide near dark onset (e.g. first half of the activity span).

Peptichemio (PTC) is a mixture of 6 peptides of *m*-[di-(2-chloroethyl)amino]-L-phenylalanine. This alkylating agent has demonstrated a promising antitumor activity in clinical phase II trials [6]. The aim of the present studies was to document any effect of dosing time upon tolerance of mice for this drug in order to provide guidelines for a further optimization of its therapeutic index. Mortality, body weight loss, leukopenia and microscopic lesions of the bone marrow, the spleen and the gastro-intestinal tract were used as toxicity endpoints.

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MATERIAL AND METHODS

Animals and synchronization

Five studies were performed between January and June 1985. A total of 559 male B6D2FI mice (IFFA-CREDO, l'Abresle, France) were used. They were housed 3 per cage, with food and water freely available. Three weeks before each study, mice were randomly distributed into 1 of 6 groups (study 1–4) or 1 of 4 groups (study 5). Each group was housed on a different shelf of an autonomous chronobiologic animal facility (E.S.I.-Flufrance, Arcueil, France). Each facility has 6 sound-proof, temperature-controlled compartments, each one having its own programmable lighting regimen. They were constantly provided with filtered air delivered at an adjustable rate (80 l/min in these studies). All mice were synchronized with a lighting regimen consisting of an alternation of 12 hr of light (L) and 12 hr of darkness (D) (LD 12 : 12).

Table 1. Main characteristics of the five studies investigating the circadian variability of tolerance of male B6D2F1 *mice for PTC (442 treated and 85 controls)*

Study	Date	No. of mice		Age (weeks)	PTC dose* (mg/kg)	No. of Time-points	Endpoints
		Controls†	Treated				
1	01/15–17/85	14	67	13	30	6‡	Survival, weight loss
2	02/5–7/85	13	77	11	24	6	id.
3	03/19–21/85	10	72	9	30	6	id.
4	05/13–15/85	48	162	10	30	6	id.
5	06/24–26/85	32	64	11	30		Weight loss, leukopenia, histology of bone marrow, spleen, intestine . . ., on day 5 and 9 after first dose

*Total given intravenously once a day for 3 consecutive days.
†Given 5% glucose i.v. (5 ml/kg) over 3 consecutive days.
‡3, 7, 11, 15, 19 and 23 hr after light onset (HALO).
§3, 9, 15 and 21 HALO.

For convenience, staggered LD regimens were used so that different circadian stages of mice were explored at similar clock hours. For example, in studies 1–4 all the groups were injected between 10:00 hr and 12:00 hr. With such a procedure, 6 circadian stages were explored: 3, 7, 11, 15, 19 and 23 Hours After Light Onset (HALO). The adequate synchronization of mice kept under such conditions for 3+ weeks is a general property of biological rhythms [7]. In the present studies it was verified by assessing the circadian rhythmicities in body weight and circulating white blood cell (WBC) count in control mice.

Drug

PTC was kindly supplied by the Istituto Sieroterapico Milanese S. Belfanti (Milan, Italy) in vials containing 40 mg of a complex of synthetic peptides equivalent to 16 mg (± 0.5) of *m*-[di-(2-chloroethyl-)amino-L-phenylalanine (m-SL). The drug was freshly prepared prior to injection by adding the appropriate amount of 5% glucose in order to achieve the desired concentration. Two dosages were administered (8 or 10 mg of complex/kg of body wt) in a fixed fluid volume (5 ml/kg body wt). Hence, drug concentrations were respectively: 1.6 and 2.0 mg/ml. Each mouse was given an intravenous injection of either drug or 5% glucose into the retro-orbital sinus daily for 3 consecutive days.

Study designs

The study designs are summarized in Table 1. Toxicity endpoints were mortality and body weight loss in study 1–4. Body weight loss, leukopenia and histologic lesions of several organs were assessed 5 and 9 days after the first dose in study 5.

Toxicity endpoints

Mortality was recorded twice a day until day 20, and once a day subsequently up to day 40. The survival rate was computed for each dose, group and study.

The following variables were measured at the same circadian time as that of injection.

Body weight was recorded 3 times per week for 30 days starting on the day of first PTC injection (day 0) in studies 1–4. In study 5, this variable was measured on days 0, 5 and 9. Body weight loss was computed as percentage difference between post-injection and initial body weight of each mouse.

In study 5, 32 PTC-treated mice and 16 controls were bled on day 5 and the same numbers on day 9 following the first PTC injection (e.g. 8 treated and 4 controls per time-point and per day). The blood obtained from the retro-orbital sinus was collected in a heparinized 1 ml syringe (Terumo) and diluted in Isoton II (Coultronics). Six drops of Zap-o-globin (Coultronics) were added, and the WBC count measured with a Coulter counter (Coultronics).

Histopathologic study (study 5)

Mice were sacrificed on day 5 or 9 after the first i.v. dose of 5% glucose (controls) or 10 mg/kg of PTC. Subgroups of mice had been injected at either of 4 different circadian stages (3, 9, 15 or 21 HALO) on day 0, 1 and 2. Bone marrow, spleen, lungs, kidney, liver, colon and small bowel were sampled, then fixed into Bouins's picroformol solution for 2 days and embedded into paraffin. Sections were stained with hematein–eosin. Each encoded slide was examined by the same histopathologist and lesions were blindly graded beteen 0 (normal) up to 5. The highest grade (5) corresponded to complete

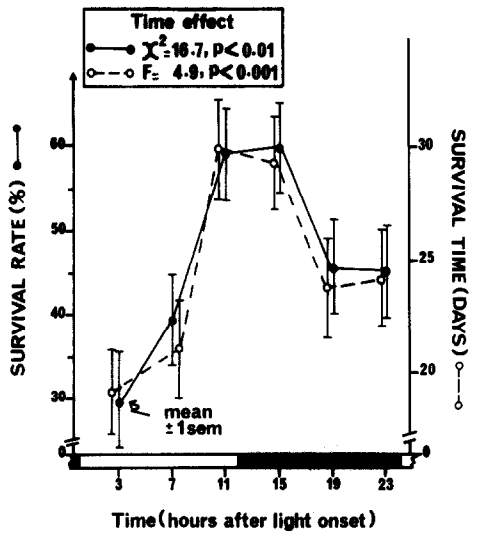

Fig. 1. Day-40 survival rate and survival time of 378 ♂ B6D2F1 mice receiving a cumulative i.v. dose of 24 or 30 mg/kg of peptichemio (PTC) at each of 6 circadian stages. Summary of 4 studies. Dosing time-related effects were statistically validated for both variables.

necrosis for the bone marrow, to complete depletion in mature lymphocytes for the spleen, to large areas of necrosis of the villi for the small bowel, and to diffuse mucous atrophy for the colon.

Statistical analysis

Means and one standard error of the mean (S.E.M.) were computed for each variable, time point dosage and study. The statistical significance of differences between groups was validated by an analysis of variance. Differences in survival rates were analyzed by χ^2. Time series were also analyzed by the cosinor method [8]. A rhythm was characterized by parameters of the fitted cosine function approximating all data with a period (τ) $\equiv$ 24 hr. The rhythm characteristics estimated by the cosinor are the mesor M (24 hr adjusted mean), the amplitude A (half the difference between the minimum and maximum) and the acrophase ϕ (time of maximum, with time of light onset as ϕ reference). A and ϕ are given with their 95% confidence limits. A rhythm is detected when A differs from zero (non-null amplitude test) with $P < 0.05$; however, A and ϕ may be approximated if $0.05 \leq P \leq 0.10$.

In all cases the concordance of several statistical methods was required to draw any conclusion.

RESULTS

Survival

Deaths occurred between day 5 and 16 following first PTC dosing. Subsequently, no death occurred until day 40, when each study was terminated. Both the survival rate and the mean survival time differed as a function of dose and dosing time. An increase in cumulative dose from 24 up to 30 mg/kg led to a 50% decrease in survival rate from 67 to 41% and a reduction in mean survival time (± 1 S.E.M.) from 32.5 (± 1.7) to 22.6 (± 1.0) days.

Whatever the dose, an effect of the circadian stage of injection on survival rate was apparent (Table 2) and statistically validated in those groups receiving 30 mg/kg ($\chi^2 = 16.7$; $P < 0.01$) and after pooling data from mice treated by either dosage ($\chi^2 = 17.0$; $P < 0.01$). The average optimal time was localized near the transition of rest to activity, e.g. near 11–15 HALO, with a marked trough in the first half of the rest span (3–7 HALO). Values at peak time were twice as high as those at through time (Fig. 1).

Differences between studies were found (Table 2). Thus the same dose appeared to be better tolerated in study 4 as compared to study 1 or 3. Moreover the dosing times associated with optimal tolerance was 11 HALO in study 1 and 15 HALO in studies 3 and 4.

Similar results were obtained for survival times (Fig. 1). Effects of both dosing time and study were indicated by 2-way-ANOVA ($P < 0.001$, with respective $F = 5.7$ and 18.5). The effect of study was not only due to the fact that one study involved a lower dosage: a 2-way-ANOVA was also performed on those survival times corresponding to the same cumulative dose of PTC (30 mg/kg); results still indicated an effect of both dosing time ($F = 4.9$; $P < 0.001$) and study ($F = 18.3$; $P < 0.001$). Moreover, an interaction term was found ($F = 2.3$; $P = 0.005$), suggesting the need for examining separately the effect of the circadian time of day in each study.

Body weight loss

Maximal weight loss was achieved on day 11 in studies 1, 2 and 3 and on day 7 in study 4. In study 5, weight loss was larger on day 9 than on day 5. Day 11 weight loss was $\cong$ 24% of the initial body weight whether mice had received 24 or 30 mg/kg of PTC, with near complete recovery achieved $\cong$ 4 weeks after treatment.

2-Way-ANOVA validated statistically significant differences in body weight loss as a function of study and dosing time on day 7 (respectively $F = 23.9$ and $F = 12.9$, $P < 0.001$) on day 9 ($F = 10.7$, and $F = 7.5$, $P < 0.001$) and on day 11 ($F = 9.6$, and $F = 10.7$, $P < 0.001$), but not on day 22 ($F = 1.4$ and $F = 1.8$, $P > 0.10$). No interaction was found with statistical significance except on day 7 ($F = 2.2$, $P < 0.01$). Thus data on body weight loss are presented on day 7, 9 and 11 as a function of dosing time and irrespective of dose (Fig. 2).

Table 2. Survival of male B6D2F1 *mice at* 40 *days after treatment with PTC at each of six different circadian stages**

Variable	Study No. (dose, mg/kg)	Time of injection (hr after light onset) 3	7	11	15	19	23	Mean (± 1 S.E.M.†)
Survival rate (%)	1 (30)	20	36	33	25	0	36	25
	2 (24)	54	69	69	77	69	64	67
	3 (30)	11	0	31	42	23	8	19
	4 (30)	26	44	78	74	63	59	57
	1–4 (24+30)	29 ± 9†	39 ±14	58 ±12	59 ±13	41 ±16	46 ±12	46 ±12
	1, 3, 4 (30)	22 ± 4	29 ±13	56 ±15	55 ±14	33 ±18	42 ±14	41 ±11
Mean survival time (days)	1 (30)	16	21	21	16	7	18	17 ± 2
	2 (24)	27	30	37	35	32	32	32 ± 2
	3 (30)	12	9	20	21	16	11	15 ± 2
	4 (30)	22	29	37	38	19	29	29 ± 2
	1–4 (24+30)	19 ± 2†	21 ± 2	30 ± 2	29 ± 2	24 ± 2	24 ± 2	24 ± 1
	1, 3, 4 (30)	16 ± 2	19 ± 2	28 ± 2	28 ± 2	22 ± 2	22 ± 2	23 ± 1

*A statistically significant effect of dosing time was indicated for both variables, respectively by χ^2 test and ANOVA with $P < 0.01$, whether data from all studies or from studies 1, 3 and 4 were pooled. A statistically significant difference ($P < 0.01$) was also found for both variables between studies 1, 3 and 4.
†Standard error of the mean.

The smallest mean body weight loss corresponded to PTC dosing near 15 HALO. Moreover, maximal weight loss was reached after a different time span according to PTC dosing time. Thus maximal weight loss was achieved on day 7 (19.9%) after dosing at 15 HALO and on day 11 after dosing at 3 HALO.

Table 3 summarizes the mean body weight loss in each study on these days. Toxicity, as gauged by this index, was larger in study 3, as compared to study 4, despite the same dose being administered.

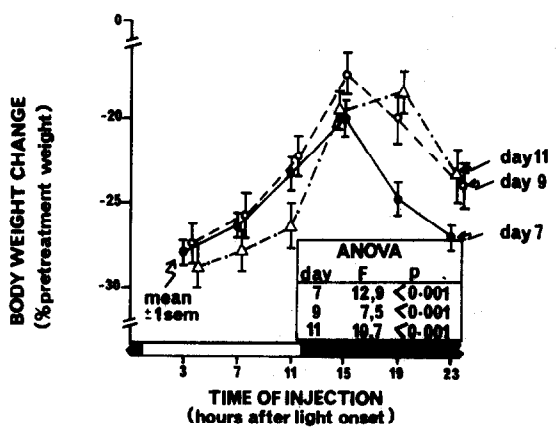

Fig. 2. Body weight loss of 378 ♂ B6D2F1 *mice injected i.v. with* 24 *or* 30 mg/kg *of peptichemio (PTC) at each of* 6 *circadian stages. Dosing time-related effects were statistically validated on those days corresponding to maximal average body weight loss, e.g. days* 7–11.

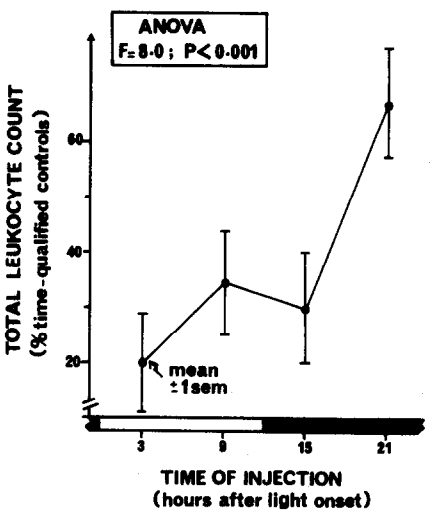

Fig. 3. Circulating leukocyte count after i.v. injection of a cumulative dose of 30 mg/kg *of peptichemio (PTC) to* 64 ♂ B6D2F1 *mice at each of* 4 *circadian stages* (3, 9, 15 *or* 21 hr *after light onset*) (*study* 5). *Data were obtained on day* 5 *and* 9 *after first PTC dose, and expressed as percentages of the corresponding mean time-qualified control value* (8 *control mice/time point*).

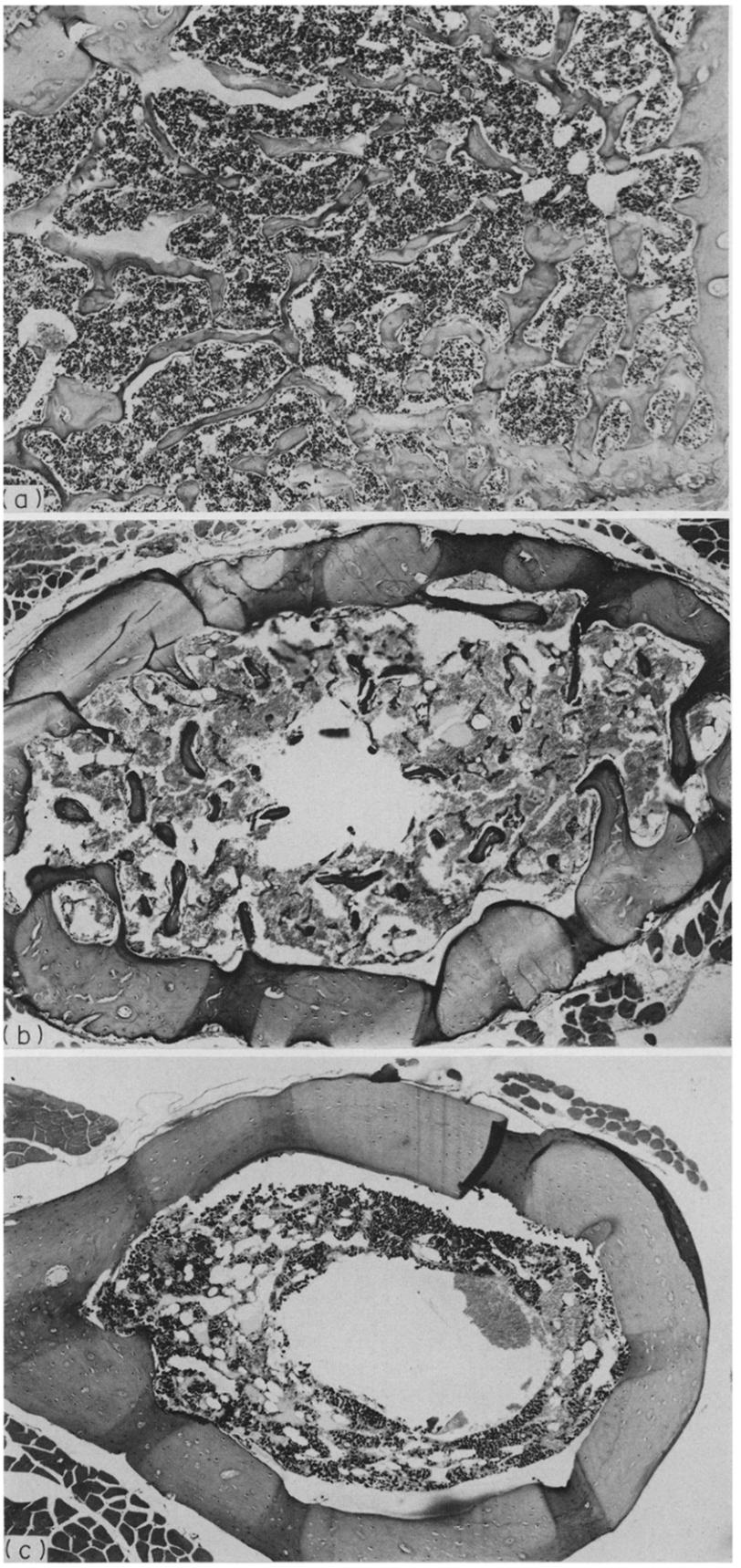

Fig. 5. *Transverse section of femoral bone marrow* 9 *days after first dose of* 5% *glucose* (*control*) *or PTC at either* 3 *or* 15 hr *after light onset* (*HALO*). *Hematein–eosin staining.*
A: *Control—Histologic score* = 0 (*magnification* × 158).
B: *PTC at* 3 *HALO—Extensive necrosis involving the whole section—Score* = 5 (× 63).
C: *PTC at* 15 *HALO—Partial regeneration of bone marrow cells—Histologic score* = 3 (× 63).

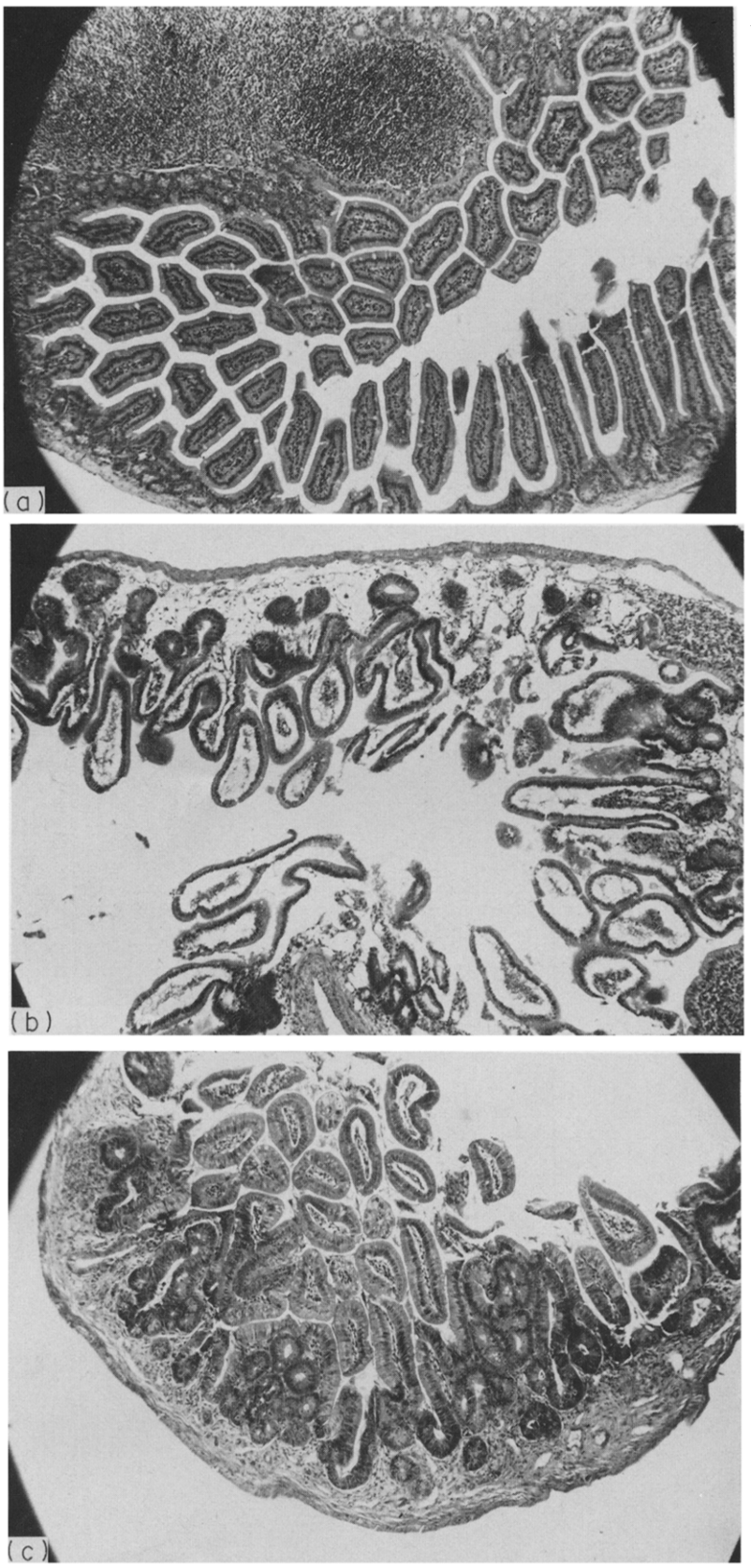

Fig. 6. Ileum 9 days after first dose of 5% glucose (control) or PTC at either 3 or 15 HALO. Hematein–eosin staining.
A: *Control—Histologic score* = 0 (*magnification* × 126).
B: *PTC at 3 HALO. Necrosis involving almost all villi—Score* = 4 (× 63).
C: *PTC at 15 HALO—Epithelial dystrophy associated to chorionic oedema in some areas. Lack of necrosis—Score* = 3 (× 63).

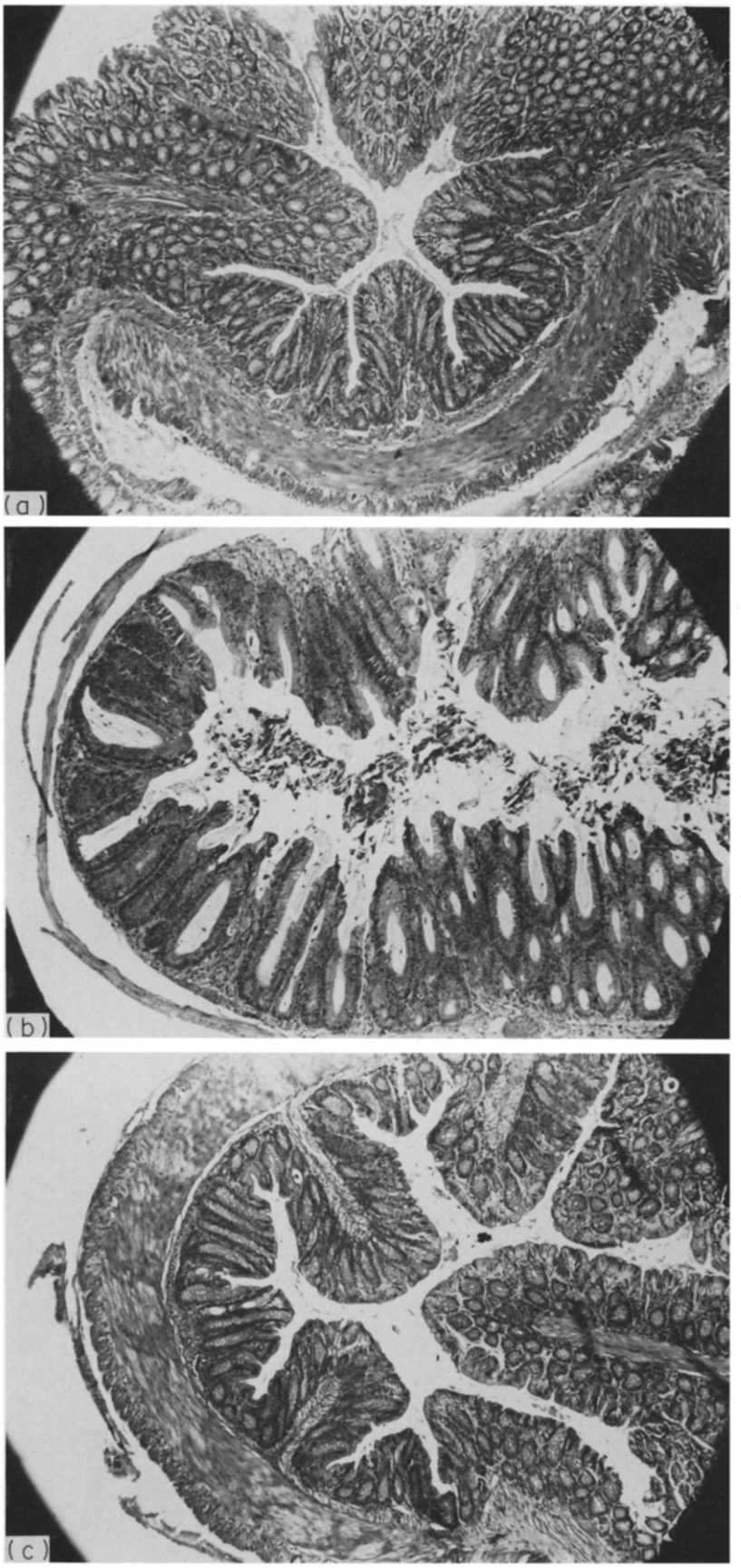

Fig. 7. Colon 9 days after first dose of 5% glucose (control) or PTC at either 3 or 15 HALO. Hematein–eosin staining.
A: *Control—Histologic score* = 0 (*magnification* × 63).
B: *PTC at* 3 *HALO—Large areas of dystrophy resulting in dedifferentiation of epithelial cells—Score* = 3 (× 63).
C: *PTC at* 15 *HALO—Decreased mucous secretion—Score* = 1 (× 63).

Table 3. Mean body weight loss (%, ± 1 S.E.M.) on days of maximal values in each study

Days after first dose	Study* 1	2	3	4	5
7	−26.2 ± 0.9 (51)	−22.8 ± 0.8 (66)	−30.5 ± 0.4 (60)	−22.6 ± 0.6 (150)	N.D.
9	−25.7 ± 1.8 (26)	−21.3 ± 1.1 (57)	−31.4 ± 0.2 (28)	−19.9 ± 0.8 (109)	−23.9 ± 1.9 (29)
11	−29.0 ± 1.6 (20)	−24.0 ± 0.9 (53)	−33.6 ± 1.4 (21)	−20.4 ± 0.7 (102)	N.D.

Numbers of mice are in parentheses.
*3 consecutive i.v. daily doses of 10 mg/kg each (studies 1, 3, 4 and 5), or of 8 mg/kg each (study 2). An effect of both dosing time and study was statistically validated on each day by two-way-ANOVA whether data were considered including or excluding those of study 2.

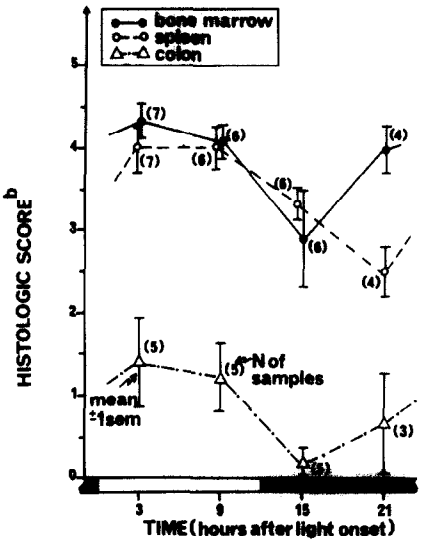

Fig. 4. Histopathologic scores of bone marrow, spleen and colon of ♂ B6D2F1 mice 9 days after the first i.v. dose of peptichemio (PTC) (cumulative dose = 30 mg/kg). Four circadian stages of PTC injection are compared. Maximal lesions correspond to grade 5 and normal aspects were graded as 0. Dosing time-related effects were statistically validated for the susceptibility of all 3 target organs by ANOVA (P < 0.01).

Immuno-hematologic toxicity

Hematologic toxicity was assessed in study 5 from the count of circulating leukocytes and the histologic scores of both the bone marrow and the spleen 5 and 9 days after the beginning of treatment.

With regard to circulating leukocytes, an effect of sampling time was statistically validated in controls by 2-way-ANOVA ($F = 3.7$, $P = 0.02$), but neither an effect of sampling day nor an interaction term was found with statistical significance ($F = 2.4$ and 1.8 respectively, $P > 0.10$). Thus, time-qualified control data were considered as reference values irrespective of sampling day. Control peak values were obtained at 3 HALO (mean ± 1 S.E.M., 9480 ± 470 cells/mm^3) and lowest ones at 15 HALO (5710 ± 1080 cells/mm^3).

In treated mice, an effect of injection time was indicated with statistical significance ($F = 4.3$, $P < 0.01$). No effect of sampling day or any interaction term was found (respective $F = 0.1$ and 1.7, $P > 0.10$). Thus, data from treated groups were also considered irrespective of sampling day. Mean leukocyte count was maximal after dosing at 21 HALO (4430 ± 930 cells/mm^3) and minimal after treatment at 3 HALO (1890 ± 340).

Because of the physiologic circadian rhythm in circulating total WBC count, data from treated mice were also expressed as percentages of the corresponding mean time qualified control value (Fig. 3). Statistically significant differences as a function of dosing time were demonstrated with ANOVA ($P < 0.001$), with highest WBC counts also corresponding to PTC dosing at 21 HALO.

Bone marrow and spleen scores were 0 in all control mice. In treated mice a complete necrosis of the bone marrow (score = 5) was found in all specimens on day 5, whereas a recovery was apparent on day 9 (mean score ± 1 S.E.M., 3.8 ± 0.2). On day 9, an effect of dosing time was statistically validated by 1-way-ANOVA ($F = 2.6$, $P < 0.05$). Recovery was achieved faster after injection at 15 HALO as compared to injection at 3 HALO (Figs. 4 and 5).

Spleen lesions were more pronounced on day 9 (mean score, 3.6 ± 0.2) as compared to day 5 (1.4 ± 0.1). Two-way-ANOVA documented statistically significant effects of both sampling day ($F = 90.4$, $P < 0.001$) and an interaction term between treatment time and sampling day ($F = 3.1$, $P = 0.03$). Circadian time-related differences were not found on day 5, but were observed on day 9. On this day, a minimal mean score corresponded to treatment at 21 HALO (2.5 ± 0.3) and a maximal score to PTC dosing at 9 HALO (4.1 ≅ 0.4) (Fig. 4).

Gastro-intestinal toxicity

The toxicity of PTC for the small bowel and that for the colon were evaluated from the histologic analysis of organs sampled 5 and 9 days after treatment onset. Lesions were more important: (a) in the small bowel (mean score: 3.9 ± 0.2) than in the colon (1.4 ± 0.2) and (b) on day 5 than on day 9 for either organ. No effect of either circadian time or day of sampling was detected with statistical significance for the small bowel by 2-way-ANOVA. This analysis revealed that both effects played a significant role for the colon (respectively, $F = 3.0$,

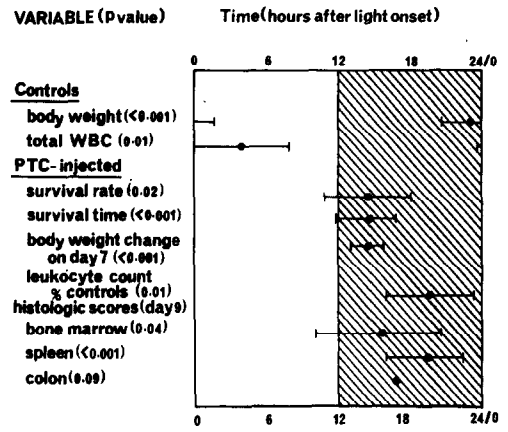

Fig. 8. Acrophase chart of chronotolerance for peptichemio (PTC) in ♂ B6D2F1 mice. Black dots correspond to the location in time of the acrophase (ϕ), and the horizontal line represents its 95% confidence interval. This parameter is given by cosinor analysis. The ϕ's of body weight and circulating total white blood cell count from control mice are shown as gauges of the adequate synchronization of the mice. The ϕ's of the several endpoints used to evaluate host tolerance for PTC are shown below. The name of each variable is followed by the P-value of rhythm detection.

$P < 0.05$ and $F = 12.9$, $P < 0.001$), with no interaction term ($F = 0.7$, $P > 0.10$).

However, the minimal mean values (± 1 S.E.M.) corresponded to PTC injection at 15 HALO for both the small bowel and the colon, and the maximal ones to treatment at 3 HALO for both organs (Figs. 6 and 7).

Other organs

No apparent lesion was found in the kidney, liver or lungs of either control or treated mice.

Chronotolerance for PTC

All data were analyzed with cosinor. Statistically significant rhythms were detected in control mice for body weight and circulating leukocyte count, and in treated mice for survival rate, body weight loss, total leukocyte count, leukopenia index, and for bone marrow, spleen, and colon scores on day 9.

Such results are summarized in Fig. 8, in the form of an acrophase chart. As can be seen, optimal chronotolerance for PTC corresponded to the first half of the dark-activity-span, e.g. near 15 HALO.

DISCUSSION AND CONCLUSION

Optimal tolerance for PTC was achieved when this drug was given in the early activity span of mice, e.g. near 15 HALO. The adequate synchronization of the mice by the 6 LD 12 : 12 schedules was demonstrated by circadian rhythms in body weight and circulating leukocytes similar to those previously reported by others [9] and us [10]. A similar optimal timing of PTC was found whatever the level of toxicity considered (survival rate, survival time, body weight loss), and whatever the target organ (bone marrow, spleen, colon). Bone marrow suppression and intestinal lesions seemed to constitute the main mechanisms of the lethal toxicity of PTC, since no apparent lesion was found in the liver, lung or kidney. Thus, optimal hematologic tolerance for PTC corresponded to dosing ≅ 6 hr later than other cytostatics such as arabinosylcytosine [11], melphalan [5], X-rays [12], 4′-*O*-tetrahydropyranyl adriamycin [10] or etoposide [13].

Large differences among the studies performed with the same dosage of PTC were observed and statistically validated for survival rate, survival times and body weight loss. Male B6D2F1 mice of similar age (9–13 weeks) were used after 3 weeks of synchronization in each study. No trend attributable to age was apparent; thus the largest differences were found between study 3 (in 9-week-old mice) and study 4 (in 10-week-old mice). No major alteration of the dosing time associated with an optimal tolerance was found among these studies (11 HALO, in study 1, 15 HALO in studies 3 and 4). The poorest tolerance, gauged by the same index, however, corresponded to treatment at 19 HALO in study 1, at 7 HALO in study 3, and again at 19 HALO in study 4. Such differences in timing were reflected by a statistically significant interaction term at the 2-way-ANOVA ($F = 2.3$, $P = 0.005$). Such inter-study differences in both overall tolerance and timing of poorest tolerance may be related to seasonal variations. A circannual rhythm was found for tolerance of mice to adriamycin and daunomycin [14] and for tolerance of rats to *cis*-dichlorodiammine platinum [15]. Optimal tolerance for both anthracyclines was found in fall and that for *cis*-dichlorodiammine platinum in winter. Thus, PTC may be better tolerated in late spring (studies 4 and 5) than in winter (studies 1 and 3). Such seasonal variations in host tolerance for anticancer drugs may be accounted for by circannual rhythms in bone marrow proliferation which were found in standardized laboratory rodents [16].

Whereas the optimal circadian dosing time for PTC may not change along the scale of the year, that of poorest tolerance for this drug may do so. Such findings may also apply to cancer patients.

On the circadian scale, the relevance of the mouse model to predict the optimal dosing time of anticancer agents in cancer patients has already been documented for adriamycin [17], 4′-*O*-tetrahydropyranyl adriamycin [18] and *cis*-dichlorodiammine platinum [17, 19, 20]. Moreover, seasonal variations in the hematologic tolerance of cancer patients for adriamycin–cisplatin chemotherapy have also been reported [20].

In summary, PTC was best tolerated by mice after dosing in the first half of the activity span. This may correspond to ≅ 14:00 hr in cancer patients. On the circannual scale, PTC may also be better tolerated in late spring than in winter.

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