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Flavonoids as protectors against doxorubicin cardiotoxicity: role of iron chelation, antioxidant activity and inhibition of carbonyl reductase

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Anthracyclines, cardioprotection, iron chelators, hydroxyl radical scavenging, lipid peroxidation, doxorubicinol

Abstract

Anthracycline antibiotics (e.g. doxorubicin and daunorubicin) are among the most effective and widely used anticancer drugs. Unfortunately, their clinical use is limited by the dose-dependent cardiotoxicity. Flavonoids represent a potentially attractive class of compounds to mitigate the anthracycline cardiotoxicity due to their iron-chelating, antioxidant and carbonyl reductase-inhibitory effects. The relative contribution of various characteristics of the flavonoids to their cardioprotective activity is, however, not known. A series of ten flavonoids including quercetin, quercitrin, 7-mono-hydroxyethylrutoside (monoHER) and seven original synthetic compounds were employed to examine the relationships between their inhibitory effects on carbonyl reduction, iron-chelation and antioxidant properties with respect to their protective potential against doxorubicin-induced cardiotoxicity. Cardioprotection was investigated in the neonatal rat ventricular cardiomyocytes whereas the H9c2 cardiomyoblast cells were used for cytotoxicity testing. Iron chelation was examined via the calcein assay and antioxidant effects and site-specific scavenging were quantified by means of inhibition of lipid peroxidation and hydroxyl radical scavenging activity, respectively. Inhibition of carbonyl reductases was assessed in cytosol from human liver. None of the flavonoids tested had better cardioprotective action than the reference cardioprotector, monoHER. However, a newly synthesized quaternary ammonium analog with comparable cardioprotective effects has been identified. No direct correlation between the iron-chelating and/or antioxidant effect and cardioprotective potential has been found. A major role of carbonyl reductase inhibition seems unlikely, as the best two cardioprotectors of the series are only weak reductase inhibitors.

1. Introduction

Anthracyclines, of which doxorubicin is the leading compound, are among the most potent anticancer drugs, however, their use is limited by the risk of severe cardiotoxicity [1]. Various plausible hypotheses have been proposed to explain doxorubicin-induced cardiotoxicity (for reviews see [2,3]). It is generally thought to be caused by free radicals generated during redox cycling of doxorubicin and/or cardiotoxic action of doxorubicinol, a C13-dihydrometabolite of doxorubicin. Therefore, pharmacological agents which would be able to suppress the formation of both doxorubicinol and reactive oxygen species merit intense investigations. Flavonoids are a group of benzo- γ -pyron derivatives, naturally found in the diet, which exhibit numerous pharmacological properties that are beneficial for human health [4]. With respect to doxorubicin cardiotoxicity, their antioxidant activity, iron-chelating properties and inhibitory effects on carbonyl reductases are of interest. Evidence has been given that the flavonoids indeed have a strong potential to relieve doxorubicin-induced cardiac side-effects [5,6,7]. However, it is not fully elucidated yet, which of their pharmacological properties are essential for their cardioprotective action.

In vivo, doxorubicin undergoes a two-electron NADPH-dependent reduction to its C13-dihydrometabolite (doxorubicinol), which has been shown to be more cardiotoxic than the parent drug [8,9]. Several ubiquitous cytosolic enzymes, such as carbonyl reductases (CR) and aldo-keto reductases participate in the formation of C13-dihydroanthracyclines. CRs, in particular, seem to play an important role in anthracycline-induced cardiotoxicity [10,11,12]. Flavonoids like quercetin or rutin are known inhibitors of CR [13] and might therefore act as pharmacological inhibitors of doxorubicinol formation.

Doxorubicin generates reactive oxygen species (ROS), which have been suggested to play an important role in the development of cardiotoxicity [3]. Free radical scavengers have therefore been proposed to protect cardiac tissue from doxorubicin-induced oxidative stress and thus to

relieve its cardiotoxicity. Most of the flavonoids possess excellent antioxidant properties and the relationships between their structure and antioxidant activity have been well described [14].

Whereas common antioxidants inactivate ROS only after they have been formed, iron chelators are able to prevent their formation. Iron can redox-cycle between its two redox states - Fe^{2+} and Fe^{3+} - and acts as a catalyst of hydroxyl radical formation (Fenton and Haber-Weiss reactions). Iron chelation is considered to be an important tool to decrease anthracycline cardiotoxicity as documented by the beneficial effect of dexrazoxane [15] as well as other chelators of iron [16,17]. In flavonoids, the antioxidant and iron chelating properties are closely related and their activity may include two steps - iron is first chelated by the flavonoid and the ROS which are formed in its vicinity are subsequently scavenged by the flavonoid. In this way, the radicals are quenched at the same place where they are formed. This concept has been called site-specific scavenging [18].

For this comparative study, we have used a series of ten differently substituted flavonoids (Fig.1), of which seven were newly synthesized [19] and three were commercially available. Some of these compounds have been well-characterized by now, for example 7-mono-hydroxyethylrutoside has already proved to be highly efficient against the cardiotoxicity of doxorubicin [5,20] and has recently entered phase II clinical trials. Quercetin and its *O*-glucoside, quercitrin, also represent extensively studied flavonoids being among the most common dietary polyphenols and the components of various food nutrients. The flavonoids were synthesized with the objective to discern the structural requirements that are essential for a good cardioprotective activity of the flavonoids.

The aim of this work was to describe and compare the inhibitory effects of the flavonoids on doxorubicin carbonyl reduction, their iron-chelating and antioxidant properties as well as their general toxicity: all in relation to their molecular structure. Most importantly, we aimed to

assess, which of those features are especially important for the cardioprotective effect of the flavonoids.

2. Materials and Methods

2.1. Chemicals

7-monoxyethylrutoside (monoHER) was kindly provided by Novartis Consumer Health, Nyon, Switzerland, quercetin and quercitrin were obtained from Sigma-Aldrich, Prague, Czech Republic and the substituted flavonoids were synthesized as described elsewhere [19]. Formulated doxorubicin (doxorubicin hydrochloride 2 mg.mL⁻¹) was obtained from TEVA (Pharmachemie B.V., Haarlem, The Netherlands). Doxorubicinol was a kind gift from Assoc. Prof. Bruce G. Charles (University of Queensland, Brisbane, Australia). SIH was obtained from Prof. P. Ponka (Mc Gill University, Montréal, Canada). All other chemicals were of the highest grade available.

2.2. Cell culture

Primary cardiomyocyte cultures were prepared from 2-day-old neonatal Wistar rats (BioTest, Konárovice, Czech Republic) according to Vlasblom et al. [21]. All the procedures have been conducted in accordance with the Declaration of Helsinki and approved and supervised by the Ethical Committee of the Faculty of Pharmacy in Hradec Králové, Charles University in Prague. The animals were anaesthetized with CO₂ and decapitated. The chests were opened and the hearts were collected in an ice-cold ADS buffer. The ventricles were thoroughly minced and serially digested with a mixture of collagenase II (0.25 mg.mL⁻¹; Gibco) and pancreatin (0.4 mg.mL⁻¹; Sigma) solution at 37°C. The obtained cell suspension was placed on a 15-cm covered Petri dish and left for 2 hours at 37°C in order to separate the myocytes

(floating in the medium) from the fibroblasts (attached to the dish). The myocyte suspension was collected and viable cells counted using trypan blue exclusion. Cells were plated on the gelatine-coated 12-well plates at a density of 800,000 cells per well in the DMEM/F12 (1:1) growth medium containing 10% horse serum, 5% FCS, 1% Pen/Strep and 4% sodium pyruvate. After 40 h, the medium was renewed and the serum concentration was lowered to 5% (FCS). The medium was changed once more after another 24 h and the experiments were performed at the 4th day after the isolation using both serum and pyruvate-free medium. For ethical reasons, primary cardiomyocytes were only used in a few major experiments (cardioprotection assessment) whereas they were replaced by rat cardiomyoblast cell line (H9c2) for routine cytotoxicity screening. The H9c2 cell line (ATCC, Manassas, VA, USA) was maintained in DMEM supplemented with 10% heat-inactivated FBS (Cambrex Inc., Walkersville, USA), 100 U.mL⁻¹ of penicillin and 100 µg.mL⁻¹ of streptomycin (PAA, Pasching, Austria) and grown under humidified atmosphere containing 5% CO₂ at 37°C. Cell passages between 25 and 40 were used for cytotoxicity experiments.

2.3. LDH leakage assay

The cardiomyocytes were preincubated with the test compounds dissolved in DMSO (0.2% final concentration) for 30 min. Thereafter, 1 µM of doxorubicin was added and the medium was sampled after 24, 48 and 72 hours. Activity of released LDH was assayed in Tris-HCl buffer pH 8.9 containing 35 mM of lactic acid (Sigma Aldrich, Prague, Czech Republic) and 5 mM of NAD⁺ (MP Biomedicals, Irvine, CA, USA). The rate of NAD⁺ reduction was monitored spectrophotometrically at 340 nm (Helios β, Unicam). LDH activity was calculated using molar absorption coefficient $\epsilon = 6.22 \cdot 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

2.4. Neutral red uptake (NRU) cytotoxicity assay

In order to assess the cytotoxicity of the tested flavonoids to the target cells, the viability of the H9c2 rat cardiomyoblast cell line was assayed by the NRU method. The cells were plated in flat-bottom microtiter plates at a cell density of 10,000 cells per well. After a 24-hour preincubation period, cells were incubated with the flavonoids for another 48 or 96 hours and tested for viability. Briefly, 40 $\mu\text{g}\cdot\text{mL}^{-1}$ of neutral red (Sigma-Aldrich, Prague, Czech Republic) was added to each well. After incubation for 3 h at 37° C, the supernatant was discarded, cells were fixated with 1% CaCl_2 in 0.5% formaldehyde for 15 min, washed twice with PBS and solubilized with 1% acetic acid in 50% ethanol. The optical density of soluble neutral red was measured at 540 nm using a BioRad microplate reader. Cytotoxicity was expressed as the IC_{50} values, which were determined using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA, USA).

2.5. Preparation of cytosolic fractions

The human liver samples from five male (18, 24, 52, 56 and 60 years old) and one female (55 year old) donors were obtained from the Cadaver Donor Program of the Transplantation Centre of the Faculty of Medicine, Charles University, Hradec Králové. Cut part of liver (*lobus hepatis sinister*) in ice-cooled Eurocollins solution was transported from the hospital to the laboratory (less than 30 min) and stored in the freezer (-80°C). Frozen liver samples were thawed at room temperature (up to 15 min) and homogenized at a 1:6 (w/v) ratio in 0.1 M sodium phosphate buffer, pH 7.4, using a Potter-Elvehjem homogeniser and sonication with Sonopuls (Bandeline, Germany). The cytosolic fractions were isolated by fractional ultracentrifugation of the resulting homogenate (the first 105,000 x g supernatant was considered the cytosolic fraction). Protein concentration was assayed using the bicinchoninic acid method [22] and the cytosolic fractions were stored at -80°C .

2.6. Doxorubicin carbonyl reduction assay

Cytosolic carbonyl reductases (CR) catalyze the formation of the most characteristic metabolite of doxorubicin – C13-dihydrodoxorubicin (doxorubicinol). We have therefore followed the rate of doxorubicinol formation in human liver cytosol to describe the potency of various flavonoids to inhibit CR. Unless otherwise indicated, C13 carbonyl reduction of doxorubicin was assayed by incubating 1 mg.mL⁻¹ of cytosolic fraction with 1 mM of doxorubicin in 50 mM sodium phosphate buffer (total volume of 150 µL) and the reaction was started with 0.5 mM NADPH (Serva, Heidelberg, Germany). To assess an inhibitory action of the flavonoids on carbonyl reduction, the test compounds were preincubated with the cytosolic fractions for 5 min prior to the addition of doxorubicin and NADPH. Control experiments were performed without biological material. The reactions were carried out at 37°C and stopped after 60 min by adding an equal volume (150 µL) of 0.2 M Na₂HPO₄ pH 8.4 while cooling the reaction mixture on ice. The anthracyclines were extracted with 1.2 mL of a 9:1 (v/v) chloroform/1-heptanol mixture. After 15 min of vigorous shaking, samples were centrifuged at 5,000 x g for 10 min to separate the layers. The lower organic phase was carefully removed to another microtube and re-extracted with 150 µL of 0.1 M *o*-phosphoric acid. After 1 min of vigorous shaking the upper aqueous layer was removed to a vial and subjected to the HPLC analysis [23]. The activity of the flavonoids was expressed as IC₅₀ being the concentration of the flavonoid causing 50% reduction in doxorubicinol formation.

2.7. Determination of doxorubicinol

Following the extraction, doxorubicin and doxorubicinol were separated and detected using the Agilent 1100 series HPLC system (Agilent Technologies, Inc.). Reverse-phase chromatography was performed with a Supelco Discovery C18 analytical column (15 cm x 4

mm, 5 μm) protected with a guard column. The analytes (25 μL) were isocratically eluted with a freshly prepared mobile phase consisting of 50 mM sodium phosphate buffer pH 4.0 and acetonitrile in a 75:25 (v/v) ratio. The flow rate was 1.5 $\text{mL}\cdot\text{min}^{-1}$. Under these conditions the substances eluted at 2.2 min (doxorubicinol) and 3.8 min (doxorubicin) as monitored spectrofluorimetrically with excitation wavelength of 480 nm and emission wavelength of 560 nm. Quantification of doxorubicinol was performed with the aid of a calibration curve constructed by using known concentrations of authentic doxorubicinol. All data are mean from at least 3 separate experiments performed in duplicates. Standard deviations were less than 10%.

2.8. Calcein assay for iron chelation

Chelator efficiency was determined using a calcein assay according to Cabantchik et al. [24] Fluorescence of free calcein (Molecular Probes, Eugene, OR, USA) was measured with a LS50B Perkin Elmer spectrofluorimeter equipped with a magnetic stirrer. The measurements ($\lambda_{\text{ex}} = 486 \text{ nm}$, $\lambda_{\text{em}} = 517 \text{ nm}$) were done at room temperature and recorded as a function of time. Due to high assay sensitivity low concentration of calcein were needed to monitor the displacement of iron from its complex with calcein and the concentration of the chelators therefore had to be adjusted accordingly to a lower level (5 μM) compared to the concentrations used in other assays described in this manuscript. Briefly, calcein (20 nM in HEPES-buffered saline, pH 7.2) was incubated for 1 h with ferrous ammonium sulphate (200 nM), after which at least 95% of fluorescence was quenched. The calcein-iron complexes were then exposed to test compounds (5 μM) for 500 s. After that, in order to obtain maximal dequenching, 5 μM of SIH was added and the reaction was followed for additional 500 s. Background fluorescence did not exceed 3% throughout the experiment. Data were analyzed using FL WinLab Software (Perkin Elmer) and normalized to the maximal fluorescence

attained by the treatment with SIH, a reference iron chelator, which was previously shown to efficiently shield the cellular labile iron pool and completely block the Fenton reaction-mediated oxidative damage [25].

2.9. Kinetics of displacement of iron from doxorubicin- Fe^{3+} complexes

The spectrophotometric assay was performed according to Hasinoff et al. [26]. Briefly, the doxorubicin- Fe^{3+} (3:1) complex was prepared by adding $FeCl_3$ in 15 mM HCl to doxorubicin solution. The resulting complex, which revealed a typical absorption band at $\lambda = 600$ nm (9-fold increase as compared to uncomplexed doxorubicin) was added to the reaction buffer (50 mM Tris / 150 mM KCl, pH = 7.4, room temperature) in the glass cuvette to yield final concentration of 45 μ M doxorubicin / 15 μ M Fe^{3+} . After the 3 minute equilibration period, tested flavonoids or reference chelators (SIH, EDTA) were added in an amount to obtain 100 μ M final concentration. The absorbance at $\lambda = 600$ nm was then followed for another 8 minutes.

2.10. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging capacity was assayed by the 2-deoxyribose (DR) method according to Halliwell et al. [27]. It is based on the competition between the flavonoids and 2-deoxyribose for hydroxyl radicals. Upon the reaction with hydroxyl radicals, 2-deoxyribose is degraded into a mixture of thiobarbituric acid (TBA)-reactive products, which can be quantified spectrophotometrically. The incubation mixture contained: H_2O_2 (2.8 mM), EDTA (when indicated, 100 μ M), 2-deoxyribose (2.8 mM), ascorbate (100 μ M) and ferric chloride (20 μ M). The concentrations of the flavonoids ranged from 100-500 μ M. The reaction mixtures were incubated at 37°C for 1 h. The reaction was stopped by the addition of TBA reagent, freshly prepared by mixing 9 parts of reagent A (2.8 mM TBA, 0.125 M HCl, 16.8%

(w/v) trichloroacetic acid) and 1 part of reagent B (1.5 mg.mL⁻¹ solution of butylated hydroxytoluene in ethanol). Subsequently, the reaction mixture was heated for 15 min at 99°C. After centrifugation (3000 x g, 7 min), the absorbance (532 nm) was measured. The second-order rate constant of the scavenger (k_s) with the hydroxyl radical can be calculated using a rate constant of $3.1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ for deoxyribose (k_{DR}). The absorbance at 532 nm (A) depends on the concentration of the scavenger ([S]) and the absorbance found without scavenger (A_0). The k_s value of the scavenger is obtained from the slope of the linear plot of the reciprocal value of A versus [S] and calculated as follows: $k_s = \text{slope} \times k_{\text{DR}} \times [\text{DR}] \times A_0$ [27].

2.11. Lipid peroxidation assay

LPO was assayed as described elsewhere [6]. Briefly, heat-inactivated microsomes from rat liver were incubated with ascorbate (200 μM) and ferrous sulphate (10 μM) at 37°C for up to 60 min. At $t = 0, 5, 10, 15, 30, 45$ and 60 min, an aliquot of 0.3 ml was mixed with 2 mL of TBA-trichloroacetic acid-HCl-butylated hydroxytoluene solution to stop the reaction. The reagent was prepared as described in the hydroxyl scavenging assay. After heating (15 min, 80°C) and centrifugation (15 min), the absorbance at 535 vs. 600 nm was determined. The IC_{50} was determined by measuring the percentage of LPO inhibition at several concentrations and calculating the concentration at which 50% inhibition was obtained.

2.12. Statistical analysis

Unless otherwise indicated, the data are given as the mean of at least three separate experiments \pm SD. One-way ANOVA with Tukey's post hoc test was performed to test for differences between groups using GraphPad Prism version 4.00 for Windows (GraphPad

Software, San Diego, CA). Pearson product moment correlation analyses were performed using SigmaStat for Windows version 3.0.1. (SPSS Inc. Chicago, IL).

3. Results

3.1. Assessment of cardiac protection

Using a model of neonatal rat ventricular cardiomyocytes we investigated the protective activity of the flavonoids against doxorubicin toxicity. Cellular damage was quantified via measurement of the time-dependent LDH release from the cells (Table 1). We found that after 24 h incubation of the cells with 1 μM of doxorubicin and 100 μM of the flavonoids, four compounds (F4, F5, F7, F10) offered more than 40% protection, four compounds (F2, F3, F6, F9) reached 20-30% protection whereas the two remaining flavonoids (F1, F8) were not protective. After 48 and 72 h incubation, only F4 and F7 maintained their high protective effect ($> 40\%$). An initial promising effect of F5 and F10 decreased dramatically, most probably due to their high intrinsic toxicity (see the cytotoxicity section, Table 2). Although the protective action of quercetin (F5) was still significant at 48 h, it disappeared completely after 72 h. On the other hand, the effect of moderate protectors – F2, F3, F6 and F9 was more stable during the incubation period, albeit weaker. In summary, none of the flavonoids was more effective than F4 (monoHER), F7 being the only one which had a comparable effect throughout the whole experiment (up to 72 h). The flavonoids F1 and F8, which lacked any protective properties from the beginning, and also F5 (quercetin) and F10, which showed a clear short-term protection that however quickly disappeared, are clearly the least interesting agents for further investigation as potential cardioprotectors.

3.2. Carbonyl reductase inhibition and cytotoxicity evaluation

First of all, we have optimized the method for doxorubicinol determination in our cytosolic samples using various substrate (doxorubicin) and coenzyme (NADPH) concentrations as well as incubation time points (Fig. 2). As a result we standardly used 1 mM of doxorubicin, 0.5 mM of NADPH and the incubations were stopped after 60 min, during which the reaction rate was linear. We have found that all of the tested flavonoids were capable of inhibiting doxorubicinol formation, although their potency strongly differed. The IC_{50} values for the inhibition of doxorubicinol formation are shown in Table 2. Quercetin and quercitrin (F5, F6) were the most effective inhibitors in the series of the present study. The only synthetic flavonoid with comparable potency was F10, indicating that neither the methylation of C3-OH nor the presence of C4-OH has influence on the CR inhibitory effect. However, the substitution of C3-OH with an aliphatic chain containing a quaternary ammonium moiety decreased the inhibitory effect (F9 vs. F10).

The viability of the H9c2 cells is shown as a function of either concentration of the flavonoids (Table 2) or time (Fig. 3). It is clear that the toxicity decreases with the degree of substitution of the hydroxyl groups, because the simplest structures, e.g. F1, F5 or F10, are the most toxic ones. Glycosylation (F2, F3, F4 and F6) or introduction of the charged and/or bulky moieties (F7, F8, and F9) lead to a decrease in the toxicity.

3.3. Iron chelation and antioxidant effects

All the tested flavonoids were found to chelate iron as demonstrated by their ability to displace iron from the iron-calcein complex. Nevertheless, their efficacy was considerably lower than the reference iron chelator SIH, which is able to displace iron from the iron-calcein complex both quickly and completely. The flavonoids did not vary strongly in their ability to

chelate iron and after 500 sec. of incubation, the activity of all the compounds reached from 12 to 39% of the maximal effect (Table 3, first column). The most efficient chelators of iron were F5 and F6. This can be explained by the presence of an additional iron-chelating moiety in their molecular structure (between 5-OH and 4-oxo groups) whereas other structures (with an exception of F4) appear to chelate iron only when the *o*-catechol group in ring B is present.

Although all the flavonoids under investigation removed iron from its complexes with calcein to some extent, none of them was capable of displacing iron from doxorubicin-iron complex in a fashion that is typical for strong chelators like SIH or EDTA (Fig. 4). This indicates higher affinity of Fe^{3+} to doxorubicin than to flavonoids.

The deoxyribose assay for hydroxyl radical scavenging was performed in the absence or presence of the strong metal chelator, EDTA and the second-order rate constants of the flavonoids with hydroxyl radicals (k_s) are presented in the second and third column of Table 3. Our results show that the k_s values of all tested flavonoids increased when EDTA was not present. This effect was particularly pronounced with F4, F5 and F6 (Table 3, fourth column) and seems to be the result of an additional chelating site in those molecules, represented by 4-oxo group combined with 5-OH.

Lipid peroxidation (LPO) inhibition assays confirm that most of the compounds studied are excellent antioxidants with F2, F5, F8, F9, F10 being particularly effective (Table 3, last column). Interestingly, the ability of the flavonoids to inhibit LPO does not seem to correlate with hydroxyl radical scavenging potency where F3, F8, F9 acted as the best scavengers. On the contrary, F5 (quercetin) promoted hydroxyl radical formation although it was very effective against LPO. The high efficiency of F8 and F9 could be attributed to the quaternary ammonium group; nevertheless, this would not explain the relatively lower efficiency of F7 and high efficiency of F3 at the same time.

4. Discussion

For many years, flavonoids attract the attention of researchers because they possess multiple pharmacological properties [28]. Some of their characteristics, namely the antioxidant, iron-chelating and carbonyl reductase-inhibitory effects, render them particularly interesting to investigate them as new protective compounds against doxorubicin cardiotoxicity. Evidence has been given that the semi-synthetic flavonoid monoHER is cardioprotective in animal models [5]. This compound was also shown not to interfere with the anticancer effects of doxorubicin [29]. Because monoHER has to be administered at a high dose and because of its low oral bioavailability, a series of new synthetic derivatives with potentially enhanced antioxidant properties and/or better intestinal absorption have been synthesized [19] in order to identify a compound with increased cardioprotective properties. To achieve this goal, various types of substitutions have been tested. An attempt was also performed to increase the cardioselectivity of the flavonoids via introduction of the quaternary ammonium moiety in various positions of the flavonoid structure [30].

Antioxidant properties were long considered to be the major or even sole determinants for efficient protectors against doxorubicin cardiotoxicity because reactive oxygen species and oxidative stress are considered to be involved in the pathophysiology of its development [31,32,33]. However, it has been shown that there are pronounced differences in the cardioprotective effects also among the flavonoids with comparable antioxidant properties [6].

Apart from their antioxidant effects, other factors are obviously involved in the protective properties of the flavonoids. We conducted this study in order to explore these other features that are likely to be involved in their cardioprotection. Iron chelation was chosen because of an apparent involvement of iron in doxorubicin-induced cardiotoxicity [34] and the effects on carbonyl reduction of doxorubicin were examined because its inhibition will prevent

formation of the cardiotoxic C13-dihydroprometabolites of the anthracyclines [11,35]. The antioxidant properties and general cytotoxicity of the compounds have also been evaluated. We attempted to determine structure-activity relationships and most importantly, the collected data were related to their cardioprotective potential in order to define the characteristics that are essential for their cardioprotective potency. This knowledge is vital for designing new efficient and safe cardioprotectors.

The cardioprotective effects of the selected flavonoids were assessed in neonatal rat cardiomyocytes. These experiments were designed with concentrations of doxorubicin (1 μM), which corresponded with plasma concentration in human patients and allowed us to prolong the incubation period (up to 72 h). In a previously used model of an electrically paced isolated mouse left atrium [6] the acute doxorubicin toxicity (1 h incubation) was measured using a higher dose of doxorubicin (35 μM), which corresponded with heart concentrations in mice [36]. The concentration of the flavonoids was based on initial cytotoxicity screening where most compounds showed acceptable cytotoxicity by 100 μM . Both models gave similar results and demonstrated that the compounds F4 (monoHER) and F7 are the best protectors of the series. For F5 and F10, a remarkable decrease in protection against the doxorubicin-induced cardiomyocyte toxicity was found during incubation (24 vs. 48 and 72 h). This might be due to their own toxicity. It is known that the metabolite of quercetin (F5), quercetin-quinone methide, is thiol-reactive and rapidly forms adducts with glutathione. This reaction may lead to toxic effects such as increased membrane permeability or altered function of the SH-containing enzymes [37]. The structures of F10 and F1 resemble that of quercetin the most and indeed, their biochemical behaviour is similar in many aspects.

Low intrinsic toxicity of the compounds is of course an important requirement to allow eventual clinical application. We have tested the cytotoxicity of the flavonoids in the H9c2 cells. With exception of F1, F5 and F10, all the compounds had acceptable toxicity. F7 was

the least toxic compound of the series. Apparently high cytotoxicity has consequences in cardioprotection: the beneficial effects of the compounds (if any) do not last for long as they are overwhelmed by another type of toxicity. In this way our study points out the limitations of short-term (acute) models of doxorubicin cardiotoxicity. For example, quercetin was previously designated as an excellent cardioprotector against anthracycline-induced toxicity in neonatal cardiomyocytes [7], however, its effects were only followed for 8 hours, which may lead to an overestimation of the beneficial effects of these types of compounds (i.e. compounds with reactive intermediates). We have found that the protection by quercetin was still high at 24 h but it decreased during the next 48 h, whereas the effect of F4 (monoHER) or F7 lasted during the whole experiment. Cytotoxicity of the flavonoids was found to be negatively correlated with the percentage of cardioprotection at 72 h ($p < 0.018$) (Table 4). Interactions of the anthracyclines with iron are well described and include not only iron-catalyzed formation of free radicals but also severe perturbations of iron homeostasis induced by the anthracyclines [38,39,40]. All the tested flavonoids were able to chelate iron and it was found that the number of chelating sites present in the molecule determines the degree of iron chelation. However, no link was found between the cardioprotective effects of the flavonoids and their iron-chelating ability. The discrepancy between the effect of monoHER (F4) in the calcein assay (low) and site-specific scavenging assay (high) may lie in a different affinity of the flavonoids for Fe^{2+} and Fe^{3+} . Although iron is introduced in its ferrous form in the calcein assay, it is rapidly oxidized to its ferric form under the conditions of the experiment. It is not possible to keep it in the ferrous form using reducing agents (e.g. ascorbate) because this leads to a degradation of the probe [41]. Therefore, the calcein assay gives information about the Fe^{3+} -chelating ability whereas in the 2-deoxyribose assay, iron is present in its Fe^{2+} form due to the presence of ascorbate. It has been suggested that the flavonoids chelate iron as Fe^{2+} and their ability to chelate Fe^{3+} is related to their capacity to reduce Fe^{3+} to Fe^{2+} before

association [42], which is apparently larger in F5 and F6 than in F4. Although we were able to identify minor nuances in the iron-chelating behaviour of various flavonoids, their inability to displace iron bound to doxorubicin indicates their overall weak chelating capacity when compared with strong non-flavonoid chelators like SIH or EDTA. On the other hand, our results suggest that displacing iron from its complexes with doxorubicin is not critical for the cardioprotective action of a compound.

The deoxyribose assay for hydroxyl radical scavenging, that has been employed to investigate iron chelation, also gives information about antioxidant behaviour of the compounds. In the presence of EDTA, information on the direct antioxidant behaviour of the flavonoids (i.e. without the participation of iron chelation) is provided. An increase of scavenging activity in the absence of EDTA is indicative for iron chelation by the flavonoids and the influence of iron chelation on total antioxidant capacity by the flavonoids. Interestingly, we have found that under the conditions of the assay, the compound F5 (quercetin) did not scavenge hydroxyl radicals in presence of EDTA (which does not allow site-specific scavenging). On the contrary, it acted as mild pro-oxidant. On the other hand, quercetin acted as a powerful protector against lipid peroxidation (LPO). This discrepancy is not very surprising, the fine balance between the anti- and pro-oxidant properties of the flavonoids is well known and it obviously depends on the exact conditions of the assays. Similar results were previously achieved by Laughton et al. [43]. As can be seen from the k_s values and the IC_{50} values for LPO, all the compounds possess good antioxidant properties. Indeed, it was previously found that the catechol moiety in combination with C2-C3 double bond and 4-oxo function are the essential structural elements for potent antioxidant activity and that 3-substituted compounds are superior to the 7-substituted compounds in LPO assay [19]. Based on our findings, we conclude that neither hydroxyl radical scavenging nor inhibition of lipid peroxidation seem to predict the cardioprotective potential of the flavonoids.

The inhibition of doxorubicin carbonyl reduction was assessed in order to evaluate the involvement of cardiotoxic metabolite formation in prevention of the doxorubicin-induced cardiotoxicity. There is one additional reason for blocking doxorubicin reduction to doxorubicinol. It has been shown that an increased carbonyl reduction, which is inducible by anthracyclines, might also lead to the development of resistance to chemotherapy [44]. To date, very few studies have been conducted on the inhibitory effects of flavonoids on carbonyl reductases [45,46], although it is known for a long time that the flavonoids quercetin, quercitrin or rutin are excellent CR inhibitors. To our knowledge, this study is the first to combine the biochemical inhibition of doxorubicinol formation with cardioprotection evaluation. Based on the IC_{50} values we have identified the compounds F5, F6 and F10 as the best inhibitors of carbonyl reductases of the series. On the contrary, F7 was the worst inhibitor. The quaternary ammonium moiety is not only bulky but it is also positively charged and thus it can affect the appropriate electrostatic interaction between enzyme and inhibitor. From the three flavonoids with a quaternary ammonium group, F9 was the best inhibitor (preserved 7-OH, substituted 3-OH) and F7 was the worst (substituted 7-OH). This means that an absence (F1, F2, F3, F8) or even a substitution (F4, F7) of C7-OH clearly decrease the degree of inhibition. The fact that F7 was a weak inhibitor further confirms the role of C7-OH in the enzyme-inhibitor interaction and the negative influence of the quaternary ammonium moiety on the inhibitory properties of the flavonoids. If we take into account that F7 and F4 (monoHER) are among the best cardioprotectors of the present study, the importance of carbonyl reductase inhibition seems unlikely. This assumption is further supported by the Pearson product moment correlation analysis, which revealed weak correlation ($p < 0.042$) between carbonyl reductase IC_{50} and cardiomyocyte protection at 72 h (Table 4).

In summary, it can be stated that the efficient cardioprotective compounds are not cytotoxic by themselves. High efficacy in carbonyl reductase inhibition is not critical for the

cardioprotective action of the flavonoids. Moreover, no correlation was found between the inhibition of LPO or hydroxyl radical scavenging and the cardioprotective effects. It was found that iron chelation increased the scavenging capacity of the flavonoids (through site-specific scavenging). The present study shows that besides monoHER also compound F7 has excellent cardioprotective properties. Cardioprotection is however not clearly associated with a single physico-chemical or biochemical property of the flavonoid.

Acknowledgement

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Figure legends

Fig. 1. Chemical structures of the flavonoids under investigation.

F4 = 7-monohydroxyethylrutoside (monoHER), F5 = quercetin, F6 = quercitrin.

Fig. 2. Optimization of the reaction conditions for the carbonyl reduction inhibition studies.

(A) Michaelis-Menten plot of doxorubicinol formation as a function of the doxorubicin concentration, (B) the effect of NADPH concentration using a fixed (1 mM) concentration of doxorubicin and (C) doxorubicinol formation in time (DOX 1 mM, NADPH 0.5 mM). All values are given as means \pm SD from three separate experiments.

Fig. 3. Time-dependent plot of the cytotoxic effects of the flavonoids (100 μ M) in H9c2 cells.

Values represent means from three separate experiments. Error bars not indicated for sake of clarity.

Fig. 4. Kinetics of the displacement of Fe^{3+} from the Fe^{3+} -doxorubicin complex by reaction

with the flavonoids and reference chelators (SIH, EDTA). The measurements were made spectrophotometrically at 600 nm in TRIS-KCl buffer pH 7.4 at 25°C. For sake of clarity, time plots of three representative flavonoids out of ten are shown: F5 represents the flavonoid with high chelating capacity whereas F1 and F4 represent those with low chelating capacity according to the calcein assay. All other flavonoids investigated displayed a similar pattern of unreactivity with Fe^{3+} -doxorubicin complexes.

Tables

Table 1. Protection of the flavonoids against doxorubicin-induced toxicity in rat neonatal ventricular cardiomyocytes (NVCM). Data express LDH activity in the extracellular media and the percentual ability of the flavonoids (100 μ M) to reverse DOX (1 μ M)-induced LDH leakage (100% = complete protection).

	24 h		48 h		72 h	
	LDH activity	Protection vs.DOX	LDH activity	Protection vs.DOX	LDH activity	Protection vs.DOX
	(mU.L ⁻¹ .min ⁻¹)	(%)	(mU.L ⁻¹ .min ⁻¹)	(%)	(mU.L ⁻¹ .min ⁻¹)	(%)
Control	18 \pm 4		26 \pm 8		43 \pm 1	
DOX	72 \pm 8		130 \pm 7		156 \pm 3	
F1 + DOX	92 \pm 2	none	138 \pm 4	none	173 \pm 10	none
F2 + DOX	53 \pm 10	37	101 \pm 14 *	29	146 \pm 4	8
F3 + DOX	54 \pm 10	35	105 \pm 9	25	142 \pm 5	12
F4 + DOX	39 \pm 10 **	58	68 \pm 7 **	55	90 \pm 10 **	58
F5 + DOX	23 \pm 3 **	91	96 \pm 15 *	34	154 \pm 10	1
F6 + DOX	57 \pm 18	24	94 \pm 10 *	30	135 \pm 0	18
F7 + DOX	47 \pm 6 *	46	86 \pm 13 **	42	109 \pm 3 **	41
F8 + DOX	69 \pm 12	8	119 \pm 8	12	151 \pm 1	5
F9 + DOX	57 \pm 9	29	108 \pm 11	22	140 \pm 4	14
F10 + DOX	36 \pm 10 **	69	114 \pm 16	17	150 \pm 1	5

Values represent means \pm SD from three to four separate experiments. *p < 0.05; **p < 0.001 (One-Way ANOVA with Tukey's post-hoc test)

Table 2. Carbonyl reductase inhibition by the flavonoids in human liver cytosol and their cytotoxicity in the rat cardiomyoblast cell line (H9c2). IC_{50} = the concentration of a flavonoid which caused 50% decrease in doxorubicinol formation or 50% decrease in cell viability, respectively.

	CR inhibition	Cytotoxicity to H9c2	
	IC_{50} (μ M)	IC_{50} 48h (μ M)	IC_{50} 96h (μ M)
F1	35 \pm 6	96 \pm 23	44 \pm 8
F2	50 \pm 7	> 500	271 \pm 29
F3	71 \pm 13	> 500	417 \pm 108
F4	84 \pm 27	340 \pm 123	463 \pm 182
F5	18 \pm 2	133 \pm 33	44 \pm 6
F6	12 \pm 4	> 500	> 500
F7	170 \pm 40	> 500	> 500
F8	67 \pm 13	341 \pm 144	146 \pm 36
F9	46 \pm 4	> 500	466 \pm 133
F10	20 \pm 2	243 \pm 38	37 \pm 10

Values represent means \pm SD from three separate experiments.

Table 3. Site-specific scavenging of hydroxyl radicals expressed as second-order rate constants of the flavonoids with hydroxyl radicals (k_s), relative iron (III) chelation expressed as percentage of the effect of the reference chelator SIH and prevention of lipid peroxidation by the flavonoids. IC_{50} = the concentration of the flavonoid at which 50% inhibition of LPO was obtained.

	Fe ³⁺ chelation*	k_s ** [$M^{-1}s^{-1}$] $\times 10^9$		w/o EDTA :	LPO***
	(% of SIH)	w/o EDTA	with EDTA	with EDTA ratio	IC_{50} (μM)
F1	15 \pm 2	57 \pm 18	10 \pm 2	6	22 \pm 6
F2	13.7 \pm 0.6	53 \pm 12	9 \pm 2	6	2.8 \pm 0.9
F3	14.8 \pm 0.5	69 \pm 5	28 \pm 2	2	16 \pm 5
F4	11.7 \pm 0.2	106 \pm 15	8 \pm 2	13	13 \pm 4
F5	39.4 \pm 0.1	20 \pm 4	-1 \pm 0	15	5.1 \pm 0.1
F6	29.1 \pm 0.9	62 \pm 11	6 \pm 2	10	n.d.
F7	22.8 \pm 0.1	53 \pm 13	10 \pm 2	5	37 \pm 2
F8	18.3 \pm 0.4	52 \pm 8	19 \pm 2	3	1.4 \pm 0.3
F9	16.4 \pm 0.8	107 \pm 16	24 \pm 2	4	3.8 \pm 0.1
F10	18.5 \pm 0.3	48 \pm 11	11 \pm 1	4	1.6 \pm 0.7

* Measured as displacement of Fe³⁺ from its complexes with calcein after 500 seconds of incubation (complete displacement by SIH).

**Hydroxyl radicals were generated in presence of H₂O₂ (2.8 mM), Fe³⁺ (20 μM), ascorbate (100 μM), EDTA (100 μM , where indicated) and 2-deoxyribose (2.8 mM), which was used as a detector molecule.

***Lipid peroxidation was induced with Fe²⁺ (10 μM) and ascorbate (200 μM) in heat-inactivated microsomes in presence/absence of various concentrations of the flavonoids.

n.d. – not determined

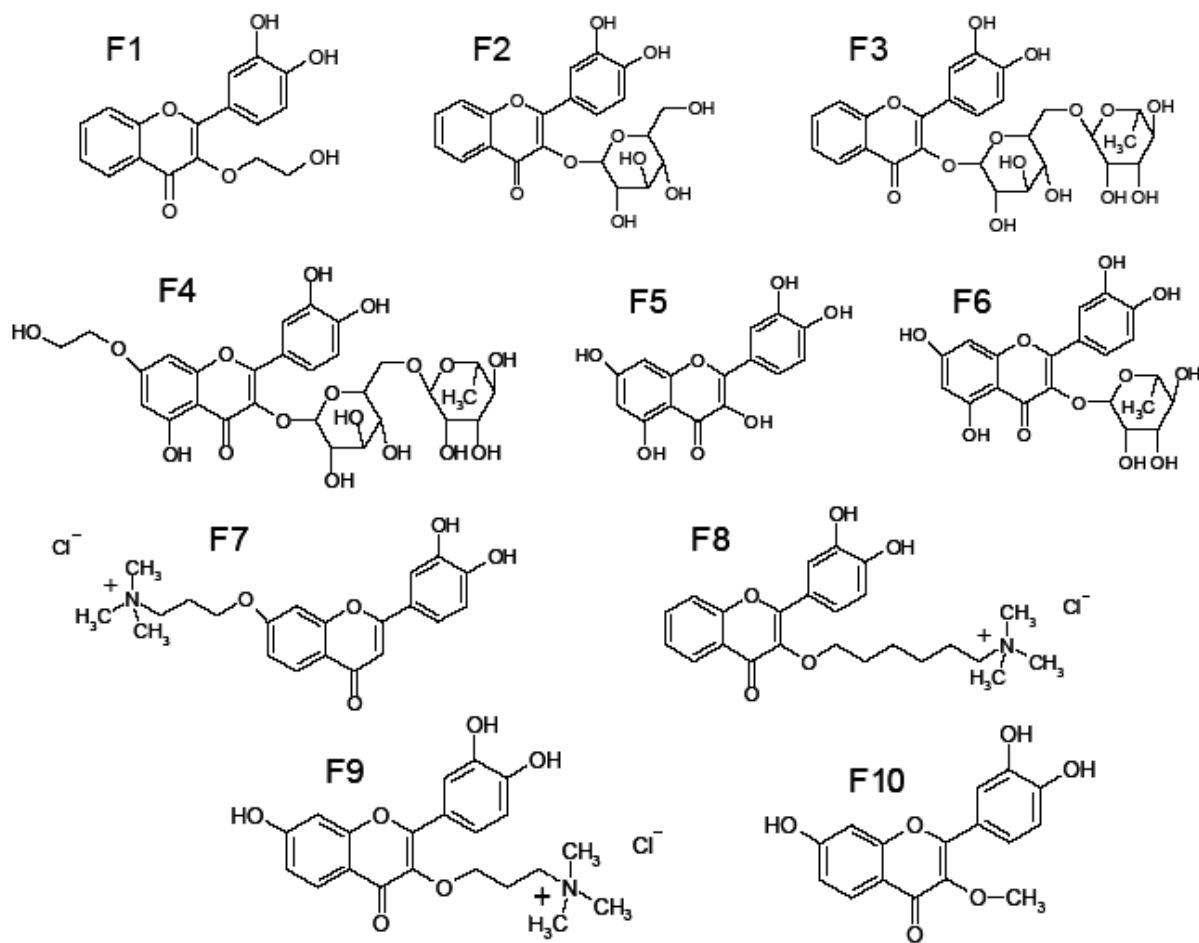
Values represent means \pm SD from at least three separate experiments.

Table 4. Correlation between the percental protection of cardiomyocytes against doxorubicin-induced LDH leakage and various biochemical parameters of the flavonoids.

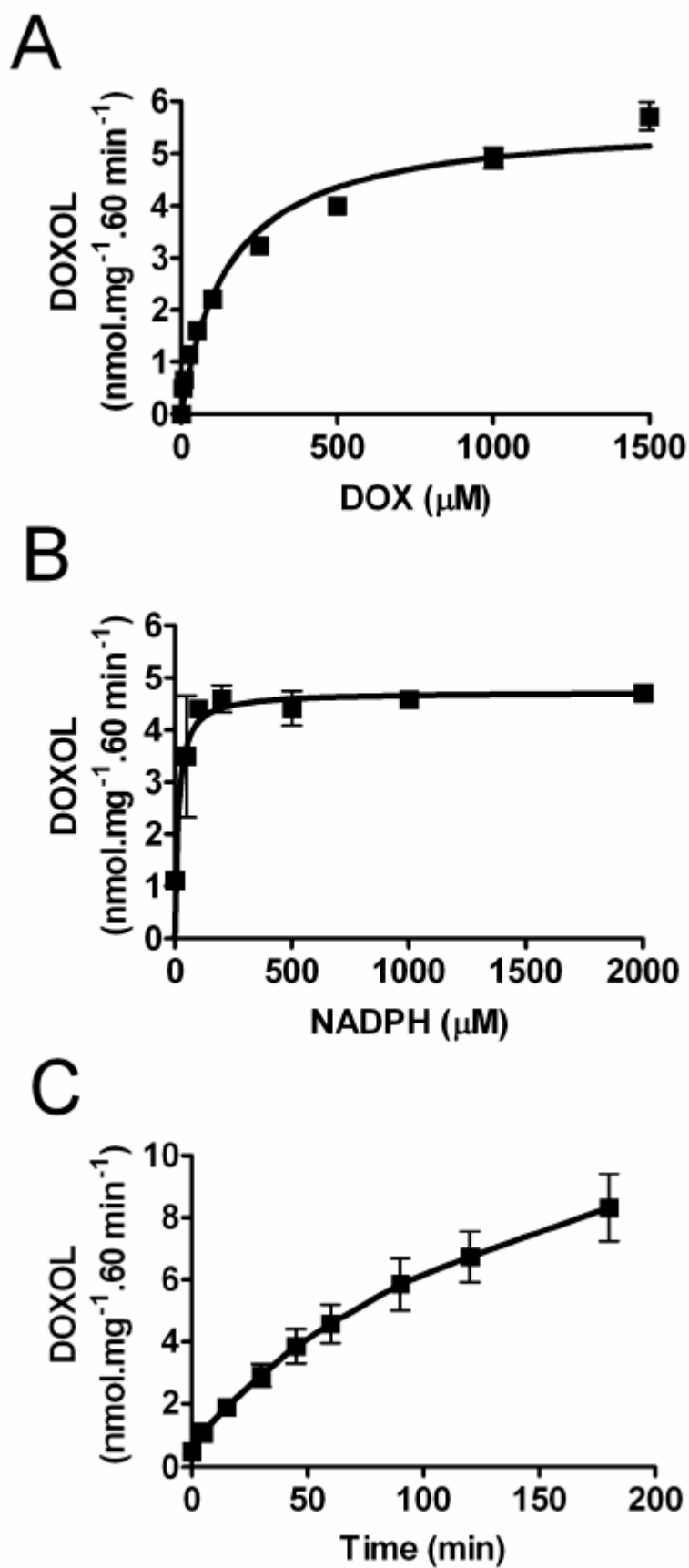
Protection %	CR inhibition	LPO inhibition	Fe ³⁺ chelation	HO [•] scavenging		Cytotoxicity IC ₅₀ (μM)
	IC ₅₀ (μM)	IC ₅₀ (μM)	%	w/o EDTA	with EDTA	
24 hours						
R	-0.047	-0.159	0.479	-0.274	-0.459	-0.093
p	0.897	0.683	0.161	0.444	0.182	0.799
48 hours						
R	0.445	0.228	0.139	0.265	-0.320	0.676
p	0.198	0.554	0.702	0.459	0.368	0.063
72 hours						
R	0.651	0.476	-0.249	0.570	-0.102	0.722
p	0.042*	0.195	0.488	0.085	0.778	0.018*

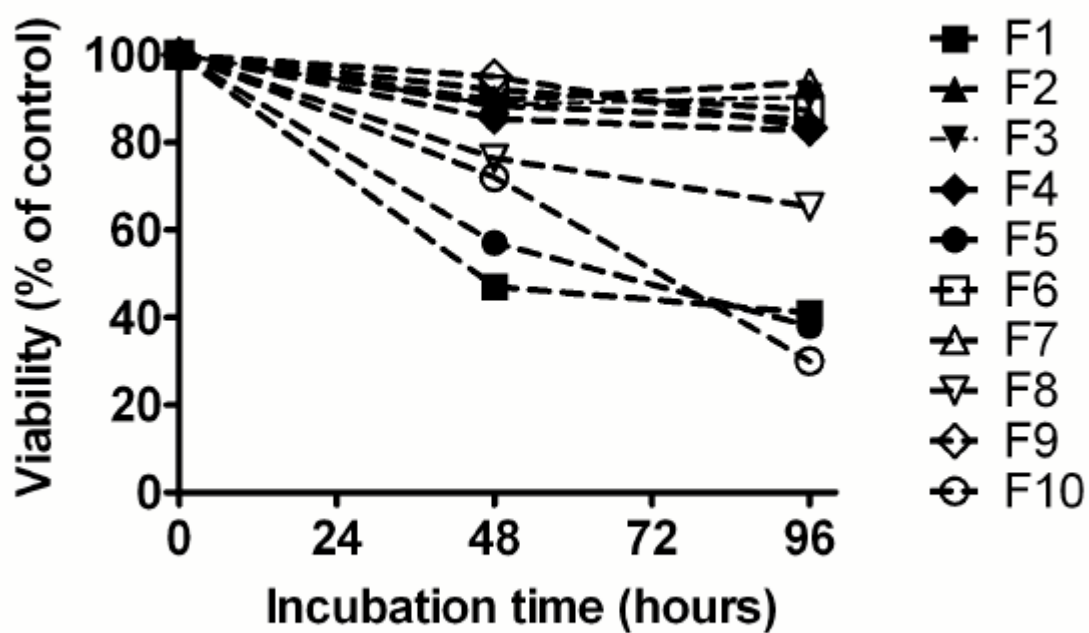
R – Pearson's product moment correlation coefficient, p – observed significance of the test

*Significant relationship between the two variables (p < 0.05)

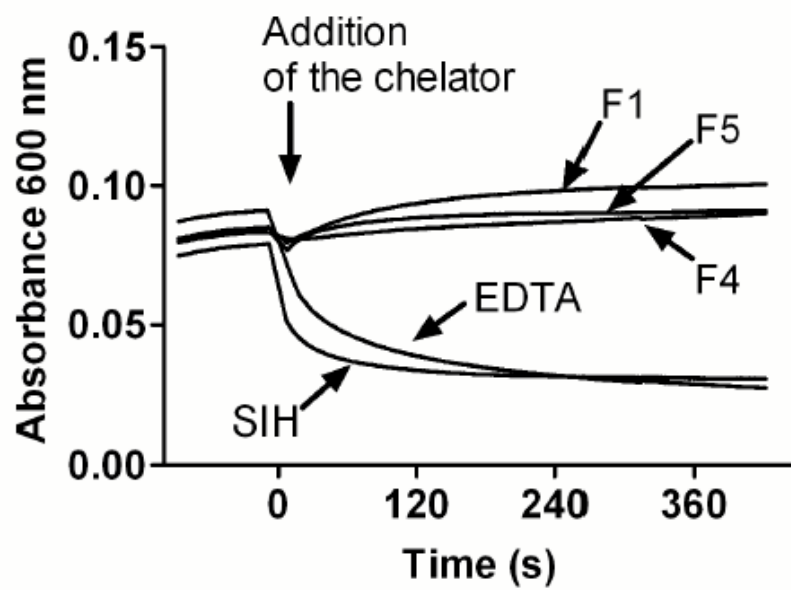


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