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Close Reproduction by Different Laboratories of Characteristics of Circadian Rhythm in 1-β-D-Arabinofuranosylcytosine Tolerance by Mice[1]

Lawrence E. Scheving, Erhard Haus, Jürgen F. W. Kühl, John E. Pauly, Franz Halberg, and Sergio Cardoso

Chronobiology Laboratories, University of Minnesota Medical School, Minneapolis, Minnesota 55455 [F. H., J. F. W. K.]; Department of Anatomy, University of Arkansas Medical Center, Little Rock, Arkansas 72201 [L. E. S., J. E. P.]; St. Paul-Ramsey Hospital, St. Paul, Minnesota 55101 [E. H.]; and Department of Pharmacology, University of Tennessee, Memphis, Tennessee 38103

SUMMARY

The tolerance of BALB/c × DBA/2 F_1 mice to the popular cytostatic drug 1-β-D-arabinofuranosylcytosine (ara-C) was tested in two laboratories about 1000 km apart. According to the same plan and on the same days in Little Rock, Ark., and Minneapolis, Minn., nine groups of 20 mice each received four courses of ara-C treatment, with 4-day intervals between them beginning February 7, 1973. In each course, a total dose of 240 mg/kg was divided among eight separate injections administered at 3-hr intervals. One group of mice received equal doses of ara-C every 3 hr (the homeostatic schedule). The eight other groups in each location received the same total dose per course but in gradually increasing and decreasing doses (the sinusoidal schedule). The timing of the highest doses on the latter schedule differed among the eight groups (by integer multiples of 3 hr). As predicted from earlier work, survival times after treatment with ara-C on different sinusoidal schedules differed drastically. However, the timing of the sinusoidal schedules yielding the longest survival was similar in the two locations. The survival times from all sinusoidal treatments from a given location were fitted by a 24-hr cosine curve. The timing of the rhythm in tolerance as a whole was thereby computed as the lag from local midnight of the peak in the cosine curve best fitting all data. The timing of this tolerance rhythm (briefly, circadian chronotolerance), computed separately for data from Arkansas and Minnesota, agreed within 1 hr. There also was close agreement in the results obtained by the 2 laboratories in mean survival time; the percentage of survivors when mice were treated according to certain of the selected sinusoidal schedules was much greater than for mice treated on the homeostatic schedule. This large and reproducible difference in tolerance and the similar timing of the overall fitted function describing chronotolerance in the hands of different investigators underlines the urgency of testing potential benefits from timed clinical treatment.

INTRODUCTION

A circadian rhythm in resistance of mice to ara-C[2] was established using a single fixed dose administered to different subgroups at 1 of 5 different time points daily for 5 days (3, 18). Subsequent work applied this finding to the treatment of leukemic mice (11) in which case ara-C was administered to each mouse as 8 doses, 3 hr apart, on each of 4 days, at 4-day intervals. The 8 doses injected each treatment day were varied among time points according to a sinusoidal pattern (14) from 7.5 mg/kg at the time point of the lowest resistance of the animals (predicted from the earlier studies) to 67.5 mg/kg at the predicted time point of highest resistance. The graded doses of this schedule had been designed so that even the smallest dose, given when the host was most susceptible, exerted some therapeutic antitumor activity with little if any toxicity. This experimental "chronotherapy" was successful in approximately doubling the survival time of leukemic mice (14, 15) in comparison with controls treated without regard for the resistance rhythms according to a scheme used and described as the best current treatment (19).

Although the advantage of leukemia treatment according to this particular sinusoidal[3] schedule was clear-cut, it seemed of interest to use a larger sample in a study limited to tolerance *per se* and to attempt an unequivocal demonstration of chronotolerance. The studies were done concurrently in 2 different laboratories (~1000 km apart).

MATERIALS AND METHODS

During the same time-span, in different geographical locations (Little Rock, Ark., and Minneapolis, Minn.) 180 male BALB/c × DBA/2 F_1 (hereafter called $CD2F_1$) mice, 7 weeks of age, were singly housed in each laboratory for 2 weeks prior to initiation of drug treatment, in rooms maintained at 24 ± 1° and illuminated daily from 6 a.m. to 6 p.m. Purina rat chow and water were available freely at all times. After stratification by weight, each group of 180 mice was divided randomly into 9 experimental groups containing 20 mice each.

Four courses of the drug were administered at both locations at 4-day intervals. Each course consisted of 8 doses given at 3-hr intervals over one 24-hr span. One group

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[2] The abbreviation used is: ara-C, 1-β-D-arabinofuranosylcytosine.

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[3] The term sinusoidal is here used with emphasis upon the Greek *eidos*, a noun having gained (as a suffix in a medical context) the connotation "similar to" (rather than identical). This is emphasized since the doses here chosen are not strictly defined by the equation of a sine curve relating the value (of a function) to time.

received equal doses [homeostatic treatment (H)]; the 8 other groups each received doses which varied sinusoidally over the 24-hr span [sinusoidal treatment (S)]. The timing of these sinusoidal schedules differed among the 8 groups by integer multiples of 3 hr (Table 1). The timing of each sinusoidal schedule also can be briefly characterized by the midpoint of its highest doses in terms of clock hr, or as degrees (with 360° = 24 hr) after midnight, after midlight, or after some biological rhythm of the animal (*e.g.*, body temperature) chosen as reference. For example, the highest doses of the sinusoidal treatment centered at 12:30 a.m. (7.5° after midnight for 1 group, at 3:30 a.m. for another group, etc.). All 9 groups received a total daily ara-C dose of 240 mg/kg. Survival times were assessed at 35 days after start of treatment when no additional mouse had died for the preceding 9 days. All animals alive on this day were counted as having survived for 35 days.

RESULTS AND DISCUSSION

The longest survival time in days (evaluated 35 days after beginning treatment) was achieved by sinusoidal treatment schedules in which the midpoint of administration of the highest doses of ara-C fell between 9:30 a.m. and 6:30 p.m. (Chart 1). Sinusoidal schedules having the midpoint of maximal doses between 9:30 p.m. and 6:30 a.m. were less favorable, as documented by a shorter survival time. These results could have been predicted on the basis of the earlier

Table 1

Details of individual doses of ara-C: homeostatic[a] treatment (H) and 8 sinusoidal schedules (S)

	Dose (mg/kg) for group								
Injection time (clock hr)	H	S-8	S-1	S-2	S-3	S-4	S-5	S-6	S-7
8 a.m.	30.0	67.5	30.0	15.0	7.5	7.5	15.0	30.0	67.5
11 a.m.	30.0	67.5	67.5	30.0	15.0	7.5	7.5	15.0	30.0
2 p.m.	30.0	30.0	67.5	67.5	30.0	15.0	7.5	7.5	15.0
5 p.m.	30.0	15.0	30.0	67.5	67.5	30.0	15.0	7.5	7.5
8 p.m.	30.0	7.5	15.0	30.0	67.5	67.5	30.0	15.0	7.5
11 p.m.	30.0	7.5	7.5	15.0	30.0	67.5	67.5	30.0	15.0
2 a.m.	30.0	15.0	7.5	7.5	15.0	30.0	67.5	67.5	30.0
5 a.m.	30.0	30.0	15.0	7.5	7.5	15.0	30.0	67.5	67.5
Clock hr[b]		9:30 a.m.	12:30 p.m.	3:30 p.m.	6:30 p.m.	9:30 p.m.	12:30 a.m.	3:30 a.m.	6:30 a.m.
Degrees from 12:00 a.m.[b]		−142.5°	−187.5°	−232.5°	−277.5°	−322.5°	−7.5°	−52.5°	−97.5°

[a] Homeostatic, fixed dose at 3-hr intervals, for 24 hr, every 4th day for 4 courses. Sinusoidal, gradually varying doses, at 3-hr intervals for 24 hr every 4th day for 4 courses. Total dose identical in all treatments.

[b] Midpoint of highest dose for sinusoidal schedule.

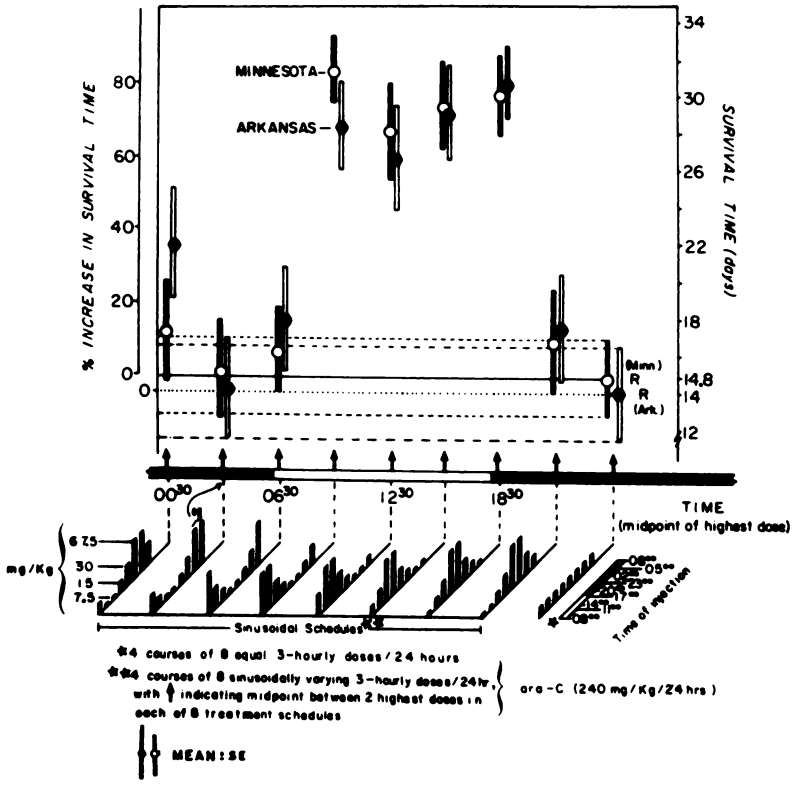


Chart 1. Survival time of $CD2F_1$ mice on different drug administration schedules (*top*) and timing of doses of ara-C (*bottom*) in sinusoidal and reference (*R*) schedules consisting of 4 courses of 240 mg/kg/24 hr each. When the same total dose of ara-C is given, certain sinusoidal drug administration schedules are definitely better tolerated by mice than are other sinusoids or a currently conventional reference treatment of 8 equal doses over a 24-hr span. Also note unequivocal reproducibility of chronotoxicity to ara-C in experiments done on the same days in different laboratories in different geographic locations.

Table 2

ara-C tolerance on different treatment schedules[a] in various geographic locations

Mean survival time evaluated 35 days after beginning treatment of 20 mice on each of 9 treatment schedules in both Arkansas and Minnesota (a total of 360 mice). Survival time in hr, mean ± S.E.

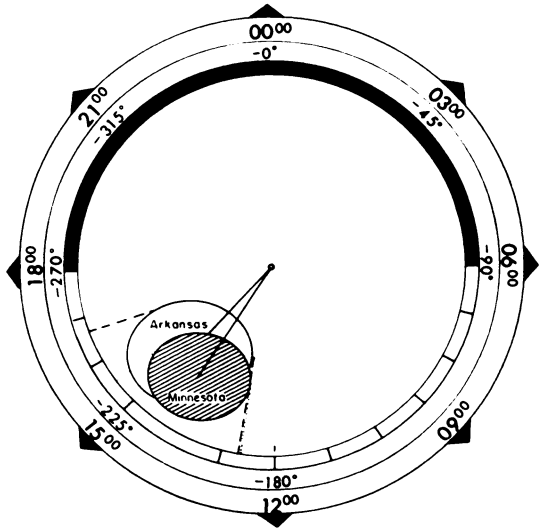
Treatment	Site and investigators: Arkansas (Schev-ing, Pauly *et al.*)	Site and investigators: Minnesota (Kühl)	Prediction
Homeostatic ("best" current Rx)	336 ± 63	355 ± 51	Unfavorable
Sinusoidal			
Schedule 8	682 ± 63	756 ± 48	Favorable
Schedule 1	641 ± 70	674 ± 66	
Schedule 2	698 ± 65	709 ± 61	
Schedule 3	738 ± 50	722 ± 55	
Schedule 4	421 ± 72	404 ± 70	Less favorable
Schedule 5	527 ± 74	414 ± 65	
Schedule 6	340 ± 68	364 ± 66	
Schedule 7	433 ± 70	389 ± 57	

[a] Homeostatic, fixed dose at 3 hr intervals, for 24 hr, every day for 4 courses. Sinusoidal, gradually varying doses, at 3-hr intervals for 24 hr, every 4th day for 4 courses. Total dose identical in all treatments.

work using single daily doses (1, 2). The homeostatic treatment compared unfavorably with most of the sinusoidal schedules (Chart 1; Table 2). [The biological rhythm used as reference need not be 24-hr synchronized (4).]

Results from the groups treated with sinusoidal schedules at the 2 laboratories were compared. The data on survival time from each laboratory, as summarized in Chart 1, were fitted by a 24-hr cosine curve by the method of least squares (10). This model provides a quantitative description of the circadian rhythm in survival time as a function of the timing of the sinusoidal schedule. A cosinor plot (10) of the results is shown in Chart 2. In this presentation, the direction of each vector represents the acrophase of the rhythm (*i.e.*, timing of highest doses for the reference sinusoidal schedule) in terms of either (*a*) clock hr (outermost circular scale), (*b*) degrees after midnight (with 360° = 24 hr), or (*c*) in relation to the light-dark regimen of the animals (innermost circular scale). The length of each vector indicates the amplitude of the fitted cosine (*i.e.*, one-half the difference between the peak and trough) in units of survival time (in this case in hr) and thus indicates the predictable extent of rhythmic response dependent upon the timing of the sinusoidal schedule. The ellipse at the tip of each vector portrays the joint 95% confidence region for the true acrophase and amplitude. The table at the bottom of Chart 2 also presents, for each laboratory, estimates and dispersion indices for the mesor of the rhythm (overall average survival time of mice on the 8 sinusoidal schedules), amplitude, and acrophase. *p* values resulting from *F* tests of the null hypothesis (zero amplitude) are indicated (10).

For comparison of results from the 2 geographic locations, a modified Bartlett test was first applied to examine the homogeneity of variances between 2 time series of survival-time data, each series being subjected to a single cosine model. The residual variances did not differ with statistical significance; an adjusted $\chi^2 = 1.08$ with 1 degree of freedom (d.f.). Therefore, one may apply tests, developed



	Mesor (M)* M ± SE	Amplitude (A)* A ± SE	Acrophase (Ø)** Ø	95% CI	% Rhythm	P-value F-test
Minnesota	554 ± 22	206 ± 31	-211°	-188°, -233°	22	.01
Arkansas	560 ± 24	167 ± 34	-221°	-191°, -251°	13	.01

N = 180 mice (20/time point) in each location

*In hours

**from local midnight

Chart 2. Cosinor summary of data on the reproducibility of chronotoxicity to ara-C in experiments done on the same days in different laboratories in different geographic locations. Polar coordinates are used to display in inferential statistical terms an extent of change of a biological rhythm (amplitude) and timing of change (acrophase) relations by the length and the angle of a directed line, respectively, shown with a bivariate statistical confidence region computed at a fixed (in this case 24-hr) trial period, first to detect a rhythm by a confidence region not overlapping the pole and second to estimate confidence intervals for the rhythm parameters. Thus, a measure of dispersion for the estimate of timing is given by radii drawn tangent to the confidence regions. The assumption underlying such a test is that the series summarized are comparable in terms of conditions of observations and/or individuals and that they are characterized by a rhythm with a similar frequency and timing. This assumption is extremely well validated (for the 2 sets of data analyzed) insofar as timing is concerned. The confidence regions of timing are similar, and the point estimates of timing vary by much less than 1 hr (since 360° are equated to 24 hr, 10° are equal to 40 min).

Table 3

Proportion of surviving mice after ara-C treatment according to sinusoidal (S) or homeostatic (H) schedules[a]

	No. treated	No. surviving		Total no.[b]	χ^2 (comparison)[c]	p
		Minnesota	Arkansas			
Favorable S (FS)						
S-8	40	17	15	32 (80)		
S-1	40	15	14	29 (73)		
S-2	40	16	16	32 (80)		
S-3	40	16	16	32 (80)		
					70.9 (FS-LS)	<0.0001
Less Favorable S (LS)						
S-4	40	6	7	13 (32.5)		
S-5	40	5	10	15 (37.5)		
S-6	40	5	5	10 (25)		
S-7	40	4	7	11 (27.5)		
					4.45 (LS-H)	<0.05
H	40	1	4	5 (12.5)		

[a] Results summarized 35 days after beginning treatment, revealing that not only is survival time prolonged (Table 2; Figs. 1 and 2) but the actual number of animals surviving is increased.

[b] Numbers in parentheses, percentage.

[c] χ^2 based on pooled data from 2 locations.

especially for the single cosine model, to examine differences in mesor (overall average) and amplitude-acrophase (16). These tests indicated no statistically significant differences between results from the 2 laboratories; F value = 0.03 with 1 and 314 d.f. in the mesor test and with F value = 0.062 with 2 and 314 d.f. in the amplitude-acrophase test. This method is based on a comparison of variances and seems more appropriate than the conventional 2-way analysis of variance when one is dealing with rhythms rather than with time-invariant phenomena. Nevertheless, conventional 2-way analysis of variance was applied to examine differences between the 2 laboratories, and the F test showed that the hypothesis of homogeneity of variance was not rejected. No statistically significant interlaboratory difference was found. The effect of the treatment schedule was significant (p = 0.01).

Table 3 presents a pool of data from both locations in terms of the number of animals surviving 35 days after beginning treatment. This pooling seems warranted in view of the agreement of results on survival time presented in Chart 1. Thus, groups of 40 animals on each treatment schedule provide information documenting the relative demerits or merits of the homeostatic treatment and each of the sinusoidal treatments. The homeostatic treatment was compatible with only 12.5% survival; 25 to 38% of the animals given treatment on "unfavorable" sinusoidal schedules survived, whereas the "favorable" sinusoids were associated with the survival of 73 to 80% of the animals.

An effect of sequencing the doses of ara-C (depending on whether one starts with high or low doses) remains unevaluated herein. It may account for the circumstance that no sinusoidal schedule was worse than the homeostatic treatment. In studies of leukemic mice treated with ara-C (15) "favorable" sinusoids indeed raised the cure rate with statistical significance, whereas unfavorable sinusoids lowered it.

Results from the studies described herein are conclusive since they were both predicted from earlier work and comparable in different locations. The cosinor presentation in Chart 2 overcomes the shortcomings of Chart 1, namely, by defining the characteristics of the rhythm in survival time and providing statistical confidence intervals. The remarkable agreement in results from 2 laboratories studying the same phenomenon comes to the fore in this chart.

The present results are limited to the exploitation of a circadian rhythm in tolerance of a drug. It is quite possible that tolerance also will vary with rhythms having frequencies higher and lower than circadian. The data suffice, however, to suggest that chronobiological concepts may be used to improve tolerance as demonstrated here for ara-C, and this is documented not only by survival times but also by the percentage of survivors. Such results must be viewed against the background of tests of chronotherapy for the entire system (host and tumor) rather than focusing upon host tolerance alone as in this study. The time seems ripe for a much broader systematic exploration of chronotherapy of leukemia with ara-C and other agents (1, 2, 5–9, 11–15, 17, 20).

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