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Experimental Chronotherapy of Mouse Mammary Adenocarcinoma MA13/C with Docetaxel and Doxorubicin as Single Agents and in Combination¹

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ABSTRACT

The therapeutic index of docetaxel, doxorubicin and their combination may be improved by an adequate selection of the circadian time of administration. The present study constitutes a prerequisite to testing the clinical relevance of chronotherapy in human breast cancer. Three experiments were performed in C3H/HeN mice. Each treatment modality was administered i.v. once a week for 3 weeks at one of six circadian stages, during the light span, when the mice were resting: 3, 7, and 11 h after light onset (HALO), or during darkness, when the mice were active: 15, 19, and 23 HALO. The circadian time dependency of single agent tolerability was investigated in healthy mice using four dose levels for docetaxel (38.8, 23.3, 14, and 8.4 mg/kg/injection) and for doxorubicin (13.8, 8.3, 5 and 3 mg/kg/injection; experiment 1). The circadian time dependency of each single agent efficacy was studied in MA13/C-bearing mice, using two dose levels of docetaxel (38.8 or 23.3 mg/kg/injection) or doxorubicin (8.3 or 5 mg/kg/injection; experiment 2). The toxicity and the efficacy of the simultaneous docetaxel-doxorubicin combination were assessed as a function of dosing time in experiment 3. Two combinations were tested (A, 16.3 mg/kg/injection of docetaxel and 2.5 mg/kg/injection of doxorubicin; and B, 11.6 and 3.5 mg/kg/injection, respectively) at each of the above six circadian times. Mortality, body weight change, and tumor size were recorded for 60–70 days in each experiment. Single agent docetaxel or doxorubicin was significantly best tolerated near the middle of the rest span (7 HALO) and most toxic in the middle of the activity phase (19 HALO). Docetaxel or doxorubicin as a single drug were also most effective at 7 HALO, irrespective of dose. Treatment at 7 HALO produced highest rates of complete tumor inhibition (81% versus 11% at 3 HALO for docetaxel, p from $\chi^2 < 0.001$, and 69% versus 44% at 11 HALO for doxorubicin, not significant) and highest day 60 survival rate (100% versus 28% at 3 HALO for docetaxel, p from $\chi^2 < 0.001$ and 89% versus 69% at 15 HALO for doxorubicin, not significant). Docetaxel-doxorubicin combinations were most effective following dosing in the beginning of the rest span or short after the onset of the activity span, with regard to the rates of both complete tumor inhibitions (45% at 3 HALO versus 15% at 19 HALO) and day 70 survival rates (85% and 80% at 3 and 7 HALO respectively, versus 20% at 19 HALO). The efficacy of single agent docetaxel or doxorubicin and that of their combination varied largely as a function of circadian dosing time. Single agent docetaxel at 7 HALO was the best treatment option in this model with regard to both tolerability and efficacy. This timing may correspond to the middle of the night in cancer patients.

INTRODUCTION

Docetaxel and doxorubicin are among the most active single agents against human breast cancer. Their combination has induced a high rate of complete regressions, with consequences on patient survival to be further assessed (1).

Doxorubicin is an anthracycline that inhibits topoisomerase II activity, intercalates between the DNA base pairs, and decreases DNA and RNA polymerase activity. Docetaxel is an antimetabolic drug that promotes tubulin assembly in microtubules and inhibits their depolymerization (2).

Bone marrow suppression and cardiac toxicity are the major dose-limiting factors of anthracyclines. In particular, doxorubicin induces irreversible congestive heart failure as a function of cumulative dose (3, 4). Docetaxel mostly produces bone marrow suppression and capillary leakage (5). Preclinical studies have reported that doxorubicin and docetaxel do not generate cross-resistance or disrupt their respective pharmacokinetic profiles (6). Their combination may have an additive activity (7).

The adaptation of chronotherapy delivery to circadian rhythms has improved tolerability and/or efficacy of anticancer agents in both rodents and cancer patients. Experimental chronopharmacology studies have successfully guided the development of chronotherapy schedules with 5-fluorouracil, leucovorin, and oxaliplatin in human colorectal cancer. The chronomodulated chemotherapy regimens have produced the highest tumor response rates and the longest survival reported in multicenter randomized trials for this disease (8–10). The chronopharmacological changes result from the circadian rhythms that modulate cellular proliferation and metabolism (11).

Mammalian circadian rhythms are generated by a set of specific genes that are expressed in most if not all normal cells (12, 13). They are coordinated by the suprachiasmatic nucleus, a central clock located in the hypothalamus (14).

The tolerability of docetaxel and that of doxorubicin varied as a function of dosing time along the 24 h time scale in C57BL/6 × DBA/2 F₁ (B6D2F₁) mice (15, 16). In particular, the highest docetaxel tolerability was found during the rest phase of these animals, in coincidence with best antitumor efficacy against transplanted PO3 pancreatic adenocarcinoma (16). The antitumor activity of the docetaxel-doxorubicin combination was further studied in B6D2F₁ mice with P388 leukemia as a function of dosing time and interval between both agents. The combination was more effective than any single agent in this model. Survival time was increased if administered simultaneously (*i.e.*, docetaxel injected 1 min before doxorubicin), rather than 12 or 24 h earlier. This combination schedule was significantly more effective if it was administered in the beginning of the rest span (17).

Here, we have investigated the circadian rhythm in the tolerability and the efficacy of docetaxel and doxorubicin, in mice bearing a syngeneic mammary cancer model (MA13/C), as a prerequisite for the development of chronotherapy schedules with these drugs in human breast cancer.

MATERIALS AND METHODS

Animals

Male C3H/HeN mice, 5 weeks old (Charles River, St. Aubin les Elbeuf, France), were housed three per cage in an autonomous chronobiological animal facility (ESI-FluFrance, Arcueil, France). The facility has six compartments each with its own programmable lighting regimen, control of temperature, and

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soundproofing. All mice were supplied food and water *ad libitum*, and they were synchronized with an alternation of 12 h of light and 12 h of darkness, before and during each experiment. Synchronization was checked by the assessment of a normal circadian variation in the rectal temperature measured before treatment initiation.

Drugs

Doxorubicin (Adriablastine; Pharmacia & Upjohn S.A., St. Quentin-Yvelines, France) was dissolved in sterile water; the final solutions were obtained by adding 0.9% NaCl.

Docetaxel (Taxotere) was provided by Aventis Pharma S.A. (Vitry sur Seine, France). It was dissolved in ethanol; polysorbate 80 and 5% glucose in water were added to obtain concentrations of 5:5:90 (v/v/v). The final dilutions (pH \approx 5) were maintained on ice until their use within 20 min after preparation.

Drug injections were made at one of six circadian times expressed in HALO³: 3, 7, and 11 HALO during the light span and 15, 19, and 23 HALO during darkness. Each drug dose was injected i.v. once a week for 3 weeks into the retroorbital venous sinus (10 ml/kg of body weight).

Tumor Model

Mammary MA13/C is a grade III adenocarcinoma from mouse origin (18), which was obtained from Aventis Pharma S.A. The tumors were dissected, and 4 \times 4-mm tumor fragments were prepared. A tumor fragment was implanted s.c. into both flanks of each mouse using a 12-gauge trocar. Two perpendicular diameters (mm) of each tumor were measured with a caliper three times weekly. Tumor weight (mg) was computed as: tumor weight = (length \times width²)/2.

Mice with tumor weight reaching 2000 mg were sacrificed for ethical reasons, and they were considered as dead from tumor progression on this day.

Toxic deaths were attributed to drug treatment in the mice that died after body weight loss >10% and tumor size <100 mg at time of death.

Electron Microscopy of the Heart

Heart ultrastructure was studied in non-tumor-bearing mice receiving the highest doxorubicin dose (13.8 mg/kg/injection) because delayed mortality was observed after administration of this dose level.

Two untreated controls and four treated mice were killed by cervical dislocation 17 days after the first of three weekly injections of vehicle or doxorubicin, respectively. Two of the treated mice displayed ascites.

The heart was rapidly removed, and the ventricular cross (apex) was immediately cut into 1–2 mm³ blocks and frozen in a 4% paraformaldehyde-1% glutaraldehyde fixative. The blocks were postfixed in 2% osmium tetroxide, dehydrated in graded ethanol, and embedded into epoxy resin. Thin sections were cut at 84 nm and examined with a Jeol 100C electron microscope.

Study Designs

Three experiments were performed in a total of 887 mice.

Experiment 1. Each drug tolerability was investigated after three weekly administrations at each dosing times (3, 7, 11, 15, 19, or 23 HALO). Four dose levels were tested for each agent: docetaxel (38.8, 23.3, 14, or 8.4 mg/kg/injection) and doxorubicin (13.8, 8.3, 5, or 3 mg/kg/injection). Each dose and time group consisted of eight mice. The endpoints, body weight change and survival, were recorded daily for 60 days.

Pilot Experiment. The antitumor efficacy of docetaxel was evaluated on MA13/C-bearing mice treated on days 10, 17, and 24 post-tumor implantation. Three doses of docetaxel were tested: 38.8, 23.3, and 14 mg/kg/injection. Body weights were followed daily until recovery and then two to three times a week. Tumor measurements were performed twice a week. Mortality was recorded daily.

Experiment 2. The antitumor efficacy of single agent docetaxel or doxorubicin was investigated as a function of circadian time of administration in mice bearing mammary adenocarcinoma MA13/C. The tumor was implanted s.c. in 281 C3H/HeN mice.

A pilot experiment led us to select two doses of docetaxel: 38.8 and 23.3

mg/kg/injection. The two doses of 8.3 and 5 mg/kg/injection for doxorubicin were selected based on previous experiments (7). Each drug was injected at one of six dosing times, once a week for 3 weeks. The first injection was performed 9 days after tumor transplantation, when tumors were palpable (<63 mg). Mouse tumor and body weight were measured three times a week, and mortality was recorded daily for 60 days.

Experiment 3. The circadian dependency of the antitumor efficacy of docetaxel-doxorubicin combination was studied. Docetaxel and doxorubicin were combined to achieve high antitumor activity without significant toxicity and to investigate whether docetaxel-doxorubicin efficacy varied as a function of circadian dosing time.

C3H/HeN mice ($n = 150$) were randomized to receive either combination: A (docetaxel, 16.3 mg/kg/injection and doxorubicin 2.5 mg/kg/injection) or combination B (docetaxel, 11.6 mg/kg/injection and doxorubicin 3.5 mg/kg/injection).

The respective drug doses in each combination were selected according to the effects of each drug given as a single agent; 70% of the dose of 23.3 mg/kg/injection of docetaxel and 50% of the dose of 5 mg/kg/injection of doxorubicin were chosen for combination A, and the reverse proportion for combination B, so as to deliver 120% of the total drug dose equivalent given for each single agent.

Mice allocated to each combination regimen were randomized to be treated at one of six circadian times. Treatment consisted of the administration of docetaxel, followed 1 min later by that of doxorubicin. Chemotherapy was given once a week for 3 weeks. Body weight and tumor size were measured three times a week, and mortality was recorded daily for 70 days.

Statistical Analyses

For each studied variable, mean and SEM were calculated. Differences between groups were analyzed by one or two way ANOVA. Differences in the rates of complete tumor inhibitions or survivors were validated by χ^2 test. Survival curves were drawn according to Kaplan-Meier and were compared with log rank test.

RESULTS

Docetaxel Tolerability. The overall docetaxel-induced mortality ranged from 4% with 38.8 mg/kg/injection to 2% with 23.3 mg/kg/injection. No toxic death was recorded after the administration of 14 or 8.4 mg/kg/injection.

The dose dependency of tolerability of docetaxel was obvious on examination of body weight changes. Maximum body weight loss occurred 9 days after last injection, *i.e.*, 11 and 5% for the 38.8- and 23.3-mg/kg/injection dosages, respectively. Body weight returned to pretreatment values 27 and 15 days after the last injection of these respective doses. Mice treated with 14 or 8.4 mg/kg/injection did not lose any weight throughout the whole experiment.

Irrespective of dose level, mean body weight loss was 3-fold as large in the mice treated near the middle of the darkness span (19 HALO) as compared with those treated near the middle of the light span (7 HALO) (Fig. 1).

Doxorubicin Tolerability. The overall lethal toxicity of doxorubicin ranged from 81% with 13.8 mg/kg/injection to 0% with 3 mg/kg/injection. Deaths began 1 week after the first injection. Doses of 8.3 and 5 mg/kg/injection caused 19 and 25% mortality, respectively, with overlapping 95% confidence intervals (8–30% and 13–37%).

Mean body weight loss was largest 7 days post-last treatment. On this day, the mice receiving 13.8 or 8.3 mg/kg/injection lost, respectively, 16 and 9% of their pretreatment weight and did not recover at the end of the experiment. The average body weight loss of the mice treated with 5 mg/kg/injection was 0.4%. Mice receiving 3 mg/kg/injection continued to gain weight despite treatment, although less than controls.

A large variation in doxorubicin toxicity was observed as a function

³ The abbreviations used are: HALO, hours after light onset; HNTD, highest nontoxic dose.

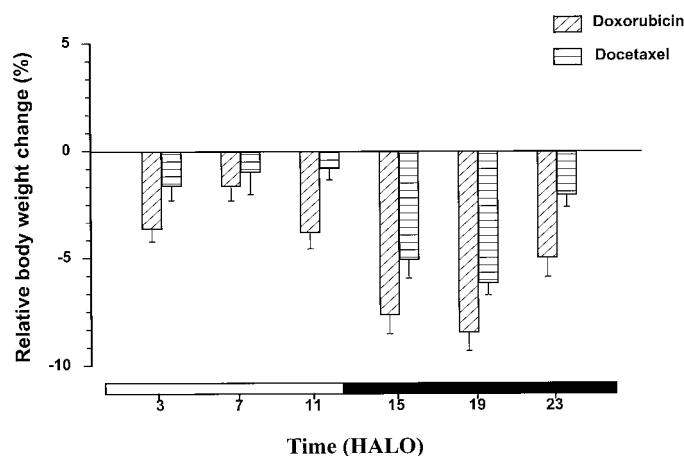


Fig. 1. Histogram of body weight change at nadir as a function of docetaxel or doxorubicin dosing time in C3H/HeN mice. Mice received three weekly administrations of docetaxel (38.8, 23.3, 14, or 8.4 mg/kg/injection) or doxorubicin (13.8, 8.3, 5, or 3 mg/kg/injection) at one of six dosing times expressed as HALO. Differences were statistically validated with two way ANOVA for both docetaxel ($F_{\text{dose}} = 584$, $P < 0.001$; $F_{\text{time}} = 93$, $P < 0.001$) and doxorubicin ($F_{\text{dose}} = 1292$, $P < 0.001$; $F_{\text{time}} = 95$, $P < 0.001$).

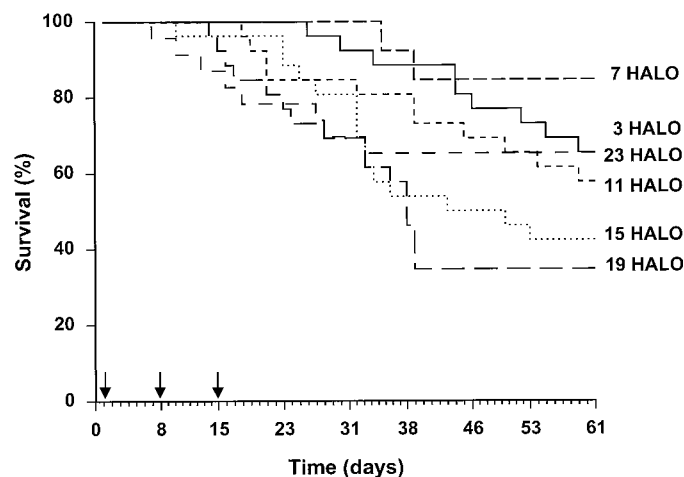


Fig. 2. Survival curves of healthy C3H/HeN mice as a function of circadian time of three weekly doxorubicin injections (\downarrow). Pooled data from four dose levels (13.8, 8.3, 5 or 3 mg/kg/injection) administered on days 1, 8, and 15 at one of six dosing times expressed in HALO. (P from log rank, < 0.001).

of dosing time, irrespective of dose level. Thus survival rate ranged between 85% at 7 HALO and 35% at 19 HALO ($\chi^2 17.2$, $P < 0.01$). Doxorubicin dosing at 7 HALO produced the best survival curve throughout the study span (P from log rank < 0.001) (Fig. 2).

Body weight loss at nadir was least in the mice receiving doxorubicin at 7 HALO, near the middle of the light span and largest in those mice dosed with doxorubicin at 19 HALO, near the middle of the dark span (Fig. 1).

Electron Microscopy of the Heart. Myocardial cells from all four doxorubicin-treated mice displayed occasional edema with many membrane fragments, moderate alterations of mitochondrial membranes, and hydropic changes around the nucleus (Fig. 3). The cell junction layers were occasionally separated by $1 \times 2\text{-}\mu\text{m}$ conical spaces containing membrane fragments. The periodic structure of the myofibrils disappeared in some myocardial cells from a doxorubicin-treated mouse with clinical ascites.

Efficacy of Docetaxel as Single Agent against MA13/C. In the pilot experiment (Table 1), the highest dose of docetaxel (38.8 mg/kg/injection) produced a maximum 24.2% body weight loss occurring 7

days post-last treatment. The HNTD of 23.3 mg/kg/injection produced a 9.1% body weight loss at nadir on day 31. This HNTD was highly active (three of five tumor-free survivors 161 days post-tumor implantation). The dosage of 14 mg/kg/injection maintained a good level of efficacy with a 3.3-log cell kill.

In experiment 2, 30 of the 52 mice receiving 38.8 mg/kg/injection (58%) and 22 of the 52 mice treated with 23.3 mg/kg/injection (42%; $\chi^2 2.5$, $P = 0.12$) were tumor-free survivors on day 60 post-tumor implantation. Large circadian time-related differences were found in the rate of mice achieving a persisting complete tumor inhibition irrespective of dose level. This was the case for 81% of the mice treated at 7 HALO as compared with 11% of those receiving docetaxel at 3 HALO ($\chi^2 21.2$, $P < 0.001$).

All of the untreated tumor-bearing mice died from tumor progression between 21 and 41 days after tumor inoculation; 36.5% of the mice receiving 38.8 mg/kg/injection of docetaxel died from lethal toxicity. Docetaxel improved survival at 23.3 mg/kg/injection; the day 60 survival rate was 69%. At this dose level, all of the recorded deaths were related to tumor progression.

Maximum body weight loss (17 days post-last injection) was 13.8% in the mice receiving 38.8 mg/kg/injection and 6% in those treated with 23.3 mg/kg/injection. Complete recovery of body weight was achieved 31 and 23 days after last injection for each respective dose.

Survival curves differed significantly as a function of the circadian stage of docetaxel administration at both dose levels (log rank = 15.7; $P = 0.008$). The survival rate on day 60 was 28% after treatment at 3 HALO as compared with 100% after treatment at 7 HALO (Fig. 4).

Efficacy of Doxorubicin as a Single Agent against MA13/C. Two dose levels were evaluated: 8.3 mg/kg/injection and 5 mg/kg/injection. At the highest dose, complete tumor inhibition was ob-

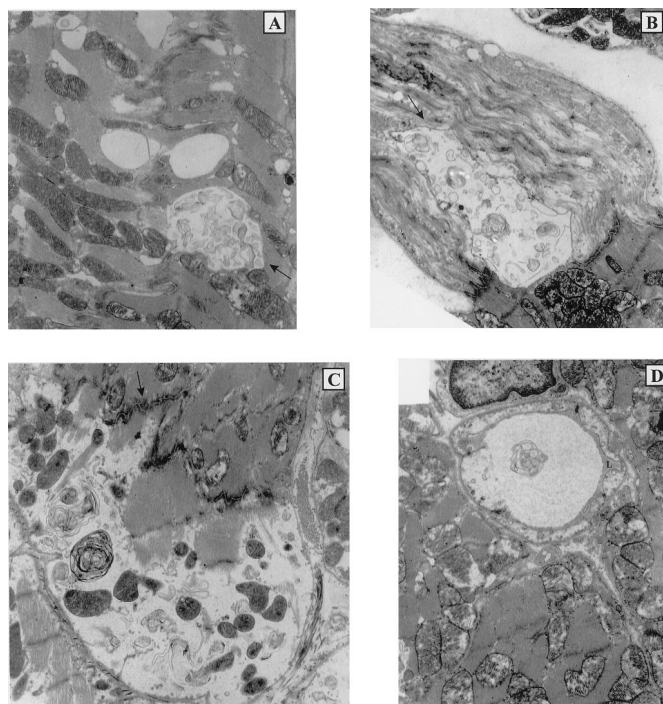


Fig. 3. Electron microscopy study of heart of non-tumor-bearing mice receiving 13.8 mg/kg/injection of doxorubicin. The main observed lesions (arrows) were: A and B, a conical localized separation along the cell junction layers of some intercalated discs, of moderate (A) or severe (B) intensity. In A, the periodic striations of myofibrils around the separation site did not show any alterations. In B, the mitochondria and the muscular myofibrils disappeared around a localized separation, whereas the endoplasmic reticulum remained normal. C, loss of myofibrils in a light lesion with edema; D, reduction of the lumen (L) in some capillaries related to the edema in some endothelial cells. Original magnifications: A and D, $\times 6600$; B and C, $\times 5000$.

Table 1 Evaluation of docetaxel against mammary adenocarcinoma MA13/C on female C3H/HeN mice

Agent ^a	Dose (mg/kg/injection)	Total dose (mg/kg)	Drug death	Av. bwl. ^b / mouse ^c (day of nadir)	Median tumor wt (mg on day 24)	% T/C day 24	Time for median tumor to reach 1000 mg	T - C (days)	Log cell kill	Tumor-free survivors day 161	Comments
Docetaxel	38.8	116.4	0/5	-24.2 (31) ^c	NTBA	NTBA	NTBA	NTBA	NTBA	NTBA	Toxic, 24% bwl HNTD, highly active Highly active
	23.3	69.9	0/5	-9.1 (31)	0 (0-0) ^d	0				3/5	
	14	42	0/5	-1.9 (31)	0 (0-71)	0	49	25.9	3.3	0/5	
Control					1250 (308-2217)		23.1			0/10	

^a Schedule: i.v. injection on days 10, 17, and 24 post-tumor implantation.

^b bwl, body weight loss (bwl $\geq 20\%$, toxic); T/C, tumor growth inhibition, determined on day 24, of control animals $\times 100$ (T/C $\leq 42\%$, active; T/C $< 10\%$ highly active); T - C, tumor growth delay, median days required for the treated and the control group to reach 1000 mg, tumor-free survivors excluded from these calculations; log cell kill, $(T - C)/3.32 \times T_d$; T_d , tumor-doubling time (2.4 days); NTBA, non-tumor-bearing animals.

^c Mouse average weight, 22.12 g.

^d Numbers in parentheses, range.

served in 50 of 52 mice (96%). Two mice of 52 died from toxicity. The average body weight loss was 11.5% at nadir, 15 days post-last treatment, without complete recovery throughout the whole treatment span.

At the 5 mg/kg/injection dosage, 62% of the animals were alive 60 days after tumor inoculation, 8 of 50 mice with complete tumor inhibition. There was no weight loss during the entire experiment and no drug-related death. When mortality occurred, it was related to tumor progression.

Regardless of dose level, the rate of day 60 complete tumor inhibition was highest at 7 HALO (69%) and lowest at 11 HALO (44%). At the lowest dose level (5 mg/kg/injection), the rate of complete tumor inhibition was 50% at 7 HALO and ranged between 0 and 22% at the other dosing times. This difference was close to statistical significance ($P = 0.06$).

The survival curves did not differ with statistical significance (log rank, 5.6, $P = 0.35$). Nevertheless, 75% of the mice receiving 5 mg/kg/injection of doxorubicin in the first half of the light span were alive on day 60 as compared with 28% of the animals treated at 15 HALO, shortly after the beginning of darkness.

Antitumor Activity of Nontoxic Single Agent Chronotherapy against MA13/C Tumor. We further examined the efficacy of single agent docetaxel (23.3 mg/kg/injection) or doxorubicin (5 mg/kg/injection) as a function of dosing time because these dose levels produced no lethal toxicity and low body weight loss.

Either agent produced moderate yet transient antitumor activity at these dose levels. Tumor growth was significantly inhibited or delayed as compared with untreated tumor-bearing mice.

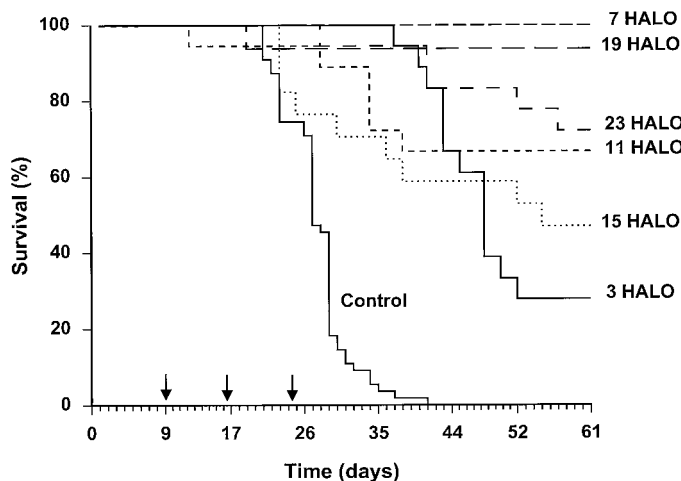


Fig. 4. Survival curves of MA13/C tumor-bearing mice receiving three weekly docetaxel injections (pooled data from two dose levels, 38.8 or 23.3 mg/kg/injection) at one of six dosing times expressed as HALO. Injections (\downarrow) were performed on days 9, 16, and 23 after tumor inoculation. (P from log rank = 0.0076).

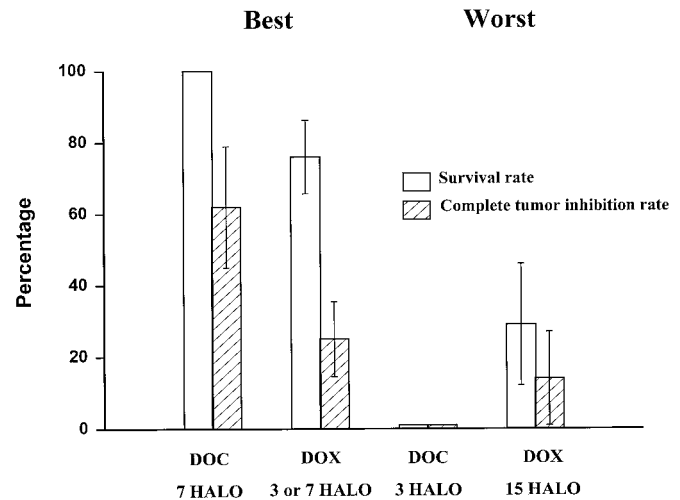


Fig. 5. Best and worst efficacy of single agent docetaxel or doxorubicin at the nontoxic dose (23.3 and 5 mg/kg/injection) expressed in rates (\pm SEM) of complete tumor inhibition and survival on day 60.

Docetaxel produced tumor growth delay in 49 of 52 mice, but tumor grew back 12 days after the third injection. A complete tumor inhibition was achieved in 42% of the mice (22 of 52).

Doxorubicin inhibited tumor growth for 24 days after treatment onset. Tumor weight increased quickly thereafter. Only 16% of the mice (8 of 50) were tumor-free survivors on day 60.

The antitumor activity of these nontoxic dose levels of docetaxel and doxorubicin varied significantly as a function of circadian dosing time. The administration of either agent at 7 HALO produced the longer tumor growth inhibition (Fig. 5, P from ANOVA, < 0.05). The rate of complete tumor inhibitions was 50% greater after administration of docetaxel or doxorubicin at 7 HALO than after 3 or 15 HALO, respectively (df 5; P from $\chi^2 < 0.01$ and $P = 0.06$, respectively). These differences translated into significant survival advantage for those mice receiving docetaxel at 7 HALO (100% day 60 survival rate) as compared with other dosing times with no day 60 survivor in the mice treated at 3 HALO (df 5, P from $\chi^2 < 0.001$). A similar trend was found for doxorubicin: 75% of the mice treated in the first half of the dark period (3-7 HALO) were alive on day 60, as compared with 28% of those injected at 15 HALO. The difference, however, was not statistically significant (df 5; P from $\chi^2 = 0.41$), possibly as a result of the limited number of mice per time point and dose level.

Efficacy of Docetaxel-Doxorubicin Combinations. The efficacy of combination A was higher than that of combination B. A complete persistent tumor inhibition on day 60 was achieved in 45% of the mice treated with combination A and in 20% of those treated with the B one ($\chi^2 = 8.6$, $P < 0.01$).

The average tolerability of both combinations was similar. No

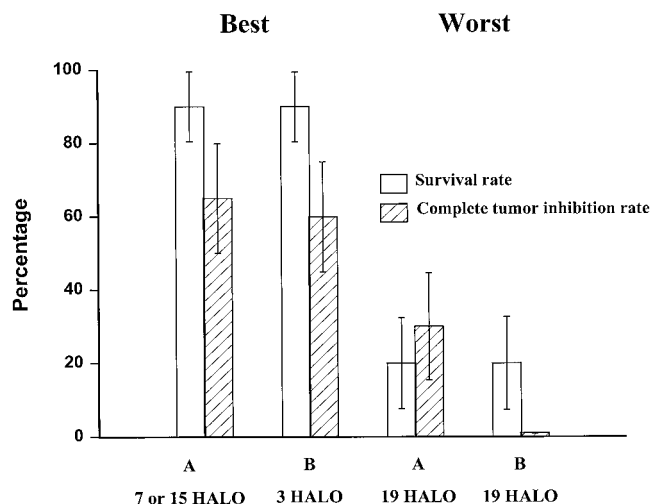


Fig. 6. Best and worst efficacy of docetaxel-doxorubicin combinations, expressed in rates (\pm SEM) of complete tumor inhibition and survival on day 60.

statistically significant difference in lethal toxicity was found between combination A (15% of the mice) and combination B (6.6%; $\chi^2 = 2.2$, $P = 0.15$). The nadir in mean body weight loss was found 13 days after treatment and reached 20.5% for combination A and 20.3% for combination B. A complete recovery of initial body weight was achieved 32 days after completion of treatment with combination A and 34 days after that of combination B.

Large differences according to dosing time of the docetaxel-doxorubicin combination were found with regard to tolerability and antitumor efficacy. The rate of complete tumor inhibitions on day 60 was 2-fold as high after the administration of combination A at 7 or 15 HALO than at 19 HALO. Conversely, combination B was up to 6-fold as active at 3 HALO as at 19 HALO (Fig. 6).

Both combinations were more toxic (10 of 60 toxic deaths, 16.6%) in the mice treated during darkness than in those receiving docetaxel-doxorubicin during the light span (3 of 60, 5%; $\chi^2 = 4.2$, $P < 0.05$). The least body weight loss and the fastest recovery was found in the mice treated at 3 HALO (P from ANOVA, <0.01).

Both drug combinations prolonged the life span of MA13/C-bearing mice as compared with controls. The day 70 survival rate was 71.6% in the mice receiving combination A and 65% in those treated with combination B, without any statistically significant difference between them ($\chi^2 0.6$, $P = 0.45$). The day 70 survival rate was 20% in the mice treated with either combination at 19 HALO as compared with 85% in those treated at 3 HALO (Fig. 7). Such improvement resulted from an overall better tolerability and antitumor activity of docetaxel-doxorubicin during the light span.

DISCUSSION

The tolerability of docetaxel and that of doxorubicin in C3H/HeN mice were several fold larger following dosing during the light span as compared with darkness. These circadian stages also corresponded to highest efficacy of these drugs against transplanted MA13/C mammary adenocarcinoma in the C3H/HeN strain. Similar results have been obtained for docetaxel in B6D2F₁ mice with PO3 pancreatic adenocarcinoma and for doxorubicin in rats with mammary cancer or plasmacytoma (16, 19). Although B6D2F₁ and C3H/HeN mice are nocturnally active, their pattern of melatonin secretion differs greatly. The average melatonin plasma concentration is ~ 10 times as high in C3H/HeN as in B6D2F₁ mice, and the peak melatonin level occurs

during darkness in C3H/HeN mice and during light in B6D2F₁ mice (20, 21).

Taken together, the results support that both host and tumor cytotoxicity mechanisms remained coupled with the rest-activity circadian cycle. Thus, the middle of the rest span of C3H/HeN mice (7 HALO) was found to be the time when single agent doxorubicin or docetaxel were both best tolerated and more effective against MA13/C mammary adenocarcinoma.

The larger the dose within the range tested, the larger was the rate of complete tumor inhibitions induced by each single agent. Nevertheless, the occurrence of lethal toxicity following the highest doses of docetaxel (38.8 mg/kg/injection) and doxorubicin (8.3 mg/kg/injection) prevented the use of these dose levels for therapeutic purposes. Furthermore, myocardial lesions were demonstrated with electron microscopy after the highest doxorubicin dose (13.8 mg/kg/injection). They were associated with clinical signs of cardiac failure, including ascites. Such lesions most likely accounted for the delayed mortality, which was observed after high dose doxorubicin.

The rate of complete tumor inhibitions displayed large variations as a function of both dose and dosing time for each single drug. Indeed, each dose level of docetaxel was more than 5-fold as active at 7 HALO as at 3 HALO. A similar difference was found for the nontoxic dose of doxorubicin, with 7 HALO being also the most effective dosing time.

Docetaxel and doxorubicin were combined with the aim of improving treatment efficacy while reducing the risk of toxicity. Two com-

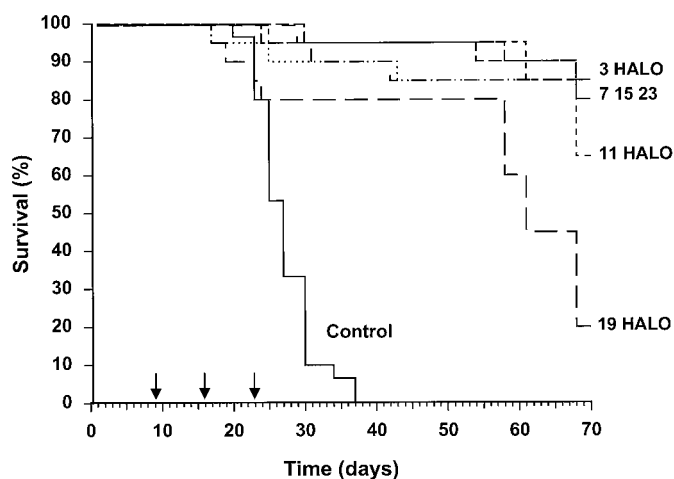


Fig. 7. Survival curves of MA13/C tumor-bearing mice receiving either docetaxel-doxorubicin combination A (16.3 mg/kg/injection + 2.5 mg/kg/injection) or B (11.6 mg/kg/injection + 3.5 mg/kg/injection) every 7 days for 3 consecutive weeks (\downarrow), starting 9 days after tumor inoculation (pooled data from both combinations). Each combination was administered at one of six dosing times expressed as HALO. P from log rank, <0.005).

Table 2. Summary of best treatment options with regard to drug, dose level, and circadian time of administration

Agent	Dose (mg/kg for 3 injections)	Best time (HALO)	Survival rate (%)
Docetaxel	38.8	7 and 19	100
	23.3	7	100
Doxorubicin	8.3	All times	93
	5	7	75
Combinations	A ^a	7 and 15	90
	B	3	90

^a A, docetaxel 16.3 mg/kg/injection + doxorubicin 2.5 mg/kg/injection; B, docetaxel 11.6 mg/kg/injection + doxorubicin 3.5 mg/kg/injection.

binations were tested with a different relative proportion of both drugs. A target dose of 120% of each single agent dose was chosen.

The tolerability of both combinations was similar with regard to toxic deaths and body weight loss. Nevertheless, they produced 11% of toxic deaths, a percentage nearly twice as high as that resulting from single agent therapy. In addition, such lethal toxicity was 3 fold as large in the mice receiving either combination during darkness as compared with the light span.

Despite the overall rate of complete tumor inhibitions was larger with combination A than with combination B (45 and 20%, respectively), the overall efficacy of docetaxel-doxorubicin was lower than that achieved with the highest dose tested for each single agent (docetaxel, 58%; doxorubicin, 96%).

The tolerability of the most effective combination (A) was worse than that of single agent docetaxel (23.3 mg/kg/injection). Yet both the rate of complete tumor inhibition (42% *versus* 45%) and that of survival on day 60 (70% *versus* 75%) were similar. Single agent doxorubicin (5 mg/kg/injection) was better tolerated but less effective than combination A (45% *versus* 15% of complete regressions). Nevertheless, the survival rates on day 60 were similar (75% and 62%). Thus, the most active combination did not improve the survival outcome of mice as compared with docetaxel or doxorubicin given as single agents at a nontoxic dose.

A marked circadian time dependency further characterized the tolerability and efficacy of both combinations. The highest rate of complete tumor inhibitions was achieved in the mice receiving combination A in the beginning of the activity span or in the beginning of the rest span in the mice treated with combination B. The poorest efficacy of either combination was obtained in the middle of the activity phase.

Even at the lowest dose levels of single agent docetaxel or doxorubicin, the efficacy was increased by taking into account the time of administration without any increase of toxicity. Table 2 summarizes the treatments that achieved the best survival rates. Single agent docetaxel at 7 HALO appeared as both optimally effective and safer than as any other tested treatment option, including any of the tested docetaxel-doxorubicin combinations. Although the survival from the highest dose of doxorubicin was similar to that produced by docetaxel at 7 HALO, this dose level was responsible for >10% body weight loss. Furthermore, the effect of the dose of 13.8 mg/kg/injection on the heart ultrastructure suggests that the cardiotoxicity from a lower dose could occur later, especially if additional injections were given.

In conclusion, the circadian dosing time of single agent docetaxel or doxorubicin or their combination profoundly influenced tolerability and antitumor efficacy in mice with MA13/C mammary adenocarcinoma. Each single agent produced the best results after administration near the middle of the rest span. Furthermore, single agent delivery at its optimal dosing time displayed better tolerability and efficacy than both tested docetaxel-doxorubicin combinations. Although these results do not rule out a favorable therapeutic index of other drug sequences or intervals in this model, they support the investigation of the clinical relevance of chronotherapy of human breast cancer. The therapeutic index of single agent docetaxel or doxorubicin would be expected to be improved after their administration at night in cancer patients.

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