Identification of the Metabolites of the Antioxidant Flavonoid 7-Mono-O-(β-hydroxyethyl)-rutoside in Mice


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ABSTRACT:

The clinical use of the anticancer drug doxorubicin is limited by severe cardiotoxicity. In mice, the semisynthetic antioxidant flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) has been successfully used as a protector against doxorubicin-induced cardiotoxicity. However, most monoHER has already been cleared from the body at the time that doxorubicin concentrations are still high. This result suggests that not only the parent compound monoHER itself but also monoHER metabolites could be responsible for the observed cardioprotective effects in mice. Therefore, in the present study, we investigated the metabolism of monoHER in mice. Mice were administered 500 mg/kg monoHER intraperitoneally. At different time points after monoHER administration, bile was collected and analyzed for the presence of monoHER metabolites. The formed metabolites were identified by liquid chromatography-diode array detection-time of flight-mass spectrometry. Thirteen different metabolites were identified. The observed routes of monoHER metabolism are methylation, glucuronidation, oxidation of its hydroxyethyl group, GSH conjugation, and hydrolysis of its disaccharide. In line with other flavonoids, methylated monoHER and the monoHER glucosides are expected to have relatively high cellular uptake and low clearance from the body. Therefore, these metabolites might contribute to the observed protection of monoHER against doxorubicin-induced cardiotoxicity.

Introduction

Doxorubicin is a very effective antitumor agent, widely used in the treatment of different types of cancer. Unfortunately, treatment with doxorubicin is limited by a cumulative dose-dependent cardiotoxicity, which manifests itself as congestive heart failure (Singal and Iliskovic, 1998; Lipshultz et al., 2005; Bast et al., 2007). Although the mechanism of doxorubicin-induced cardiotoxicity is still not fully understood, the formation of free radicals by doxorubicin semiquinones seems to be implicated (Horenstein et al., 2000; Xu et al., 2001). Cardiac tissue is particularly vulnerable to free-radical-induced injury because its antioxidant protection by enzymes such as superoxide dismutase and catalase is markedly reduced compared with that of other tissues in the body (Doroshov et al., 1980; Julicher et al., 1988; Iarussi et al., 2000).

The semisynthetic flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) is a constituent of the registered drug Venoruton, which is used in the treatment of chronic venous insufficiency (Petruzelli et al., 2002). In a series of structurally related flavonoids, monoHER was found to be the most potent antioxidant (Haenen et al., 1993). As for most flavonoids, monoHER consists of three rings referred to as the A, B, and C rings (Fig. 1). It also contains a 3',4'-catechol moiety in the B ring and a C2–C3 double bond and 4-oxo function in the C ring, which contribute to its high antioxidant activity. Further characteristic structural features of monoHER are the rutinose group (glucose + rhamnose) at the 3-O position in the C ring and the hydroxyethyl group at the 7-O position in the A ring.

Preclinical experiments in mice have shown that monoHER administration before doxorubicin effectively protects against doxorubicin-induced cardiotoxicity, without interfering with its antitumor activity (van Acker et al., 1997, 2000). Of interest, the half-lives of monoHER in plasma and heart tissue (11.8 and 16.2 min, respectively) are much lower than those of doxorubicin (12.6 and 11.5 h, respectively) (Abou El Hassan et al., 2003a,b). In addition, monoHER cannot be detected for longer than 2 h after administration, whereas doxorubicin is present in plasma for at least 48 h after administration. This result means that monoHER has already been cleared from the body at the time that doxorubicin concentrations are still relatively high and suggests that not only the parent compound monoHER itself but also monoHER metabolites might be responsible for the observed cardioprotective effects in mice. Moreover, evidence with other polype-
nols that metabolites may mediate or substantially contribute to the pharmacological efficacy of the parent molecule is accumulating, as is observed with flavonoids in grapes and wine (Gescher and Steward, 2003; Forester and Waterhouse, 2009). Therefore, identifying the metabolites of compounds is of great importance. Up to the present no metabolites were found in plasma or urine from mice and humans in our studies with monoHER (Abou El Hassan et al., 2003b; Willems et al., 2006). Earlier studies with radiolabeled monoHER in mice and rats reported that the liver was the main drug-eliminating organ. A major unidentified metabolite was found in bile, which was suggested to be a glucuronide of monoHER (Barrow and Griffiths, 1974; Hackett and Griffiths, 1977a). In addition, enterohepatic cycling has been described previously (Hackett and Griffiths, 1977b).

To further elucidate the protective effect of monoHER, the objective of this study was to investigate the metabolism of monoHER in mice and characterize its metabolites. This was realized by analyzing the bile fluid of mice that received monoHER for the presence of monoHER and possible monoHER metabolites using LC-DAD-TOF-MS analysis.

**Materials and Methods**

**Chemicals.** 7-Mono-O-(β-hydroxyethyl)-rutioside was 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. Catechol-O-methyltransferase (COMT), S-adenosyl-L-methionine (SAM), dithiothreitol, and MgCl₂ were obtained from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA) and NaOH were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile HPLC grade, methanol, and dimethyl sulfoxide were obtained from Biosolve (Valkenswaard, The Netherlands).

**Animals.** Eighteen male BALB/c mice (8 weeks old, 20–25 g) purchased from Charles River (Maastricht, The Netherlands) were kept in a light- and temperature-controlled room (21–22°C, humidity 60–65%). The animals were fed a standard diet and were allowed to eat and drink tap water ad libitum. The animals were obtained from Biosolve (Valkenswaard, The Netherlands).

**Experimental Design.** The protocol was approved by the Ethics Committee for Animal Experiments of Maastricht University (Maastricht, The Netherlands). The mice were administered 500 mg/kg monoHER intraperitoneally. At different time points after monoHER administration (15, 60, 120, 180, and 240 min), three mice were sacrificed, and the gallbladder was collected. One group of mice received no monoHER; the bile of these mice served as a blank.

**Sample Preparation.** Each gallbladder was perforated and mixed with 50 μl of dimethyl sulfoxide-methanol (1:4, v/v). The mixture was vortexed and centrifuged (17,060g, 15 min). The supernatant was removed and injected onto the LC column.

**LC-DAD Analysis.** LC-DAD of the bile samples was performed on an Agilent 1100 series LC-DAD system (Agilent Technologies, Santa Clara, CA). Analytical separations were achieved using an ODS-3 column (5 μm, 250 × 3 mm) (Inertsil; Varian Inc., Palo Alto, CA) at 40°C. The mobile phase consisted of ultrapure water containing 0.1% (v/v) TFA (mobile phase A) and acetonitrile containing 0.1% (v/v) TFA (mobile phase B). The gradient was started at t = 0 min with 85% (v/v) A, was changed linearly over the first 20 min to 30% (v/v) B followed by an increase to 90% (v/v) B at t = 25 min, and was then stationary for 1 min. The column was reequilibrated with 15% (v/v) B for 5 min. A flow rate of 1 ml/min and an injection volume of 10 μl were used. Detection was performed with a DAD. The chromatograms presented are based on detection at 355 nm.

**LC-DAD-TOF-MS Analysis.** LC-DAD-TOF-MS, including fragmentation and exact mass measurements, was performed using an Agilent 1100 LC-DAD-TOF-MS system (Agilent Technologies) to further characterize the peaks in the LC chromatogram. The UV signal at 355 nm was collected. Electrospray ionization was performed in positive mode with the following conditions: m/z range 50 to 3000, 175 V fragmentor (varied for fragmentation experiments), 0.1 m/z step size, 1,013 cycles/s, 350°C drying gas temperature, 10 liters of N₂/min drying gas, 35 psig nebulizer pressure, and 2 kV capillary voltage. The MS data were collected using internal reference mass correction. The reference substances [purine at 121.050873 Da and hexakis-(1H,1H,3H-tetrafluoro-pentoxy)phosphazene (HPK-921) at 922.09798 Da] were constantly injected in the electrospray ion source equipped with a dual-sprayer mechanism. The LC conditions were the same as described above. The LC-DAD-TOF-MS system was controlled using MassHunter qualitative analysis software (B03.01; Agilent Technologies).

Elemental composition calculations from the exact masses were performed off-line using MassHunter. This software was used to work with the spectrum manually generated for every peak. Potential assignments were calculated using the monoisotopic masses with specifications of a tolerance of 10 ppm deviation. The number and types of expected atoms were set as follows: carbons <60, hydrogens <120, oxygens <30, nitrogens <30, and sulfurs <5, whereas the double bond equivalent was set to <50.

**Quantification of MonoHER Metabolites.** Quantification of the different metabolites was achieved by their UV response at 355 nm. Quantification of metabolites is normally achieved using external calibration with reference standards. However, no commercial reference standards were available for these metabolites. Therefore, it was assumed that the UV response at 355 nm is equal to that of monoHER for all metabolites. This is a reasonable assumption because the UV spectra in the 355 nm region of all metabolites are similar to that of monoHER.

The relative amounts of the metabolites were estimated by calculating the area under the curve (AUC) from the concentration-time curve of each metabolite, using the trapezoidal rule, and expressing this as percentage of the total AUC of all detected compounds together.

**Synthesis of 4'-Methyl-MonoHER.** MonoHER was enzymatically methylated using the enzyme COMT and the methyl donor SAM. The reaction mixture for the enzymatic O-methylation of monoHER consisted of 100 μM monoHER, 20 μM of COMT, 1 mM SAM, 1.2 mM MgCl₂, and 1 mM dithiothreitol in a final volume of 1 ml of Tris-HCl buffer. The mixture was incubated at 37°C for 24 h and then analyzed by the LC-DAD procedure described above. 1H NMR analysis identified the formed metabolite as 4'-methyl-monoHER. To verify the presence of 4'-methyl-monoHER in the collected bile samples, bile fluid was spiked with this metabolite and analyzed by LC-DAD.
**Results**

Bile samples collected after monoHER administration contain several compounds that are absent in the blank bile (Fig. 2). The DAD spectra of these compounds and the absorption maxima at 355 nm are similar to those of monoHER, indicating that these compounds are metabolites of monoHER. LC-DAD-TOF-MS was used to further identify these metabolites. Six major metabolites (M1–M6), representing more than 90% of all detected compounds, and seven minor metabolites (M7–M13) were detected. The mass ([M + H]+), main fragment ions, and the proposed compound name of each metabolite are listed in Table 1. In Table 2, the exact mass determination data and the corresponding chemical formulas are summarized.

**Parent.** The peak in the LC chromatogram with a retention time of 8.5 min elutes at the same retention time as the parent compound monoHER. LC-MS analysis revealed m/z of 655, which corresponds to a molecular mass of 654 Da (Table 1). Tandem mass spectrometry fragmentation produced three key fragments: m/z 509 (−145, loss of rhamnose sugar), m/z 347 (−307, loss of rhamnose and glucose sugar), and m/z 303 (−351, loss of rhamnose, glucose, and the hydroxyethyl group of the A ring) (Fig. 1). This fragmentation pattern is similar to the fragmentation of monoHER. In addition, the elemental composition (C29H34O17) generated by exact mass measurements (Table 2) is identical to that of the parent compound, identifying this compound as monoHER.

**Major Metabolites.** M1 and M2. Metabolites M1 and M2 have an m/z 176 Da higher than monoHER. This finding indicates that the metabolites are glucuronidated derivatives of monoHER. In addition, the fragments are similar to that of monoHER, only 176 Da higher (Table 1). The chemical formula (C30H36O17) generated by exact mass measurements confirms the formation of monoHER glucuronide (Table 2).

M3. LC-MS analysis revealed that the main fragments of metabolite M3 are 14 Da higher than monoHER (m/z 523 and 361) (Table 1). The chemical formula (C29H33O17) indicates that this metabolite has two hydrogen atoms less and one oxygen atom more (−H + O) than monoHER (Table 2). This finding points to the oxidation of the hydroxyethyl group in the A ring of monoHER to a carboxyethyl group, thereby generating 7-mono-O-(β-carboxyethyl)-rutoside (monoCER).

M4. The main fragments of metabolite M4 are 14 Da higher than monoHER (m/z 523 and 361) (Table 1). The chemical formula (C29H34O17) generated by exact mass measurements confirms the formation of monoHER glucuronide (Table 2). This peak as methyl-monoHER glucuronide.

M5. Metabolite M5 shows main fragments similar to monoHER, only 14 Da higher (m/z 523, 361, and 317) (Table 1). On the basis of the mass spectral data and the chemical formula (C29H34O17), this metabolite was identified as the methylated metabolite of monoHER (Table 2). Moreover, the peak area of M5 in the chromatogram

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TABLE 1

<table>
<thead>
<tr>
<th>t_R (min)</th>
<th>Compound</th>
<th>[M + H]+ (m/z)</th>
<th>Mass Change from Parent</th>
<th>Main Fragment Ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>MonoHER</td>
<td>655</td>
<td>0</td>
<td>509, 347, 303</td>
</tr>
<tr>
<td>M1</td>
<td>MonoHER glucuronide</td>
<td>831</td>
<td>+176</td>
<td>765, 685, 523, 347, 303</td>
</tr>
<tr>
<td>M2</td>
<td>MonoHER glucuronide</td>
<td>831</td>
<td>+176</td>
<td>765, 685, 523, 347, 303</td>
</tr>
<tr>
<td>M3</td>
<td>Methyl-monoHER</td>
<td>845</td>
<td>+190</td>
<td>699, 537, 361, 317</td>
</tr>
<tr>
<td>M4</td>
<td>MonoCER</td>
<td>669</td>
<td>+14</td>
<td>523, 463, 361</td>
</tr>
<tr>
<td>M5</td>
<td>Methyl-monoHER</td>
<td>669</td>
<td>+14</td>
<td>523, 361, 317</td>
</tr>
<tr>
<td>M6</td>
<td>Methyl-monoCER</td>
<td>683</td>
<td>+28</td>
<td>537, 375</td>
</tr>
<tr>
<td>M7</td>
<td>Dimethyl-monoHER</td>
<td>537</td>
<td>−118</td>
<td>375</td>
</tr>
<tr>
<td>M8</td>
<td>Methyl-monoCER</td>
<td>859</td>
<td>+204</td>
<td>551, 375</td>
</tr>
<tr>
<td>M9</td>
<td>Dimethyl-monoHEG</td>
<td>523</td>
<td>−132</td>
<td>479, 361, 303</td>
</tr>
<tr>
<td>M10</td>
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<td>845</td>
<td>+190</td>
<td>537, 361</td>
</tr>
<tr>
<td>M11</td>
<td>Dimethyl-monoHER</td>
<td>859</td>
<td>+204</td>
<td>551, 375</td>
</tr>
<tr>
<td>M12</td>
<td>Dimethyl-monoHEG</td>
<td>537</td>
<td>−118</td>
<td>375</td>
</tr>
<tr>
<td>M13</td>
<td>Dimethyl-monoCER</td>
<td>683</td>
<td>+28</td>
<td>537, 375</td>
</tr>
<tr>
<td>M14</td>
<td>GSH-monoHER</td>
<td>960</td>
<td>+305</td>
<td>814, 705, 652, 523, 371</td>
</tr>
<tr>
<td>M15</td>
<td>Methyl-monoCER</td>
<td>1007</td>
<td>+352</td>
<td>861, 845, 831, 699, 523, 347</td>
</tr>
<tr>
<td>M16</td>
<td>Methyl-diHER</td>
<td>523</td>
<td>−132</td>
<td>479, 361, 303</td>
</tr>
<tr>
<td>M17</td>
<td>DiHER</td>
<td>699</td>
<td>+44</td>
<td>655, 523, 347</td>
</tr>
</tbody>
</table>

**Minor Metabolites.** M7, M8, M9, M10, M11, M12, M13, M14, M15, M16, and M17 are listed in Table 1. In Table 2, the exact mass determination data and the corresponding chemical formulas are summarized.

**Impurities/metabolites.** M14, M15, M16, and M17 are listed in Table 1. In Table 2, the exact mass determination data and the corresponding chemical formulas are summarized.

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**FIG. 2.** Representative LC chromatograms of (A) blank bile and (B) bile collected from mice 60 min after monoHER administration. The numbered peaks represent monoHER [parent (P)] and its six major metabolites (M1–M6). The chromatograms presented are based on detection at 355 nm.
increased after the bile sample was spiked with enzymatically synthesized 4'-methyl-monoHER.

**M6.** Metabolite M6 has an $m/z$ 28 Da higher than monoHER (Table 1). From its chemical formula (C$_{36}$H$_{42}$O$_{24}$) it was deduced that the hydroxyethyl group in the A ring was oxidized together with the methylation of one of the hydroxyl groups of the catechol of monoHER (Table 2). This metabolite was therefore identified as methyl-monoCER.

**Minor Metabolites.** M7. LC-MS analysis of metabolite M7 shows an $m/z$ 305 Da higher than monoHER, which is indicative of GSH conjugation (Table 1). The peak area of M7 in the chromatogram increased after the bile sample was spiked with the synthetic reference standard, 2'-GSH-monoHER (Jacobs et al., 2009), identifying the peak as such.

**M8.** Metabolite M8 shows an $m/z$ 352 (or 2 × 176) Da higher than the parent compound (Table 1). This indicates the addition of two glucuronic acid groups to monoHER and identifies this metabolite as methyl-monoHER diglucuronide.

**M9.** Metabolite M9 shows an $m/z$ 146 Da lower than methylated monoHER (Table 1). The chemical formula (C$_{29}$H$_{32}$O$_{18}$) shows the loss of C$_6$H$_{10}$O$_4$ (rhamnose sugar) compared with methyl-monoHER (Table 2). This can be explained by hydrolisis of the sugar group at the B ring of methyl-monoHER, thereby generating methyl-7-mono-O-(β-hydroxyethyl)-glucoside (methyl-monoHEG).

**M10.** On the basis of the exact mass determination data and the corresponding chemical composition (C$_{30}$H$_{38}$O$_{18}$) (Table 2), metabolite M10 was identified as monoCER glucuronide.

**M11.** LC-MS analysis of metabolite M11 shows the same main fragments as monoCER-glucuronide, only 14 Da higher ($m/z$ 551 and 375) (Table 1). On the basis of the mass spectral data and its chemical composition (C$_{32}$H$_{40}$O$_{18}$) (Table 2), this metabolite was identified as methyl-monoCER glucuronide.

**M12.** Metabolite M12 shows an $m/z$ 146 Da lower than dimethyl-monoHER (Table 1). Accurate mass measurements indicate the loss of the rhamnose sugar (C$_{6}$H$_{10}$O$_{4}$) compared with dimethyl-monoHER (Table 2). This metabolite was therefore identified as dimethyl-monoHEG.

**M13.** Metabolite M13 shows an $m/z$ 28 Da higher than the parent compound (Table 1). The chemical formula (C$_{32}$H$_{36}$O$_{17}$) generated by exact mass measurements shows the addition of two methyl groups (+2 × CH$_3$) (Table 2), identifying this metabolite as dimethyl-monoHER.

**Impurities/Metabolites.** Besides monoHER itself and monoHER metabolites, other compounds were also detected in bile, accounting for less than 0.5% of all compounds. On the basis of their mass spectral data, these compounds were identified as impurities of monoHER, and metabolites of these impurities, i.e., diHER (M14), triHER (M15), methyl-diHER (M16), and dimethyl-diHER (M17). MonoHER was isolated from the hydroxyethylrutoside mixture Venoruton (consisting of monoHER, diHER, triHER, and tetraHER). Analysis of monoHER used in the present study confirmed the presence of diHER and triHER in very small amounts.

**Time Course of MonoHER Metabolite Formation.** As shown in Fig. 3, the concentrations of most metabolites peaked at 60 min after monoHER administration. Exceptions are the metabolites in which the disaccharide has been hydrolyzed, i.e., the glucosides methyl-monoHEG (M9) and dimethyl-monoHEG (M12). They are gradually formed over time, and 240 min after monoHER administration their concentrations are higher than those of the other metabolites.
FIG. 4. Proposed metabolism of monoHER in mice. The percentages in parentheses indicate the relative amounts of each metabolite. The relative amounts of the metabolites were estimated by calculating the AUC from the concentration-time curve of each metabolite, using the trapezoidal rule, and expressing this as a percentage of the total AUC of all detected compounds together.
Discussion

The main objective of the present study was to completely characterize the metabolites of the semisynthetic antioxidant flavonoid monoHER in mice. Because the liver is the main drug-eliminating organ, characterization was done in the bile fluid of mice. Our results show that monoHER is extensively metabolized. A scheme of the identified metabolites is shown in Fig. 4. The observed routes of monoHER metabolism are methylation, glucuronidation, oxidation of its hydroxyethyl group, GSH conjugation, and hydrolysis of its disaccharide.

The primary metabolic route appeared to be methylation, yielding a 4′-O-methylated metabolite of monoHER (M5) that represents approximately 40% of all detected compounds. The O-methylation of catechols is mainly catalyzed by the enzyme COMT (Zhu, 2002). It has been shown that methylation of flavonoids makes them more lipophilic, thereby improving their transport over biological membranes and increasing their cellular uptake (Spencer et al., 2003, 2004). Moreover, blocking the free hydroxyl group by methylation prevents other conjugation reactions such as glucuronidation at this group and thus increases the metabolic stability of flavonoids (Walle, 2009). Methylation of the hydroxy group also decreases the antioxidant capacity of flavonoids (Spencer et al., 2003). However, this decreased activity is compensated for by their increased intracellular concentration and metabolic stability (Spencer et al., 2003). Moreover, after their uptake into the cells, cytochrome P450-dependent demethylation can take place, thereby generating the parent compound (Breinholt et al., 2002). In addition, their high concentration formed and their relatively slow elimination suggests that the O-methylated metabolites of monoHER contribute to the antioxidant effect of monoHER.

Several hours after monoHER administration, the glucosides methylmonoHEG (M9) and dimethyl-monoHEG (M12) have the highest concentrations of all metabolites. In addition to methylation, the rhannose sugar has been removed in these metabolites. The time course of their formation indicates that they are primarily formed out of methyl-monoHER (M5) (Fig. 3). These metabolites contain a terminal glucose moiety. Flavonoid glucosides are suggested to enter cardiac cells via the glucose transporter 4 (Strobel et al., 2005; Passamonti et al., 2009), which transports glucose in the heart (Huang and Czech, 2007). Because cellular access is required to exert a protective effect, the methylated metabolites of monoHER as well as the monoHER glucosides are believed to contribute to the protective effect of monoHER.

The monoHER metabolites formed out of the three conjugation reactions, i.e., glucuronidation, GSH conjugation, and oxidation, are charged at physiological pH. These metabolites therefore probably have decreased cellular uptake and are unlikely to be involved in the protective effect of monoHER. In addition, Spencer et al. (2003) reported that the glucuronidated metabolites of flavonoids are unable to protect against oxidative stress-induced damage, presumably because of their inability to enter cells. The presence of GSH-monoHER conjugates indicates that monoHER has functioned as an antioxidant. GSH-monoHER formation is preceded by the oxidation of the catechol of monoHER, e.g., when it acts as an antioxidant by scavenging free radicals. Relatively low amounts of the GSH-monoHER conjugate were found, probably because there was no oxidative stress in these mice and because, as recently shown, the monoHER quinone reacts with the antioxidant ascorbate rather than with GSH (Jacobs et al., 2010).

To summarize, it has been observed that even though monoHER is practically cleared from the body at the time that doxorubicin concentrations are still relatively high, the antioxidant monoHER does protect against doxorubicin-induced cardiotoxicity in mice (van Acker et al., 2000). Three possible explanations can be given for this prolonged protective effect: first, it could mean that to have a protective effect monoHER only has to be present when the doxorubicin concentration peaks, i.e., shortly after doxorubicin administration; second, monoHER could have a “memory effect” on cells by inducing a protection that lasts after the monoHER has been cleared from the body; or third, as the results of the present study suggest, monoHER metabolites might contribute to the observed cardioprotective effects. Although further research is needed, the methylated metabolites and the glucosides in particular are expected to contribute to the antioxidant activity of monoHER, which is involved in its protective effect against doxorubicin-induced cardiotoxicity.

Authorship Contributions

Participated in research design: Jacobs, van der Vijgh, Bast, and Haenen.
Conducted experiments: Jacobs, den Hartog and Haenen.
Contributed new reagents or analytic tools: Jacobs and Peters.
Performed data analysis: Jacobs and Peters.
Wrote or contributed to the writing of the manuscript: Jacobs, Peters, den Hartog, van der Vijgh, Bast, and Haenen.

References


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