Frederine, a New and Promising Protector Against Doxorubicin-induced Cardiotoxicity

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INTRODUCTION

Anthracyclines constitute a major class of cytotoxic agents for the treatment of cancer. Among these, doxorubicin possesses a broad spectrum of antitumor activity. The reduction of doxorubicin-induced cumulative cardiomyopathy represents a major goal in improving the clinical application of doxorubicin. It has been recognized at an early stage that anthracycline-induced cardiotoxicity is a problem observed in 2 to 20% of patients receiving anthracyclines (1). Despite extensive research, the mechanism underlying the cardiac damage induced by doxorubicin is still not completely elucidated. One of the leading hypotheses suggests that doxorubicin increases free-radical production (2–4), whereas heart tissue has only a limited defense system.

Recently, we have shown in mice that the semisynthetic flavonoid monoHER2 (Fig. 1) protects against doxorubicin-induced cardiotoxicity (5, 6) without compromising the antitumor activity (7). This strongly supports the free radical hypothesis, because this flavonoid is an excellent antioxidant. Apart from vitamin C and the fat-soluble tocopherols, flavonoids are the most abundant and active antioxidant compounds naturally occurring in food (8).

Although monoHER was found to completely protect against doxorubicin-induced cardiotoxicity, a relatively high dose of 500 mg/kg (administered i.p. to mice) was needed to obtain complete protection. To find more potent protectors than monoHER, we recently synthesized a series of new flavonoids based on the structure of monoHER (9). The general structure of the new compounds consists of a flavone backbone with a C2-C3 double bond and a catechol moiety on ring B. According to structure-activity relationships of commercially available flavonoids published previously (10–14), incorporation of these structural elements led to potent antioxidants. We selected substituents that besides modifying antioxidant activity also improved water solubility and cardioselectivity.

To select the most promising candidate from this series, we screened these new flavonoids for their antioxidant activity, their cardioprotective properties in vitro, and their possible cytotoxic effects.3 Frederine (Fig. 1B) was selected from the series because this compound is a potent antioxidant, protects against doxorubicin-induced cardiotoxicity in vitro, and is nontoxic to hepatocytes (at 250 μM).

The purpose of the present study was to investigate Frederine for its toxicity in mice and for its cardioprotective prop-

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2 The abbreviations used are: monoHER, 7-monohydroxyethylrutoside; ECG, electrocardiogram; LPO, lipid peroxidation.


ABSTRACT

The flavonoid 7-monohydroxyethylrutoside (monoHER) can protect against doxorubicin-induced cardiotoxicity. A drawback of monoHER therapy would be the relatively high dose needed to obtain complete protection (500 mg/kg in mice). Therefore, we synthesized a series of new compounds with improved antioxidant properties. After characterization of antioxidant activity, cardioprotection in vitro, and possible toxic properties in hepatocytes, we selected Frederine for additional investigations in vivo. In the present study, it was found that this compound did not induce weight loss or (gross) organ changes in mice in a treatment schedule of 170 mg/kg i.p., 5 times/week during 2 weeks. We recorded the electrocardiogram telemetrically in mice during and 2 weeks after the combined treatment with doxorubicin (4 mg/kg, i.v.) and 5 times Frederine (68 mg/kg, i.p.; equimolar to 100 mg/kg monoHER) for 6 weeks. Complete protection against doxorubicin-induced cardiotoxicity was found, indicating that Frederine is at least 5 times more potent than monoHER. Frederine did not have a negative influence on the antiproliferative effects of doxorubicin on A2780, OVCAR-3, and MCF-7 cells in vitro and on OVCAR-3 xenografts grown in nude mice when administered 5 min before doxorubicin (8 mg/kg i.v.) and 4 days thereafter with an interval of 24 h. It can be concluded that we succeeded in designing a better cardioprotector than monoHER. Therefore, Frederine merits further investigation as a possible protector against doxorubicin-induced cardiotoxicity in cancer patients.
properties in mice treated with doxorubicin using telemetry. Frederine was also investigated for its possible influence on the antitumor activity of doxorubicin in tumor cell lines in vitro as well as in human ovarian cancer xenografts grown in nude mice.

MATERIALS AND METHODS

Methods

Chemicals

MonoHER (MW 654.6) was kindly provided by Novartis Consumer Health, Nyon, Switzerland. MonoHER was dissolved in 36 mM NaOH in sterile water, giving a final concentration of 33 mg/ml (pH 7.8–8). Frederine (3',4'-dihydroxy-3-glucosylflavone; MW 432.4) was synthesized as described earlier (9). For the toxicity study in mice, Frederine was dissolved in 18 mM NaOH in sterile water, giving a final concentration of 20 mg/ml. For the cardiotoxicity study and antitumor activity studies in mice, Frederine was dissolved in 5 mM NaOH in sterile water, giving a final concentration of 10 mg/ml (pH 7.8–8). Formulated doxorubicin (doxorubicin hydrochloride; 2 mg/ml) was obtained from Pharmachemie B.V. (Haarlem, the Netherlands). Before injection, the contents of the vial were dissolved in 0.9% NaCl solution (sterile).

Animals

All of the protocols were approved by the Ethics Committee for Animal Experiments of the Vrije Universiteit in Amsterdam. Thirty-six male BALB/c mice (20–25 g) obtained from Harlan Nederland B.V. (Horst, 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). Tap water and food pellets were provided ad libitum. The animals were allowed to adapt to the laboratory housing conditions for at least 1 week before starting the experiment. Nude female mice (Hsd:athymic-nu) were obtained from Harlan Nederland B.V. (Horst, the Netherlands) at the age of 6 weeks. The animals were maintained in isolation under controlled atmospheric conditions (temperature, 23–25°C; humidity, 50–60%). Animal handling was carried out under sterile conditions.

Toxicity Study

In the toxicity study, a dose of 170 mg/kg Frederine was used to compensate for the shorter period of the experiment (2 weeks) compared with the cardiotoxicity study (68 mg/kg for 6 weeks). BALB/c mice were treated 5 times/week with 170 mg/kg Frederine (n = 5), which is equimolar to 250 mg/kg monoHER, or 0.9% NaCl solution (n = 5) i.p. for 2 weeks. During treatment, body weight was determined daily as a measure of general toxicity. After 2 weeks, mice were killed by decapitation, and heart, liver, and kidneys were removed quickly after visual inspection. Organ weights were determined as a measure for organ toxicity. Organ weight was expressed as relative organ weight, i.e., the weight of an organ relative to the body weight of the mouse (g/g body weight × 100%).

Cardiotoxicity Study

Telemetry System

The telemetry system, which consisted of implantable transmitters (TA10ETA-F20) and telemetry receivers (RPC-1), was obtained from DATA Sciences International (St. Paul, MN). The frequency output (Hz) from the transmitter was monitored by the telemetry receivers placed underneath the animal’s cage. Samples were taken every 10 min using the Data Sciences data acquisition system (Dataquest A.R.T. 1.01).

Surgery

BALB/c mice were anesthetized with 0.07 ml/10 g i.p. of a mixture of Hypnorm (0.315 mg/ml fentanyl and 10 mg/ml fluanisone), Dormicum (5 mg/ml midazolam), and sterile water in the ratio 1:1:2. Surgery was performed as described in detail by Kramer et al. (15). In short, the transmitter was implanted in the peritoneal cavity of each mouse 2 weeks before the start of the treatment. The leads of the transmitter were sutured s.c. in lead II position [the (--) lead at the right shoulder and the (+) lead toward the lower left chest].

Treatment

After surgery, the mice were allowed to recover for 2 weeks, after which they were submitted to one of the following weekly dose schedules for 6 weeks: group 1, (n = 7) 0.9% NaCl solution i.v.; group 2, (n = 7) 0.9% NaCl solution i.p., followed by 4 mg/kg doxorubicin i.v. after 1 h; group 3, (n = 5) 100 mg/kg monoHER i.p., followed by 4 mg/kg doxorubicin i.v. after 1 h and 100 mg/kg monoHER i.p. every 24 h for 4 days (7); group 4, (n = 6) 68 mg/kg Frederine i.p., followed by 4 mg/kg doxorubicin i.v. after 1 h and 68 mg/kg Frederine i.p. every 24 h for 4 days; and group 5, (n = 5) 68 mg/kg Frederine i.p. and 68 mg/kg Frederine i.p. every 24 h for 4 days.

To compare the potency of Frederine with monoHER, we administered 68 mg/kg Frederine, which is an equimolar dose of 100 mg/kg monoHER. Using this concentration, a decreased or increased potency would be identifiable, because 100 mg/kg monoHER provided approximately 50% protection in the pres-
ent dose schedule. For the monoHER group (group 3), data were used, which were presented earlier (7).

i.v. injections were administered in the tail vein. After the treatments, the animals were observed for 2 additional weeks. During treatment and the observation period, body weight was determined once/week as a measure of general toxicity. ECG and heart rate were registered telemetrically in the freely moving animals once/week until the end of the study.

Parameters

Changes in weight were taken as an indication of general toxicity of the treatments, as were behavior of the animal and general impression of the condition. For interpretation of the ECG, five complexes were analyzed in detail. The ST interval was determined as mean ± SD of these five complexes. At the end of the study, the animals were sacrificed by decapitation.

Antitumor Activity Studies

Cell Culture

Three cell lines were used, i.e., the human ovarian cancer cell lines, A2780 (16) and OVCAR-3 (17), and the human breast cancer cell line, MCF-7 (18). All of the cell lines were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% penicillin, and 50 g/ml streptomycin (Flow, Irvine, United Kingdom) in an incubator with a humidified atmosphere containing 5% CO₂ at 37°C.

A stock solution of Frederine was prepared in DMSO. Doxorubicin was dissolved in sterile water. Drugs were diluted in culture medium immediately before their addition to the culture plates. The DMSO concentration never exceeded 0.55%, which did not influence cell growth.

Exponentially growing cells were harvested and plated as single cell suspensions in 96-well flat-bottomed microtiter plates (Greiner, Solingen, Germany). Cells were seeded in quadruplicate at a density of 5000 cells/well for MCF-7 and OVCAR-3 and 3000 cells/well for A2780. After 24 h (day 1), Frederine was added in a final concentration of 100 μM in the presence or absence of doxorubicin (final concentrations ranging from 10⁻¹⁴ to 10⁻⁵ M; dosing interval, 5 min before doxorubicin and, for OVCAR-3 cells, 1 h before doxorubicin). The total exposure time was 96 h. At the end of the drug exposure period (day 5), growth inhibitory effects were evaluated with the standard tetrazolium test (19). Briefly, after removal of the supernatant, 50 μl of tetrazolium-solution (0.4 mg/ml) was added to each well, and the cells were incubated at 37°C and 5% CO₂ for at least 1.5 h. Subsequently, 200 μl of DMSO with 0.5% FCS was added to the cells, and the absorption of the formed formazan product was measured using a multiscan bichromatic platereader (A₅₄₀ nm-A₆₅₀ nm; Reader Microelisa System; Organon Teknika, Turnhout, Belgium). Data were collected and analyzed using Excel. The results were expressed as the IC₅₀, which is the concentration of the drug giving a 50% inhibition of cell growth of treated cells when compared with the growth of control cells.

In Vivo Antitumor Activity

Experimental Design. The drug tolerance and antitumor activity studies were performed with the same dose as that for the cardiotoxicity study. For drug tolerance studies, nontumor-bearing nude mice (8 weeks of age) were used. Each group contained three mice. After treatment, which was the same as for the antitumor studies, the mice were weighed daily to determine gross toxicity.

For the antitumor studies, 10⁶ cells from OVCAR-3 were inoculated s.c. into both flanks of 8-week-old mice. The solid tumors arising were used for transplantation of small fragments in subsequent recipients. Treatment was started when the tumor size was approximately 100 mm³; the first treatment day was designated as day 0. Treatment and control groups consisted of five to six mice each. Mice were weighed, and tumors were measured twice/week.

Treatment. Treatments are as follows: group 1, no treatment; group 2, 8 mg/kg doxorubicin i.v. on days 0 and 7; group 3, 8 mg/kg doxorubicin i.v. on days 0 and 7, 68 mg/kg Frederine i.p. on days 0 and 7 (5 min before doxorubicin), and in 24-h intervals on days 1–4 and days 8–11; group 4, 8 mg/kg doxorubicin i.v. on days 0 and 7, 68 mg/kg Frederine i.p. on days 0 and 7 (1 h before doxorubicin), and in 24-h intervals on days 1–4 and days 8–11; and group 5, 68 mg/kg Frederine i.p. on days 0–4 and in 24-h intervals on days 7–11.

Previously, we established that the maximum tolerated dose for one i.v. injection of doxorubicin/week during 2 weeks was 8 mg/kg. At this schedule, tumor-bearing nude mice showed a reversible weight loss of approximately 10% of the initial weight within 2 weeks after the first injection (20).

Tumor volumes were calculated according to the formula 0.5 × length × width × height (21). The tumor volume from the start of the treatment (V₀) until the value at any given time (Vₜ) was calculated for each tumor and expressed as the relative tumor volume (Vₜ/V₀) on the day of measurement. The mean of these values was used to calculate the efficacy of the treatment as a ratio of the relative tumor values between treated (T) and control (C) tumors (T/C × 100%). Growth inhibition was expressed as 100% – (T/C × 100%).

Statistical Analysis

All of the parameters were expressed as mean ± SE unless stated otherwise. Parameters were evaluated using Student’s t test in Excel or ANOVA with Fisher’s LSD test for multiple comparisons when ANOVA indicated significant differences between groups. The program used for this ANOVA analysis was “NCSS” (by Dr. J. L. Hintze, Kaysville, Utah). For weekly comparisons between groups (multiple comparisons), the level of significance chosen was 99% (P < 0.01).

RESULTS

Toxicity Study

Behavior appeared normal in both the Frederine-treated group as well as the control group. All of the animals appeared lively throughout the study, and no weight loss was detected. Weight gain on day 12 of the experiment was 4.0 ± 0.6% and 8.2 ± 1.1% for the saline group and the Frederine treated group (P < 0.01 relative to saline), respectively.
Cardiotoxicity Study

General Toxicity. After surgery, recovery of the animals was indicated by an increase in weight after an initial decrease (15) and by normal behavior such as building a nest of the available paper towels. Animals appeared lively throughout the study, and no behavioral changes were observed compared with available controls.

Weight Gain. A trend was observed that weight gain in the doxorubicin-treated group was somewhat less than that in the saline-treated group, which would indicate general toxicity. No visible abnormalities of the organs were detected. The organ weights of the heart, liver, and kidneys were not significantly different between the two groups. For the saline-treated group, relative weights were 4.07 ± 0.16%, 1.46 ± 0.04%, and 0.44 ± 0.02% for liver, kidneys, and heart, respectively. In case of the Frederine-treated group, relative organ weights were 4.22 ± 0.05%, 1.53 ± 0.03%, and 0.45 ± 0.02% for liver, kidneys, and heart, respectively.

Pathology. Three deaths occurred: one in the control group (group 1) during week 8, one in the doxorubicin group (group 2) during week 4, and one in the doxorubicin + Frederine group (group 4) during week 3. No explanation could be found for these three deaths after necropsy. The abdominal organs, such as kidney, liver, and intestine, appeared normal in all of the mice, including the three deaths. This indicates that both the transmitter and the cardioprotectors did not cause any visible abnormalities.

ECG Measurements. The ECGs of the control animals did not change during the course of the study. Doxorubicin had a profound influence on the shape of the ECG. The ST interval increased with time by 16.9 ms after 8 weeks (P < 0.01, relative to doxorubicin and control; Ref. 7). In contrast to monoHER, an equimolar dose of Frederine was able to reduce the increase in ST interval completely to 1.7 ms after 8 weeks (P < 0.01, relative to doxorubicin; P < 0.001, relative to monoHER; not significantly different from control). Frederine alone did not result in a change of the ST interval.

Antitumor Activity Study

Cell Culture. A2780, OVCAR-3, and MCF-7 cells were exposed to doxorubicin in the presence or absence of Frederine for 96 h. IC_{50} values are summarized in Table 1. All of the three cell lines showed a similar sensitivity to doxorubicin. Frederine alone (100 µM) showed limited inhibition of cell growth, in particular in MCF-7 and OVCAR-3 cells (growth of 83.8% and 86.8%, respectively in MCF-7 and OVCAR-3, respectively).

More importantly, Frederine given 5 min before doxorubicin did not reduce the antitumor activity of doxorubicin in the investigated cell lines. In the case of OVCAR-3 cells, no difference was found between the 5-min and 1-h dosing intervals.
Cardioprotection by the New Flavonoid Frederine

**Antitumor Activity.** First, we investigated the weight loss induced by Frederine with or without 8 mg/kg doxorubicin. In groups of three nontumor-bearing nude mice, mice given 68 mg/kg Frederine on days 0–4 and 7–11 did not show a weight loss. Coadministration of Frederine and doxorubicin did not lead to a more pronounced weight loss than doxorubicin alone (Table 2).

The results of the antitumor activity study are shown in Table 3 and Fig. 3. Combination of Frederine with doxorubicin did not lead to a more pronounced weight loss than doxorubicin alone, whereas mice receiving Frederine alone did not show a significant weight loss. On day 15 of the experiment, both the doxorubicin-treated group and the Frederine-doxorubicin-treated groups had recovered from their weight loss. The Frederine-treated group showed a weight gain similar to the control group. However, when Frederine was administered 1 h before doxorubicin, Frederine significantly reduced the growth delay caused by doxorubicin alone (day 39, \( P < 0.05 \)).

**DISCUSSION**

The involvement of free radicals in the mechanism of doxorubicin-induced cardiotoxicity has been the subject of a number of reviews (1, 22–25). Several mechanisms have been suggested for doxorubicin-induced cardiotoxicity, but the free radical theory is still the most favored. Doxorubicin can generate oxygen radicals in several ways, either by itself or by forming a complex with iron ions. Production of radicals is generally attributable to redox cycling of doxorubicin. The fact that the antioxidant and iron chelator monoHER can protect against the doxorubicin-induced cardiotoxicity underlines this theory.

Because a relatively high dose of monoHER is needed to provide complete protection (500 mg/kg, in mice), we have recently synthesized a series of new flavonoids, using monoHER as a lead compound. The combination of parameters such as antioxidant activity (lipid peroxidation), cytotoxicity, and cardioprotection against doxorubicin in vitro (isolated mouse left atrium model) is thought to be an efficient method for the screening of potential cardioprotectors. From this consecutive set of in vitro assays, the synthetic flavonoid Frederine has been selected. The antioxidant activity was established using LPO and trolox equivalent antioxidant capacity assays. Frederine is 5 times more potent in the LPO assay, provides similar protection against doxorubicin in the isolated mouse left atrium, and shows a slightly higher lactate dehydrogenase leakage and a slightly larger decrease in reduced glutathione concentration in hepatocytes, when compared with monoHER. The present study deals with possible toxic effects of Frederine in mice and its effect on the cardiotoxicity and antitumor activity of doxorubicin.

Frederine did not induce weight loss, significant changes in the relative organ weight, or visible changes to the organs when administered at the dose of 170 mg/kg, which is equimolar to 250 mg/kg monoHER. To the contrary, a significantly larger weight gain was observed with respect to controls, whereas heart, liver, and kidneys were not different from control non-treated organs. This weight gain could also be an indication for toxicity, because organ toxicity might lead to an increased organ toxicity.
weight and, thus, an increased body weight. Therefore, changes of relative organ weights are often used as an indication of organ toxicity (26, 27). As shown, the relative organ weights of heart, liver, and kidneys did not change. As a result, Frederine is considered to be safe at the dosing schedule used.

As a measure for cardiotoxicity, we have used the changes in ST interval, which correlate with the degree of cardiotoxicity in both rats (28) and mice (29). We previously validated (30) this model by correlating the increase in ST interval with the histological score using Billingham’s grading scale. The histological scores/individual mouse were found to be in agreement with the increase in ST interval (31). In a previous study, 500 mg/kg monoHER given as an i.p. injection 5 days a week was needed to completely prevent the cardiotoxicity in mice that was caused by 4 mg/kg doxorubicin given in six weekly i.v. injections. This treatment resulted in an increase in ST interval of only 1.7 ± 0.8 ms after 8 weeks versus 16.7 ± 2.7 ms for doxorubicin (P < 0.001, relative to doxorubicin; not significantly different from control; Ref. 5). A combination of doxorubicin with 100 mg/kg monoHER gave approximately 53% protection against the toxicity of doxorubicin (increase in ST interval, 7.8 ± 1.7 ms).

To compare the potency of Frederine with monoHER, we administered 68 mg/kg Frederine, which is equimolar to 100 mg/kg monoHER. At this dose, both a higher or lower potency of Frederine compared with monoHER would be identifiable. We found that 68 mg/kg Frederine given as an i.p. injection 5 times a week was able to completely protect against doxorubicin-induced cardiotoxicity. This treatment resulted in an increase in ST interval of only 1.7 ± 0.3 ms after 8 weeks (P < 0.001, relative to doxorubicin; not significantly different from control). Thus, 68 mg/kg Frederine provided at least the same protection as 500 mg/kg monoHER. This means that our new compound is at least 5 times more potent than monoHER. The increased potency for Frederine might be explained by the increased antioxidant potency compared with monoHER (IC_{50} values in the LPO assay for Frederine and monoHER were 2.8 ± 0.9 and 12.7 ± 3.7 μM, respectively), although Frederine did not show an increased protection on the isolated mouse left atrium. Other factors such as differences in pharmacokinetics, metabolism, or mechanisms of action may also play a role.

In the antitumor activity experiments with the human OVCAR-3 xenograft described here, 68 mg/kg Frederine administered 5 min before doxorubicin was found not to influence the growth inhibition of doxorubicin. In addition, Frederine did not increase weight loss caused by doxorubicin. However, if Frederine was administered 1 h before doxorubicin, it seemed to reduce the growth delay caused by doxorubicin alone. After 29 days, Frederine did not significantly influence the antitumor activity of doxorubicin, but after 39 days, a small but significant decrease in antitumor activity could be observed (P < 0.05). This is in contrast to the data obtained from the in vitro experiments, where Frederine did not affect the antitumor activity of doxorubicin in OVCAR-3 cells with both dosing intervals.

The effect on the growth delay of doxorubicin is not necessarily a drawback in the further development of Frederine as a cardioprotector. When Frederine was administered 5 min before doxorubicin, no significant effect on the antitumor activity was observed even after 39 days. This means that the dosing schedule could play an important role. Cardioprotective properties of Frederine could be investigated in mice using a 5-min dosing interval instead of the current interval of 1 h. Furthermore, it is possible that the lowest dose of Frederine that is needed to give complete protection is well below 68 mg/kg.

Doroshow (32, 33) reported that the antitumor activity of doxorubicin is also free-radical mediated, meaning that the ratio of the protector and doxorubicin determines the effect on the antitumor activity. A reduction of the dose of Frederine might abolish its possible effect on the antitumor activity of doxorubicin.

In summary, we found that Frederine is an attractive compound, suggesting that the consecutive set of assays (LPO, cytotoxicity, and isolated mouse left atrium model) used for the screening of these compounds is an efficient method for the selection of potential cardioprotectors. The selected compound Frederine is not toxic to mice under the tested conditions and is at least 5 times more potent as a cardioprotector than monoHER in mice. Frederine did not reduce the antiproliferative effects of doxorubicin in A2780, OVCAR-3, and MCF-7 cells in vitro and in OVCAR-3 xenografts grown in nude mice when administered 5 min before doxorubicin. Therefore, it can be concluded that Frederine merits further investigation as a possible protector against doxorubicin-induced chronic cardiotoxicity in cancer patients.

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