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Doxorubicin-induced cardiotoxicity monitored by ECG in freely moving mice

A new model to test potential protectors

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Abstract In laboratory animals, histology is most commonly used to study doxorubicin-induced cardiotoxicity. However, for monitoring during treatment, large numbers of animals are needed. Recently we developed a new method to measure ECG values in freely moving mice by telemetry. With this model we investigated the effect of chronic doxorubicin administration on the ECG of freely moving BALB/c mice and the efficacy of ICRF-187 as a protective agent. The ST interval significantly widened from 15.0 ± 1.5 to 56.8 ± 11.8 ms in week 10 (7 weekly doses of 4 mg/kg doxorubicin given i.v. plus 3 weeks of observation). The ECG of the control animals did not change during the entire study. After sacrifice the hearts of doxorubicin-treated animals were enlarged and the atria were hypertrophic. As this schedule exerted more toxicity than needed to investigate protective agents, the protection of ICRF-187 was determined using a dose schedule with lower general toxicity (6 weekly doses of 4 mg/kg doxorubicin given i.v. plus 2 weeks of observation). On this schedule, the animals' hearts appeared normal after sacrifice and ICRF-187 (50 mg/kg given i.p. 1 h before doxorubicin) provided almost full protection. These data were confirmed by histology. The results indicate that this new model is very sensitive and enables monitoring of the development of cardiotoxicity with time. These findings result in a model that allows the testing of protectors against doxorubicin-induced cardiotoxicity as demonstrated by the protection provided by ICRF-187.

Key words Telemetry · ECG · Cardiotoxicity · Doxorubicin · Mice

Abbreviations *ECG* Electrocardiogram · *bpm* beats per minute

Introduction

Doxorubicin is a very effective antitumor agent that is used against a variety of human malignancies. The major acute toxicity is bone marrow suppression, but the long-term clinical usefulness is limited by dose-dependent irreversible chronic cardiotoxicity, which manifests as congestive heart failure. In patients, doxorubicin-induced cardiac damage is monitored by measurement of the left ventricular ejection fraction and/or by endomyocardial biopsy, of which the latter is the most reliable but has the disadvantage of being an invasive method [7].

In laboratory animals that are used to study doxorubicin-induced cardiotoxicity, histology is most commonly used to determine the extent of myocardial damage because doxorubicin, as all other anthracyclines, causes very specific changes in heart tissue [2]. In addition, biochemical and functional parameters are used to record cardiotoxicity [5, 14, 15, 17].

Another approach to monitor doxorubicin-induced cardiotoxicity in both humans and laboratory animals is by means of the electrocardiogram (ECG).

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Significant changes in the ECG, such as widening of the QRS complex and bradycardia [21] as well as ST-segment widening and T-wave flattening [6], have been reported for chronic cardiotoxicity in vivo in laboratory animals, whereas the QT interval has been found to be shorter after the addition of doxorubicin to perfusate of isolated Langendorff-perfused guinea pig hearts [18]. The severity of the ECG changes correlate with the known cardiotoxicity of several anthracyclines [6, 21].

In laboratory animals a serious limitation of the value of ECG analysis is that ECGs can be measured only in animals placed under anesthesia, which might interfere with the experiment, or under severe stress caused by restraint of the animals and the insertion of needles, which introduces stress artefacts and animal-management problems [4]. Furthermore, it is difficult to measure the ECG in small laboratory animals such as mice; therefore, these studies are usually performed in rats. However, it is known that rats develop a nephrotic syndrome after receiving doxorubicin, which may influence the severity of the cardiotoxicity [20]. Mice, as well as humans, do not readily develop a nephrotic syndrome, which makes the mouse the species of choice in doxorubicin-induced chronic cardiotoxicity studies [20].

With the telemetry system, which we recently developed for mice, it is now possible to measure the ECG, heart rate, and temperature in freely moving mice [11]. The aim of the present investigation was to determine whether the ECG and heart rate measured by telemetry in freely moving BALB/c mice can serve as a model to study doxorubicin-induced cardiotoxicity and to test possible cardioprotectors. The new telemetry model was evaluated using the clinically successful cardioprotector ICRF-187 and by comparison of the ECG parameters with the established method of histological evaluation.

Materials and methods

Animals

A total of 20 male BALB/c mice (20–25 g) obtained from Harlan Olac CPB (Zeist, The Netherlands) were kept in a light- and temperature-controlled room (21–22° C, humidity 60–65%). The animals were fed a standard diet (Hope Farms, Woerden, The Netherlands) and were allowed tap water ad libitum. Animals were kept in quarantine for at least 1 week before surgery.

Telemetry system

The telemetry system, which consisted of implantable transmitters (TA10ETA-F20), a telemetry receiver (RA1010), and an analog ECG adapter (Option R08), was obtained from Data Sciences (DSI, St. Paul, Minn., USA). The data-acquisition system consisted of a MacLab (ML020 MacLab/8, ADInstruments Ltd, London, England), which was connected to an Apple Macintosh LCII 4/80 computer with the program Chart from MacLab. The transmitter was activated by a magnet, after which the output of the transmitter (frequency in hertz) was received by an antenna mounted in a receiver board placed under the animal's cage. This board was connected to the data-acquisition system.

Surgery

The mice were anesthetized i.p. with 0.07 ml/10 g of a mixture of fentanyl and fluanisone (Hypnorm, 0.2 mg/ml), midazolam (Dormicum, 5 mg/ml), and sterilized water at a volume ratio of 1:1:2. Surgery was performed as described in detail by Kramer et al. [11]. In short, the transmitter was implanted in the peritoneal cavity of each mouse at least 2 weeks before the start of the treatment. The leads of the transmitter were sutured s.c. in the lead II position, the negative lead being placed at the right shoulder and the positive lead, toward the lower left chest.

Experimental setup

After surgery the mice were allowed to recover for 2 weeks, after which they were subjected to administration of one of the following dose schedules.

Schedule I

Two mice served as control animals and received 0.05 ml saline i.v. once per week for 7 weeks, and four mice received 4 mg/kg doxorubicin (Adriblastina, 2 mg/ml, Free University Hospital) i.v. once per week for 7 weeks, rendering a cumulative dose of 28 mg/kg.

Schedule II

As schedule I exerted too much toxicity, the dose schedule of doxorubicin was adjusted as follows to investigate the protection of ICRF-187:

1. Doxorubicin ($n = 5$): 0.1 ml saline was given i.p. 1 h before i.v. administration of 4 mg/kg doxorubicin once per week for 6 weeks.
2. ICRF-187 ($n = 5$): 50 mg/kg ICRF-187 (Cardioxane, a gift from EuroCetus Amsterdam, The Netherlands) was injected i.p. 1 h before i.v. administration of 4 mg/kg doxorubicin once per week for 6 weeks.
3. Control ($n = 4$): 0.1 ml saline was given i.p. 1 h before i.v. administration of 0.05 ml saline once per week for 6 weeks to a group of six mice, including the controls from schedule I.

After treatment the animals were observed for another 3 (schedule I) or 2 weeks (schedule II) and then sacrificed by decapitation. The hearts were removed and subjected to histological evaluation for the mice treated with schedule II; the hearts of the remaining animals were subjected to pathological evaluation.

After surgery the mice were weighed every day to follow their recovery; during treatment their weight was determined three times per week as a measure of general toxicity. The ECG and heart rate were registered after surgery before recovery from anesthesia and then three times per week in the freely moving animal (on Monday, Wednesday, and Friday) until the end of the study. To ensure that the ECG would be recorded under the same conditions throughout the study, especially with a reproducible heart rate, ECG tracings were obtained just after the animals had been placed in a different cage. This resulted in a maximal heart rate, that remained at 700–750 bpm during the entire study.

Parameters

Telemetry parameters

For interpretation of the ECG, four consecutive complexes were analyzed in detail. The PR segment, QRS complex, QT interval, and ST interval were determined as mean values ± SD of these four complexes. In case the noise caused by movement of the animal resulted in an unacceptable variation among the complexes (SD > 10%), four other complexes were selected for evaluation. The "ratemeter" from the program Chart was used for automatic calculation of the heart rate from the ECG input.

Other parameters

Changes in weight were taken as a measure of general toxicity, as were the behavior of the animal and the general impression of its condition. After sacrifice the animals were investigated for organ abnormalities due either to doxorubicin or to the transmitter.

Histological evaluation

Longitudinal sections of the hearts of mice treated with schedule II were embedded in 2-hydroxyethyl metacrylate. Sections (1 μm) were prepared and stained with Giemsa for light microscopic evaluation. The hearts of the doxorubicin-treated, doxorubicin/ICRF-187-treated, and control (saline) groups were presented to the pathologist in a completely blinded fashion. Scoring was performed according to Billingham's semiquantitative grading scale [3]. The grading was based on the number of muscle cells showing myofibrillar loss and cytoplasmic vacuolization.

Statistical evaluation

All parameters were expressed as mean values ± SEM unless stated otherwise. The differences in parameters between the groups were evaluated using analysis of variance (ANOVA), with Fisher's LSD test for multiple comparisons being applied when ANOVA indicated significant differences between groups. The histological parameters were evaluated using Kruskal-Wallis nonparametric ANOVA analysis. The program used for these analyses was NCSS (by Dr J. L. Hintze, Kaysville, Utah, USA). The level of significance chosen was 99% ($P < 0.01$) to correct for comparisons between groups every week (multiple comparisons). As two animals on schedule I died during week 9, the end point of this study with respect to the means and the statistical evaluation was week 9.

Results

Behavior/general toxicity

After surgery, recovery of the animals was indicated by an increase in weight after an initial decrease and by changes in their behavior such as building a nest from available paper towels. Animals appeared lively throughout the study, and no behavioral change was observed relative to mice without transmitters. The doxorubicin-treated animals treated on schedule I showed decreased liveliness during the last 2 weeks,

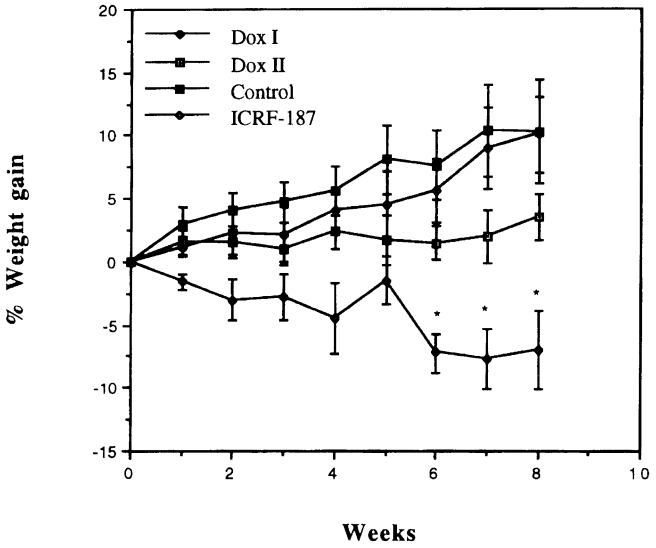

Fig. 1 Percentage of weight change per treatment group (mean values ± SEM): control ($n = 6$), doxorubicin schedule I (*Dox* I, $n = 4$), Dox II ($n = 5$), and ICRF-187 ($n = 5$). *$P < 0.005$ relative to controls

which was not encountered with schedule II. During treatment with doxorubicin the body weight slowly dropped to a mean weight loss of 10.7 ± 3.2% during week 9 of schedule I (Fig. 1). With schedule II, no significant difference in weight gain was observed between the groups (3.48 ± 1.87%, 10.02 ± 2.98%, and 10.26 ± 4.13% for the doxorubicin, ICRF-187, and control groups, respectively: Fig. 1).

Pathology

At the date of sacrifice, all doxorubicin-treated animals (schedule I) suffered from ascites and pleural effusion. None of the animals on schedule II showed any of these signs of toxicity.

In our original setup we planned to determine the remaining functionality of the atria in an organ-bath study, as this had been found to be a useful parameter for cardiotoxicity in our previous study [19]. However, in the present study the hearts of doxorubicin-treated mice (schedule I) were dilated and the atria were enlarged and hypertrophic to such an extent that they were nonfunctional (Fig. 2); therefore, no functional parameter could be measured. With schedule II the atria of all animals were functional, but no difference in functional parameters was detected between the groups, probably because the differences were too small to be detected by this sample size (data not shown).

All abdominal organs such as the kidney, liver, and intestine appeared normal in control, doxorubicin treated and doxorubicin/ICRF-187-treated mice. This indicates that the transmitter did not cause any abnormality.

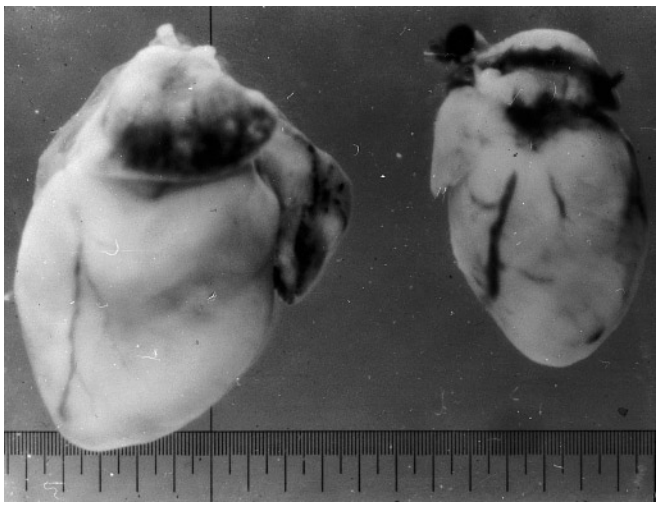

Fig. 2 Effect of doxorubicin treatment on the heart of a mouse receiving 4 mg/kg doxorubicin once per week for 7 weeks followed by 3 weeks of observation (*left*) and on the heart, of a mouse receiving saline (*right*)

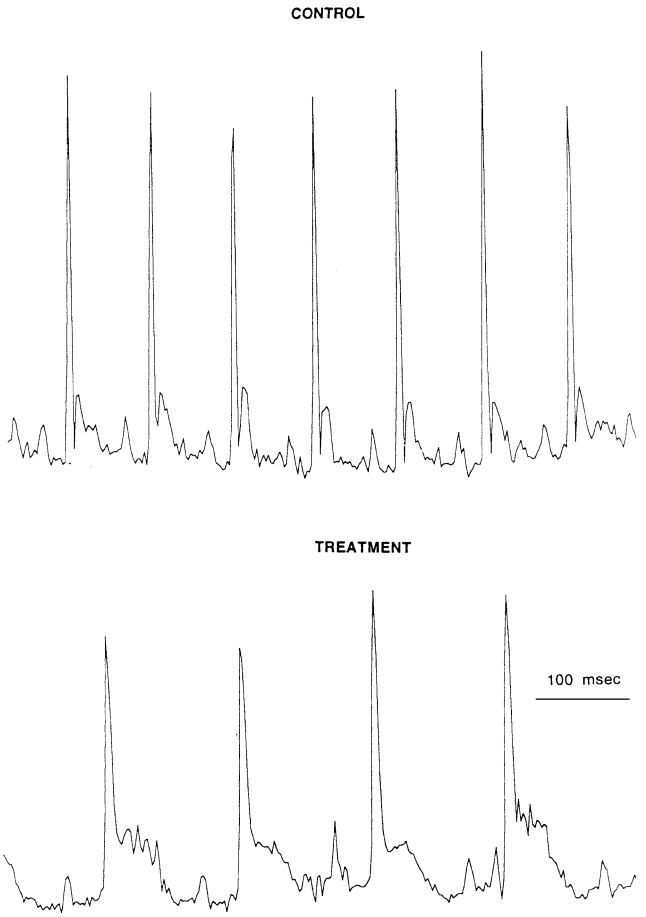

Fig. 3 Typical ECG trace recorded for a mouse before (CONTROL) and after (TREATMENT) the administration of 4 mg/kg doxorubicin once per week for 7 weeks

ECG findings

The ECG signal in the lead-II deflection of mice is somewhat different from that of humans, as has been confirmed by the conventional methods used to measure the ECG, such as the application of anesthesia or restraint [13, 16]. In mice the T-wave immediately follows the QRS complex; thus, no ST segment can be found (Fig. 3). The signal was very clear and reproducible, although severe movement of the animal led to minor and temporary changes in the lead position and, thus, in deflection. The ECGs of the control animals did not alter during the course of the study. The PR segment, QRS complex, ST interval, and QT interval remained constant and the form of the ECG did not change.

Doxorubicin had a profound influence on the shape of the ECG (figure 3). The QT and ST intervals increased with time (Fig. 4), whereas the QRS complex remained constant. No arrhythmia was seen in animals of any of the groups. The ST interval in doxorubicin-treated animals on schedule I increased from 15.0 ± 1.5 ms during week 1 to 56.8 ± 11.8 ms during week 9 (significant from week 3, $P < 0.005$). During the last 2 weeks of this study (schedule I), large differences were noted among the treated mice, causing the difference between the groups to lose statistical significance for both parameters.

With schedule II the ST interval of doxorubicin-treated animals increased by only 16.7 ± 1.8 ms during week 8. The differences relative to control values were significant ($P < 0.005$) beginning from week 3. ICRF-187 completely blocked this increase in ST interval; no difference relative to control values was seen up to week 8 (increase of only 1.8 ± 0.9 ms).

When the data obtained from the doxorubicin-treated animals on schedules I and II were combined until week 6 (until which point both schedules were equal), the widening of the ST interval was significant beginning from week 2 (doxorubicin $n = 9$, control $n = 6$, $P < 0.001$; Fig. 4B).

Heart rate

In a previous investigation using telemetry in mice [11], we found that handling of the mouse and its placement in a new cage led to a stress situation for the animal and, hence, to an increased heart rate (700–800 bpm). This heart rate could not be increased any further with the positive chronotropic drug isoprenaline. Therefore, the observed heart rate of 700–800 bpm was considered to be the maximal heart rate. When the heart rate was measured in the home cage, it was lower ($\pm$ 600 bpm), and in animals at rest (lying in their nests) it was 400–450 bpm. Differences observed not only between individual mice but also within the same animal were considerable for the heart

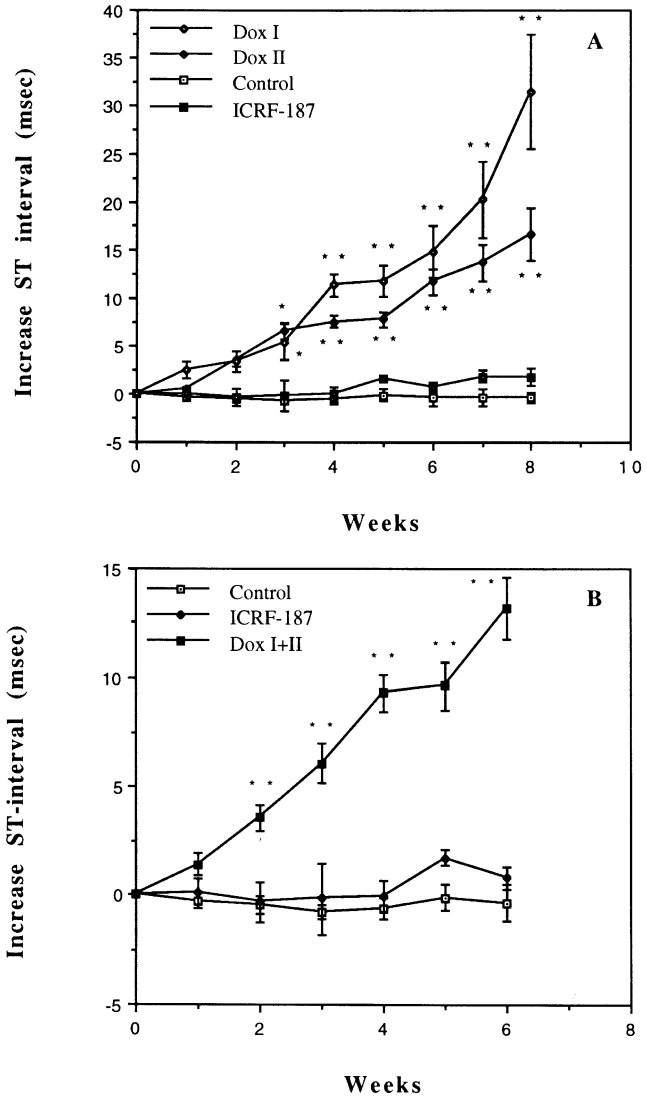

Fig. 4A, B Increase in the ST interval with time. **A** Per treatment group: control ($n = 6$), doxorubicin schedule I (*Dox I*, $n = 4$), and Dox II ($n = 5$), and ICRF-187 ($n = 5$). **B** All doxorubicin-treated mice (cumulative dose 24 mg/kg, $n = 9$) as compared with controls ($n = 6$) and ICRF-187-treated mice ($n = 5$). Data represent mean values ± SEM. $^{*}P < 0.005$, $^{**}P < 0.001$ relative to control

rate at rest; thus, these heart-rate values are considered to be of little use to indicate effects of treatment.

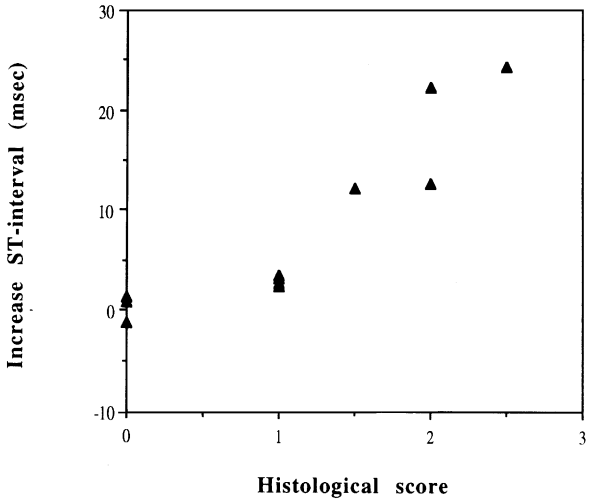

Fig. 5 Comparison of the ST interval and the histological score per individual mouse treated on schedule II

In all animals the maximal heart rate was 700–750 bpm during the entire study. Only in the doxorubicin-treated animals on schedule I did the maximal heart rate remain constant up to week 8, after which it suddenly dropped to 400–500 bpm during week 10.

Histology

The scores recorded for the myocardial lesions resulting from doxorubicin administration according to schedule II are presented in Table 1. ICRF-187 coadministration protected mice significantly against doxorubicin-induced cardiotoxicity ($P < 0.005$). The protection was not complete, as the score of the doxorubicin/ICRF-187-treated group also significantly differed from the saline control value ($P < 0.003$). The histological scores per individual mouse were found to be in agreement with the increase in ST interval (Fig. 5).

Discussion

Doxorubicin is known to cause a decrease in heart functionality in both humans and laboratory animals

Table 1 Cardiomyopathy scores recorded for mice given 4 mg/kg doxorubicin once per week for 6 weeks with or without 50 mg/kg ICRF-187

Treatment	Number of animals	Cardiomyopathy score						
		0	1	1.5	2	2.5	3	Score ≤ 1.5[a]
Doxorubicin	5	0	0	1	3	1	0	0/5
Doxorubicin/ICRF-187	5	0	5	0	0	0	0	5/5*,**
Saline Control	4	4	0	0	0	0	0	4/4

$^{*}P < 0.005$ versus doxorubicin-alone values
$^{**}P < 0.003$ versus control values
[a] Number of animals with a cardiomyopathy score of 1.5 or less/number of animals examined

[7]. In humans, changes in the ECG are too unspecific to be of diagnostic value. Although the ECG is not a functional parameter, several authors have described changes in the ECG of laboratory animals upon administration of anthracyclines. Danesi et al. [6] and Zbinden et al. [21] demonstrated for several anthracyclines that the severity of the changes in ECG paralleled the drugs' known cardiotoxicity in patients.

Therefore, we decided to follow closely the ECG changes occurring upon doxorubicin administration and to monitor the ECG 3 days per week for 10 weeks. Widening of the ST interval became significant during the 3rd week ($P < 0.005$) and continued to increase during the treatment period (Fig. 4A). Even after doxorubicin treatment had stopped, ST-interval widening continued to such an extent that one of the animals on schedule I died of heart problems. During the last few weeks of the study according to schedule I, the individual differences between treated animals became so large that the differences between the groups lost their statistical significance.

These results clearly indicate that the development of doxorubicin-induced cardiotoxicity does not stop after therapy. It is not known whether this is caused by the remaining presence of small amounts of doxorubicin or its metabolite, doxorubicinol, in the heart tissue or by a vicious circle of compensating mechanisms in an already damaged heart. Both observations, i.e., late cardiotoxicity and a large variance in toxicity, are also found in patients [7].

The widening of the ST interval, which stands for the prolongation of the repolarization phase, may be explained by the prolongation of the action potential. The action potential has been prolonged in Purkinje fibers after incubation with doxorubicin [12], and in isolated myocytes it has been found that oxygen-derived free radicals, which are generated by doxorubicin-iron complexes, can increase the duration of the action potential [9].

The heart rate did not change until the last stage of the study (schedule I, significant decrease beginning from week 9, $P < 0.05$). During this period the doxorubicin-treated animals were severely ill. Thus, a drop in the heart rate might be explained not only by a decrease in the functionality of the heart but also by a decrease in liveliness and a response to external stress. Control animals and animals treated on schedule II did not show changes in heart rate. To answer the question as to what causes the drop in heart rate, one must know the functional parameters of the atria. With the β-agonist isoprenaline, the maximal heart rate of the right atrium can be determined ex vivo in an organ-bath study. However, as described above in Results, the in vitro functionality of the atria could not be measured.

ICRF-187 is the only clinically successful cardiotoxicity modulator [10]. It is hydrolyzed intracellularly into an ethylenediaminetetracetic acid (EDTA)-like iron chelator. By chelating iron, it can inhibit the formation of the oxygen free radicals mentioned above. Therefore, ICRF-187 was used to test whether our model would be suitable for testing of potential protectors.

The cumulative dose of 28 mg/kg and the 3 weeks of observation resulted in severe cardiac toxicity and cardiomyopathy-related general toxicity. All doxorubicin-treated animals showed strong dilatation of their hearts and hypertrophic atria. Therefore, we decreased the cumulative dose for the second part of the study to 24 mg/kg and reduced the observation period to 2 weeks (schedule II). The final cardiotoxicity observed in animals treated on this schedule appeared acceptable for the animals and sufficient to establish protection as demonstrated by the significant increase in ST interval seen in the doxorubicin-treated animals and the protection provided by ICRF-187 (not significantly different from controls during the entire study; Fig. 4). This protection is confirmed by the histological data (Table 1) and is in accordance with animal studies and clinical data [8, 10]. ICRF-187 alone was not tested, as there have been numerous reports that it does not have an effect on the heart by itself [8, 15]. Moreover, we have demonstrated in an earlier study that ICRF-187 itself has no influence on the hearts of BALB/c mice [19]. Therefore, it is very unlikely that ICRF-187 would have an effect opposite to that of doxorubicin and thus "protect" by counteracting a doxorubicin-induced increase in the ST interval with a decrease in this interval.

Because histology is the most widely used method to determine doxorubicin-induced cardiotoxicity in animals, we decided to validate our new ECG parameters with this established parameter. The parameters recorded for the individual animals are in good agreement with each other. The disadvantage of histology is that it is time-consuming, labor-intensive, and results in a score at only one time point per animal. Therefore, as interindividual variation is usually quite large, it is possible to "monitor" the development of cardiotoxicity in time only by using large groups of animals for each time point. The histological data show that ICRF-187 does not fully protect against cardiotoxicity as demonstrated by a statistically significant difference from the control group ($P < 0.003$). These results are equivalent with the ST-interval data, where the curve of the ICRF-187-treated group lies slightly above the control curve.

To test the reproducibility of the method we combined the data obtained from the doxorubicin-treated and control animals for the two schedules. Until week 6, both schedules are equal and all doxorubicin-treated animals show the same widening of the ST and QT intervals. Statistical analysis performed on this combined group of doxorubicin-treated animals in comparison with the combined control group shows a highly significant increase in ST interval beginning as early as week 2 ($P < 0.001$, Fig. 4B). Power analysis of schedule II revealed that with this model we need only five

animals per group to be capable of detecting 45% protection or more as tested during week 8 only ($\alpha = 0.05$, power $= 0.90$, one-sided test). Less protection is not relevant, as screening aims only at powerful protective agents.

Several years ago, Bartoli Klugmann et al. [1] investigated the effects of acute and chronic doxorubicin administration in mice on the ECG (under anesthesia) and morphology. In contrast with our findings, they found no correlation between ECG changes and histopathological data. They therefore concluded that the ECG cannot be used in mice to monitor the progression of cardiotoxicity. This contradiction with our data might be caused by the observation that in their experiments the ECG was measured in mice under anesthesia and in different animals on days 15 and 25. Because of the variability in standard values between animals, changes occurring in less than 4 weeks after the first dose of doxorubicin might be too small to detect using five animals per group. Within this short period, the aforementioned mice received a high cumulative dose (32.4 and 40 mg/kg). This might also account for the encountered high mortality and high degree of general (non-heart-related) toxicity. In our steady, doses of 24 or 28 mg/kg were spread over a longer period.

The advantages of our new method are clear. First, a highly significant difference between doxorubicin-treated and protector-treated groups can be achieved early in the study on a 6×4-mg/kg dose schedule, which results in toxicity sufficient to investigate protectors yet is acceptable for the animal. Furthermore, only five animals per group are necessary to detect 45% protection or more, and monitoring is possible within the same animal and without the introduction of interfering factors such as anesthesia, to which BALB/c mice are very sensitive. Therefore, our model saves animals and time, as morphometry or histology is much more time-consuming than measurement of the ECG and can be performed only once per animal. Furthermore, it is a sensitive and accurate method because each animal can be its own control and changes are significant as early as after 2 weeks at a cumulative dose of 12 mg/kg.

As judged from the present results, the ECG measured by telemetry can be considered a valuable and sensitive tool for measuring the cardiotoxic effects of anticancer agents and protectors by monitoring of the animals as often as necessary during treatment. In addition, telemetry makes it possible to monitor without introducing interfering factors.

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