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# Monohydroxyethylrutoside as protector against chronic doxorubicin-induced cardiotoxicity

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**1** The clinical use of the antitumour agent, doxorubicin, is largely limited by the development of a cumulative dose-related cardiotoxicity. This toxicity is generally believed to be caused by the formation of oxygen free radicals. In earlier studies it was established that flavonoids, naturally occurring antioxidants, can provide some degree of protection. In this study we investigated whether 7-monohydroxyethylrutoside (monoHER), a powerful antioxidative flavonoid with extremely low toxicity, can provide protection to an extent comparable to the clinically successful Cardioxane (ICRF-187).

**2** Balb/c mice of 20–25 g were equipped i.p. with a telemeter to measure ECG. They were given 6 i.v. doses of doxorubicin (4 mg $kg^{-1}$) at weekly intervals. ICRF-187 (50 mg $kg^{-1}$) or monoHER (500 mg $kg^{-1}$) were administered i.p. 1 h before doxorubicin administration. In the 2 monoHER groups the treatment continued with either 1 or 4 additional injections per week. A saline and monoHER treated group served as controls. After these 6 weeks, they were observed for another 2 weeks.

**3** At the end of this study (week 8) the ST interval had increased by $16.7 \pm 2.7$ ms (mean ± s.e.mean) in doxorubicin-treated mice. At that time, the ST interval had increased by only $1.8 \pm 0.9$ ms in ICRF-187 co-mediated mice and in monoHER co-medicated mice by only $1.7 \pm 0.8$ and $5.1 \pm 1.7$ ms (5- and 2-day schedule, respectively, all $P < 0.001$ relative to doxorubicin and not significantly different from control). The ECG of the control animals did not change during the entire study. The QRS complex did not change in either group.

**4** It can be concluded that monoHER protects against doxorubicin-induced cardiotoxicity and merits further evaluation in this respect.

**Keywords:** Doxorubicin; cardiotoxicity; free radicals; antioxidant; flavonoid

## Introduction

Doxorubicin, an anthracycline originally isolated from *Streptomyces peucetius*, has potent antitumour activity against a wide range of human malignancies. The major acute toxicity is bone marrow suppression, but the long-term clinical usefulness is limited by a cumulative dose-related cardiotoxicity, which manifests itself as congestive heart failure. Although other mechanisms of toxicity have been suggested, doxorubicin-related cardiotoxicity is generally believed to be caused by the formation of oxygen free radicals (Myers *et al.*, 1977). Cardiac tissue appears to be particularly sensitive to free radical-induced toxicities because the enzymes which protect against oxidative damage are markedly reduced compared to other tissues in the body (Green *et al.*, 1990; Keizer *et al.*, 1990; Takács *et al.*, 1992).

Iron chelators and free radical scavengers might provide protection by preventing the formation of the extremely reactive hydroxyl radical in the Fenton reaction and by scavenging radicals which are already formed. In this respect Cardioxane (ICRF-187, Figure 1), which is hydrolysed in the cell into a strong iron chelator, has clinical success in anthracycline therapy. As a result of ICRF-187 co-administration, the maximum cumulative dose of doxorubicin can be raised four fold in rats (Koning *et al.*, 1991) and at least two fold in human subjects (Speyer *et al.*, 1988).

Flavonoids are a group of naturally occurring compounds with both excellent iron chelating and radical scavenging properties (Havsteen 1983; Rekka & Kourounakis 1991; Haenen *et al.*, 1993) and may therefore be of interest as possible modulators of doxorubicin-induced cardiotoxicity. A standardized mixture of flavonoids, Venoruton (O-(β-hydroxyethyl)-rutosides), has been shown to protect *in vivo* against doxorubicin-induced cardiotoxicity (Van Acker *et al.*, 1993). The main constituents of Venoruton are 7-monohydroxyethylrutoside (monoHER, Figure 1), 7,4′-diHER, 7,3′,4′-triHER, 5,7,3′,4′-tetraHER and 7,3′,4′-trihydroxyethyl quercetin (triHEQ), of which monoHER is the most powerful antioxidant (Haenen *et al.*, 1993). Furthermore, hydroxyethylrutosides are known to have very low toxicity at high concentrations (Leuschner *et al.*, 1973). These observations make monoHER an interesting compound to investigate its protecting abilities in doxorubicin-induced chronic cardiotoxicity.

The aim of the present investigation was to determine whether monoHER has protective properties against myocardial damage caused by doxorubicin. Because ICRF-187 has up to now been the only drug with significant cardioprotection in clinical studies, a comparison was made with this compound.

## Methods

### *Animals*

28 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 one week before surgery.

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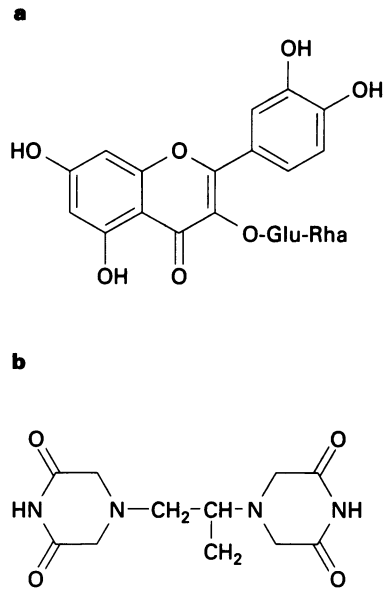


**Figure 1** The chemical structures of 7-monohydroxyethylrutoside (MonoHER) (a) and ICRF-187 (b).

### *Telemetry system*

As described earlier (Kramer *et al.*, 1993), 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, MN, U.S.A.). 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 programme Chart from MacLab. The transmitter was activated by a magnet, after which the output of the transmitter 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. The sampling rate was 400 samples $s^{-1}$.

### *Surgery*

As approved by the Vrije Universiteit Ethics Committee, the mice were anaesthetized with 0.07 ml per 10 g i.p. of a mixture of 0.315 mg $kg^{-1}$ fentanyl and 10 mg $ml^{-1}$ fluanisone (Hypnorm), midazolam (Dormicum, 5 mg $ml^{-1}$) and sterilized water in the ratio 1 : 1 : 2. Surgery was performed as described in detail by Kramer *et al.* (1993). In short, the transmitter was implanted in the peritoneal cavity of each mouse at least two weeks before the start of the treatment. The leads to the transmitter were studied subcutaneously in lead II position (the (−) lead at the right shoulder and the (+) lead towards the lower left chest).

### *Experimental set-up*

After surgery, the mice were allowed to recover for two weeks after which they were submitted to one of the following weekly dose-schedules for 6 weeks: group 1 ($n=6$): 0.05 ml 0.9% NaCl solution intravenously (i.v.); group 2 ($n=5$): 4 mg $kg^{-1}$ doxorubicin, i.v.; group 3 ($n=5$): 500 mg $kg^{-1}$ monoHER i.p., 4 mg $kg^{-1}$ doxorubicin i.v. after 1 h, followed by 500 mg $kg^{-1}$ monoHER i.p. every 24 h for 4 days (MonoHER 5); group 4 ($n=5$): 500 mg $kg^{-1}$ monoHER i.p., 4 mg $kg^{-1}$ doxorubicin i.v. after 1 h, followed by 500 mg $kg^{-1}$ monoHER i.p. after 24 h (MonoHER 2); group 5 ($n=2$): 500 mg $kg^{-1}$ monoHER i.p. followed by a second dose after 24 h; group 6 ($n=5$): 50 mg $kg^{-1}$ ICRF-187 i.p., 4 mg $kg^{-1}$ doxorubicin i.v. after 1 h. I.v. injections were administered in the tail vein. After this treatment the animals were observed for another two weeks. During treatment and the observation period, body weight was determined once per week as a measure of general toxicity. ECG and heart rate were registered after surgery when still under anaesthesia and then in the freely moving animal once per week until the end of the study.

At the end of the study the animals were killed by decapitation and submitted to pathological evaluation. They were investigated for organ abnormalities either due to doxorubicin or the transmitter.

### *Parameters*

*Telemetry parameters* For interpretation of the ECG four consecutive complexes were analysed in detail. The PR segment, QRS complex, QT interval and ST interval were determined as mean ± s.d. of these four complexes. In case of an unacceptable variation in the complexes due to noise caused by movement of the animal (coefficient of variation >10%), a different part of the ECG was selected for evaluation. The 'ratemeter' from the program 'Chart' was used to automatically calculate the heart rate from the ECG-input.

*Other parameters* Changes in weight were taken as a measure of general toxicity, as were behaviour of the animal and general impression of the condition.

### *Chemicals*

MonoHER (7-monohydroxyethyl rutoside, mol.wt. 654.6) was kindly donated by Zyma S.A., Nyon, Switzerland. Doxorubicin (Adriblastina, 2 mg $ml^{-1}$) was obtained from Farmitalia Carlo Erba (Milan, Italy). ICRF-187 (Cardioxane 20 mg $ml^{-1}$, (+)-1,2-*bis*(3,5dioxopiperazinyl-1yl)propane, mol.wt. 304.3) was kindly provided by EuroCetus (Amsterdam, The Netherlands). Before injection, the compounds were dissolved in 0.9% NaCl solution, except for monoHER, which was dissolved in 36 mM NaOH in sterile water, in a final concentration of 33 mg $ml^{-1}$ (pH = 7.8–8).

### *Statistical analysis*

All parameters were expressed as mean ± s.e. mean unless stated otherwise. All parameters were evaluated using analysis of variance (ANOVA) with Fisher's LSD test for multiple comparisons when ANOVA indicated significant differences between groups. The programme used for this analysis was 'NCSS' (by Dr J.L. Hintze, Kaysville, Utah, U.S.A.). The level of significance chosen was 99% ($P<0.01$) to correct for comparisons between groups every week (multiple comparisons).

## Results

### *General toxicity*

After surgery, recovery of the animals was indicated by an increase in weight after an initial decrease and by changes in their behaviour such as building a nest of the available paper towels. Animals appeared lively throughout the study and no behavioural changes were observed compared to the mice without transmitters. Behaviour appeared normal in all treatment groups. There were no signs of decreased activity, which indicates low general toxicity This was confirmed by the growth curves, which reveal no significant differences in weight gain between groups. However, there was a trend that weight gain in the doxorubicin-treated group was somewhat less than in the doxorubicin/ICRF-187, doxorubicin/monoHER, monoHER and saline groups (% weight gain at the end of the study, mean ± s.e.mean, was 3.5 ± 1.9, 10.0 ± 3.0, 14.1 ± 3.5 (5 day), 9.3 ± 3.9 (2 day), 8.3 ± 2.7 and 10.3 ± 4.1%, respectively).

### *Pathology*

One non-treatment related death occured in the monoHER 5 group in week 6. An i.p. injection error caused a fatal internal bleeding. One of the doxorubicin/monoHER mice on the 2 day schedule developed a hypertrophic left atrium. This was accompanied by an increase in ST interval. The abdominal organs, such as kidney, liver and intestine appeared normal in all mice. This indicates that the transmitter did not cause any abnormalities.

### *ECG*

As described earlier, the ECG signal in lead II deflection of a mouse is somewhat different from that of man (Van Acker *et al.*, 1995), but corresponds with the ECG measured in mice under anaesthesia or restained (Monath *et al.*, 1978; Postan *et al.*, 1987). The T wave immediately follows the QRS complex, thus there is no ST segment (Figure 2). The ECGs of the control animals did not change during the course of the study. 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. The QT- and ST interval increased with time (Figure 3) by $17.7 \pm 2.9$ and $16.7 \pm 2.7$ ms, respectively, in week 8, while the PR segment and the QRS complex remained constant. Both ICRF-187 and monoHER were able to protect against these ECG changes, which have earlier been found to correlate with the development of cardiotoxicity in both rats (Parachini *et al.*, 1993) and mice (Seyedin *et al.*, 1994; Van Acker *et al.*, 1995). ICRF-187 reduced the increase of the ST interval to $1.9 \pm 0.9$ ms and the monoHER 5 schedule to $1.7 \pm 0.8$ ($P < 0.001$ relative to doxorubicin, not significantly different from control). The monoHER 2 schedule showed somewhat less protection, the increase in ST interval was $5.1 \pm 1.7$ ms ($P < 0.001$ relative to doxorubicin, not significantly different from control). MonoHER alone in a 2 day schedule did not exert any effect on the ECG. No arrhythmias were seen in animals of any of the groups.

### *Heart rate*

In a previous investigation with telemetry in mice (Kramer *et al.*, 1993), we found that handling of the mouse and placing it in a new cage led to a stress situation for the animal and to an increased heart rate (700–800 beats per min (b.p.m.)). This

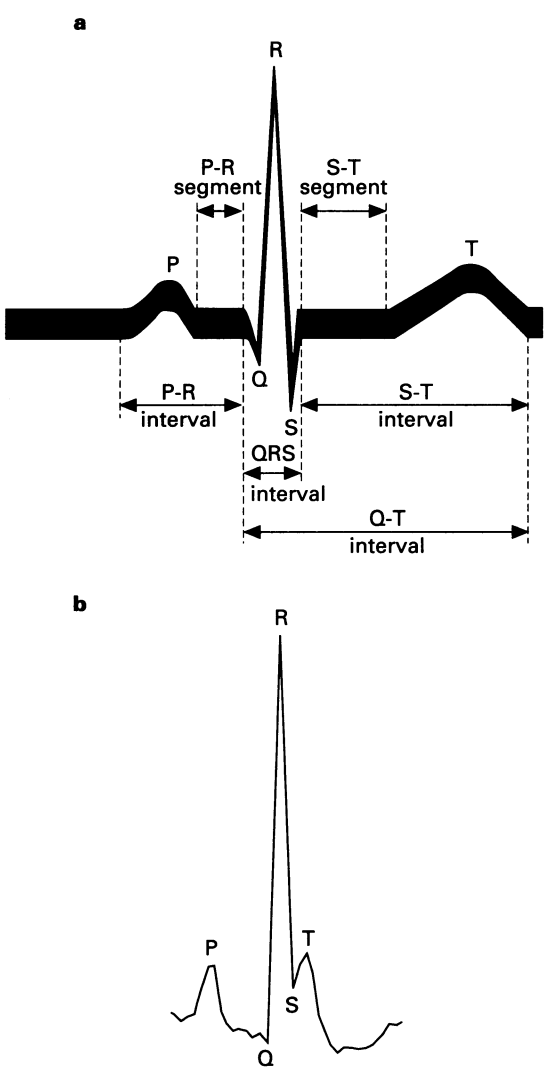


**Figure 2** ECG (lead II deflection) of human subject (a, schematic) compared to mouse (b, typical trace).

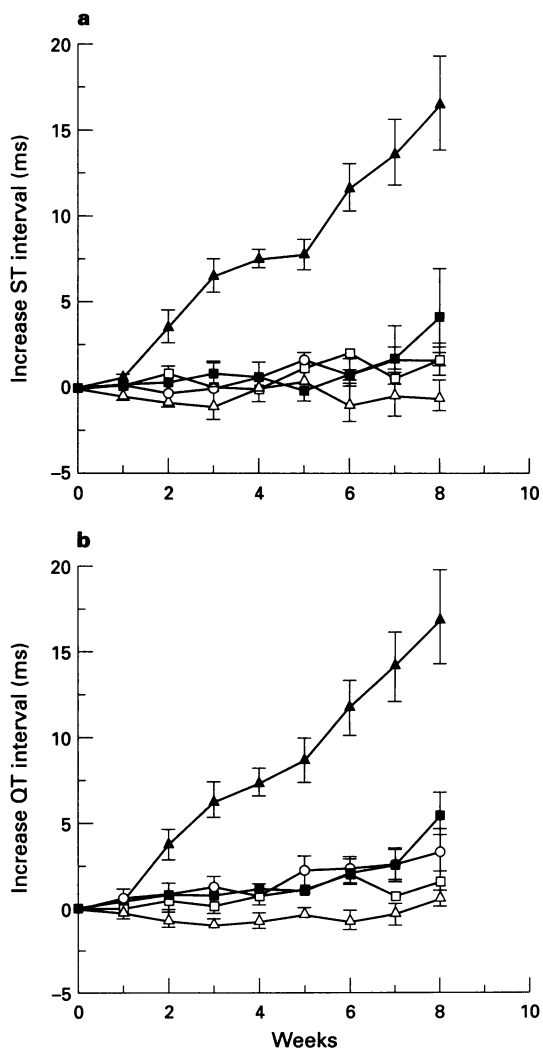


**Figure 3** Increase in ST interval (a) and QT interval (b) per treatment group. All treatment groups relative to doxorubicin $P < 0.001$ from week 2. (▲) Doxorubicin: $4\,mg\,kg^{-1}$, i.v. 1x per week for 6 weeks; (△) control: saline i.v. 1x per week for 6 weeks; (○) ICRF-187: $50\,mg\,kg^{-1}$, i.p. 1 h before doxorubicin, (□) MonoHER 5: $500\,mg\,kg^{-1}$, i.p. 1 h before doxorubicin and the next 4 days; (■) MonoHER 2: $500\,mg\,kg^{-1}$, i.p. 1 h before doxorubicin and the next day. Baseline value ST interval $13.0 \pm 0.5$ ms, QT interval $23.8 \pm 0.8$ ms.

heart rate could not be increased any further with the positive chronotropic drug isoprenaline. Therefore, the observed heart rate of 700–800 b.p.m. is considered to be the maximum heart rate. When the heart rate was measured in the home case, it was lower (±600 b.p.m.) and at rest (lying in their nests) the heart rate was 400–450 b.p.m. Differences between individual mice but also within the same animal were considerable for heart rate at rest; thus these heart rate values are considered of little use to indicate effects of treatment. In all animals the maximum heart rate was 650–750 b.p.m. during the entire study.

## Discussion

The formation of oxygen free radicals is still generally believed to be the case of doxorubicin-induced cardiotoxicity, although therapy with compounds limiting free radical formation, such as antioxidants and iron-chelators has had only limited success (Herman & Ferrans 1983; McGinness *et al.*, 1986; Myers *et al.*, 1991; Pritsos *et al.*, 1992). Up till now, only ICRF-187, which is hydrolysed intracellularly into an EDTA-like iron chelator, has been used in clinical practise (Speyer *et al.*, 1988; Koning *et al.*, 1991). However, the success of ICRF-187 indicates that free radicals are very likely to be involved and it demonstrates that antitumour activity and cardiotoxicity are probably caused by different mechanisms, or at least, can be separated, because ICRF-187 does not influence the antitumour activity of doxorubicin.

Problems with other potential protectors, such as superoxide dismutase, desferrioxamine and vitamin E are probably caused by the fact that they are taken up very poorly into the cell and therefore cannot reach the site of action of doxorubicin. N-acetyl cysteine (NAC) was used in clinical trial, but failed to show protection (Myers *et al.*, 1991). NAC is a precursor of glutathione (GSH), an endogenous thiol with antioxidant activity. Unfortunately, both NAC and GSH are not very good 'direct' scavengers and they do not have chelate iron. The antioxidant activity of GSH is mainly based on being a cofactor for enzymes as GSH-peroxidase and free radical reductase (Bast *et al.*, 1991). Ebselen and α-phenyl-tert-butylnitrone (PBN), both antioxidants, have been shown to have protective properties in animals (Pritsos *et al.*, 1992; Parachini *et al.*, 1993).

Flavonoids are excellent transition metal chelators and radical scavengers. The combination of these properties enables 'site-specific' scavenging, i.e. oxygen radicals are, if they can still be formed around the iron, formed in the vicinity of the flavonoid and can be scavenged immediately (Haenen *et al.*, 1993).

In an earlier study we demonstrated the potential of the ECG, especially the ST interval to study doxorubicin-induced chronic cardiotoxicity (unpublished observations). It was found that 5 animals per group was enough to investigate putative protecting agents, because at least 45% protection can be detected with this number of mice. In a study by Seyedin *et al.* (1994) measuring ECG in mice with conventional methods (with needle electrodes under anaesthesia), similar ECG changes were described (Seyedin *et al.*, 1994). However, they predicted the need of groups of 9–14 animals to see significant differences between the doxorubicin-treated and the control group. This was probably because they compared means of the ST interval per group after treatment. The interindividual variation in the ST interval in the mice before treatment is relatively large compared to the increase in the ST interval due to treatment. There is, however, only small interindividual variability when the increase in ST interval per mouse is used, thus normalised for the initial value. Parachini *et al.* (1993) demonstrated the protective effect of several spintraps by measuring changes in ST- and QT interval with conventional methods in rats.

It appeared that the monoHER 5 schedule is somewhat more active against cardiotoxicity than the monoHER 2 schedule. This is most pronounced in weeks 7 and 8, where the ST interval of the 2 day group seems to increase. A 5 day schedule had earlier been proved successful with Venoruton (Van Acker *et al.*, 1993), but from known pharmacokinetics of both doxorubicin (Van der Vijgh *et al.*, 1990) and monoHER (Hackett & Griffiths 1977) it seemed possible to use a 2-day schedule. Since most of the doxorubicin and its major metabolite doxorubicinol is cleared from the heart within 48 h (Van der Vijgh *et al.*, 1990), it was suggested that monoHER had to be present only during this period of time. This data is supported by the observation that cardiotoxicity could be decreased by reducing peak plasma levels from long-term infusion of doxorubicin. Due to the fast clearance of the flavonoid (Hackett & Griffiths 1977), it was administered twice, whereas ICRF-187 was administered only once, as this was described as the optimal dose schedule (Herman & Ferrans, 1986).

The increase of ST interval in the monoHER 2 group after the last week of treatment (week 6) indicated that peak plasma levels are clearly not the only cause of cardiotoxicity. The difference between the two monoHER schedules might perhaps be based on a pharmacokinetic background. Low levels of doxorubicin and doxorubicinol, still persisting in the heart after a few days, might continue to cause radical damage, whereas the remaining concentration of monoHER from the 2-day schedule was too low to provide total protection. This would mean that in the case of the 5-day schedule there was still enough monoHER present to 'block' any radical damage. A pharmacokinetic study with radiolabelled monoHER showed almost total excretion of the radioactivity at 48 h after one i.p. injection of 25 mg $kg^{-1}$. Because we gave 20 times as much for five days per week, it is tempting to speculate that accumulation occurs, which might be substantially larger in the 5-day schedule than in the 2-day schedule. Thus, the monoHER 5 schedule can provide protection for a longer period of time.

The flavonoid most suitable for *in vivo* protection against doxorubicin-induced cardiotoxicity has to be taken up intracellularly in large amounts and must reach the site of action of doxorubicin. Furthermore, it must be a powerful chelator and scavenger, but it must not be able to redox-cycle and generate oxygen radicals in physiological conditions, as is known for quercetagetin, myricetin and quercetin (Laughton *et al.*, 1989; Canada *et al.*, 1990). It must also not decrease the antitumour activity of doxorubicin.

MonoHER is a powerful antioxidant, the best of the Venoruton constituents (Rekka & Kourounakis 1991; Haenen *et al.*, 1993). To our knowledge, there are no reports of redox-cycling abilities. Whether monoHER has any influence on the antitumour activity of doxorubicin *in vitro* or *in vivo* has to be investigated in the future. However, Venoruton, which contains about 10% of monoHER, was found not to have an influence on antitumour activity of doxorubicin *in vitro* in L1210 and p388 cells and *in vivo* (McGinness *et al.*, 1986; Bull *et al.*, 1988). Furthermore, it was also found not to influence the antitumour activity of cisplatin and bleomycin against L1210 and B16 cells *in vitro* and *in vivo* (Bull *et al.*, 1988).

In this investigation, monoHER has been shown to protect *in vivo* against chronic doxorubicin-induced cardiotoxicity. It appeared to be at least as effective as ICRF-187, which is successfully used in the clinic. It is known that hydroxyethylrutosides have little toxicity of their own, whereas ICRF-187 shows bone marrow suppression. Therefore, as it is expected that monoHER will not show any negative influence on doxorubicin cytotoxicity *in vitro* and *in vivo*, it can be concluded that monoHER merits further investigation as a possible protector against doxorubicin-induced chronic cardiotoxicity.

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