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Insights into the redox cycle of human quinone reductase 2

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Abstract

NRH:quinone oxidoreductase 2 (QR2) is a cytosolic enzyme that catalyzes the reduction of quinones, such as menadione and co-enzymes Q. With the aim of understanding better the mechanisms of action of QR2, we approached this enzyme catalysis via electron paramagnetic resonance (EPR) measurements of the by-products of the QR2 redox cycle. The variation in the production of oxidative species such as H_2O_2, and subsequent hydroxyl radical generation, was measured during the course of QR2 activity under aerobic conditions and using pure human enzyme. The effects on the activity of the following were compared: (i) synthetic (*N*-benzyldihydronicotinamide, BNAH) or natural (nicotinamide riboside, NRH) co-substrates; (ii) synthetic (menadione) or natural (co-enzyme Q0, Q2) substrates; (iii) QR2 modulators and inhibitors (melatonin, resveratrol and S29434); (iv) a pro-drug activated via a redox cycle [CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide]. The results were also compared with those obtained with human QR1. The production of hydroxyl radicals is: (i) observed whatever the substrate/co-substrate used; ii) quenched by adding catalase; (iii) not observed with the specific QR2 inhibitor S29434; (iv) observed with the pro-drug CB1954. While QR2 produced free radicals with this pro-drug, QR1 gave no EPR signal showing the strong reducing capacity of QR2. In conclusion, EPR analysis of QR2 enzyme activity through free radical production enables modulators and effective inhibitors to be distinguished.

Keywords: *quinone reductase 1, quinone reductase 2, menadione, electron paramagnetic resonance, reactive oxygen species, free radicals*

Introduction

Quinone oxidoreductases (QR1 and QR2) are flavoproteins that catalyze the reductive metabolism of quinones and their analogues [1,2,3,4]. The dicoumarol-sensitive QR1, a cytosolic protein ubiquitously present in all tissues types, catalyzes the two-electron reduction of quinones [1,2,3]. This reduction produces hydroquinones, which can be removed by conjugation with glutathione, UDP-glucuronic acid and other compounds, thus avoiding the formation of free radicals and highly reactive oxygen species (ROS) in the cell. The two-electron reduction competes with the one-electron reduction to semiquinone radicals, catalyzed by cytochromes P450 and P450 reductases. These semiquinone radicals can produce ROS and can be reoxidized to the original quinones and initiate a new redox-cycle [5,6]. It is also known that the QR1-mediated reduction of quinones is exploited for the bioactivation of anti-cancer drugs (doxorubicin, daunorubicin and mitomycin C) [3,7]. QR1 is thus implicated both in protection against carcinogenesis [8] and exploited in cancer chemotherapy [9], making it difficult to generalize as to whether QR1 is a detoxification or a toxifying enzyme for individual quinones or pseudo-quinones [10].

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NRH:QR2 is a homologue of the more fully characterized QR1[11]. Amino acid sequence alignment of QR1 and QR2 indicates 49% sequence identity, with the major difference being the lack of a 43-residue C-terminal tail in QR2. The crystal structures of QR1 [12] and QR2 [13] are highly similar, both forming homodimers with two independent and equivalent active sites each containing a FAD cofactor. The analysis of the crystal structure of QR2 revealed that it contains a specific metal binding site, which is not present in QR1 [13]. Whereas the two proteins retain similar substrate specificities, structural differences result in differences in specificity for electron donating cofactors. Indeed, QR1 can use either NADH or NADPH. By contrast, QR2 can use neither NADH nor NADPH efficiently and instead requires dihydronicotinamide riboside (NRH) or its related molecules to reveal what can be considered as a latent enzyme activity [14]. There is only limited information available on the role of QR2 in metabolism and detoxification and/or activation of quinones and anti-tumour drugs. In contrast to $QR1^{-/-}$mice, $QR2^{-/-}$ mice showed decreased sensitivity to menadione induced-hepatic toxicity, suggesting that QR2 catalyzed menadione metabolism [15]. This observation was also confirmed independently on our strain of mice (P. Delagrange and J.A. Boutin, unpublished). Moreover, recent studies have demonstrated the significance of quinone reductases 1 and also 2 in limiting chemically-based skin carcinogenesis [8].

This non-exhaustive bibliographic review shows that the biological roles of QR1 and QR2 are still not clearly understood. Because QR2 is an enzyme with many enigmatic features [16], additional studies are required to understand its catalytic capability and roles in various patho-physiological conditions. By-products of the QR2 redox cycle, such as H_2O_2, and subsequent hydroxyl radical generation, may be responsible for the propagation of quinone toxicity. Therefore in the present study, electron paramagnetic resonance (EPR) was used to study the formation of radical species by pure human QR2 in aerobic conditions, by comparison with human QR1. To document the variation of the free radical production during QR2 activity, experiments were carried out with the following: (i) synthetic (BNAH) or natural (NRH) co-substrates; (ii) synthetic (menadione) or natural (co-enzyme Q0, Q2) substrates; (iii) QR2 modulators and inhibitors (melatonin [17], resveratrol [18] and S29434 [19]) (Figure 1); (iv) an anti-cancer pro-drug activated via a redox cycle (CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide).

Experimental procedures

Chemicals

5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO), superoxide dismutase (SOD-Mn, reference S5689), catalase (reference C40), H_2O_2, tris(hydroxymethyl) aminomethane buffer solutions (Tris buffer), n-octyl-beta-glucopyranoside (octyl-GP), menadione, resveratrol, melatonin, NADH, co-enzymes Q0 and Q2, ethylendiaminetetraacetic acid (EDTA), dicoumarol and CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide were purchased from Sigma-Aldrich-Fluka Co. (Saint Quentin Fallavier, France). $Fe(NH_4)_2(SO_4)_2.6\ H_2O$, came from VWR International (Strasbourg, France). *N*-benzyldihydronicotinamide (BNAH) and nicotinamide riboside (NRH) were custom-synthesized by Tebu-Bio, Paris. S29434 synthesis has been described and characterized in Mailliet *et al* [20].

Reactant and substrate preparations for EPR experiments

All experiments were carried out with demineralized (18.2 MΩ.cm) water containing 50 mM tris(hydroxymethyl)amino-methane and 1 mM ß-octyl-glucoside, final pH = 8.5. The DMPO spin trap stock solution (1 M) was prepared in water. To purify the solution, 250 mg of active charcoal was added to 10 mL of solution. The suspension was then filtered through 0.22 µm filters and stored at − 20°C until required. Cell homogenates from CHO-QR1, CHO-QR2 and naïve CHO cells were prepared as follows: 2.25 mL of buffer was added to the cells (30 µL/million cells). The suspension was agitated for 1 h at 4°C and then centrifuged for 10 min at 25,000 rpm (4°C). The supernatant was aliquoted (4 mg protein/mL) and stored at − 80°C. Menadione, BNAH and NRH solutions were prepared in DMSO. NADH was dissolved in Tris buffer solution. Inhibitor solutions (melatonin, resveratrol and S29434) were first dissolved in DMSO (5 or 10 mM) and then diluted 100-fold in DMSO/Tris 50/50 (v/v). Dicoumarol was first diluted in DMSO (5 mM) then diluted 100-fold in Tris buffer. Co-enzyme Q2 was dissolved in DMSO. All other solutions were prepared in Tris buffer.

Expression of 6His-hQR2 in Sf9 cells

6His-hQR2 cDNA was obtained by PCR amplification of the pcDNA3.1(+)/hQR2 plasmid [21] [using forward primer (5′-gattccaccatgcatcaccatcaccatcacgcaggttaagaaagtactc-3′) and reverse primer (3′-ggtgaccg-tgaagcccgttattgtagac-5′) to add six histidine residues between the first and second hQR2 amino acids and a Bgl II site in the 3′ end of cDNA. The pelleted cells were stored at − 80°C. The pellets were suspended in 150 mL of buffer (50 mM Tris-HCl, pH 8.5, with 1 mM octyl-GP) in the presence of anti-protease, EDTA-free, cocktail (Roche), vigourously mixed at 4°C and centrifuged at 100,000 × g, 1 h, at 4°C. The process was repeated twice. The supernatants were combined and applied

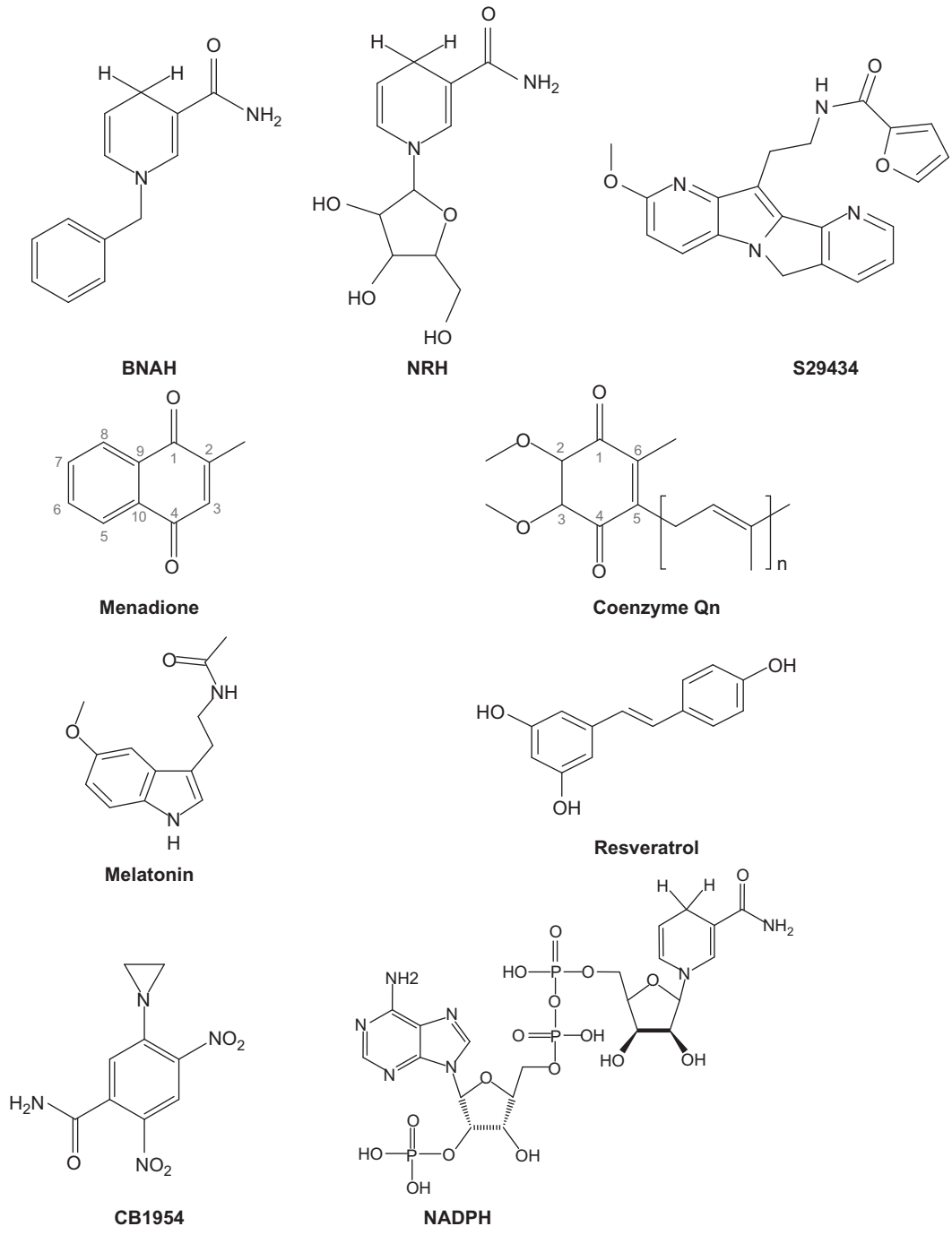

Figure 1. Structures of the substrates, co-substrates and modulators of quinone reductase used in the current study.

at 1 mL/min onto a 1.5 mL Ni-NTA chromatography column, previously equilibrated with the extraction buffer supplemented with 10 mM imidazole. The column was washed with 10 volumes of the same buffer. The protein was eluted stepwise with increasing concentrations of imidazole in the same buffer at 50, 100, 200, 300 and 500 mM. The fractions with QR2 activity were pooled and dialyzed overnight against the Tris buffer (50 mM Tris, pH 8.5, with 1 mM octyl-GP). The enzyme preparation was then aliquoted and snap-frozen at − 80°C until further use. CHO-QR1 was a gift from Professor David Ross (Denver, Co).

EPR experiments

EPR spectra were obtained at X-band and at room temperature (RT) on a Bruker EMX-8/2.7 (9.86 GHz) equipped with a high-sensitivity cavity (4119/HS 0205) and a gaussmeter (Bruker, Wissembourg, France). A flat quartz cell FZK160-5 × 0.3 mm (Magnettech, Berlin, Germany) was used for analysis. EPR data

processing and spectrum computer simulation were performed using WINEPR and SIMFONIA software (Bruker, Wissembourg, France). Typical scanning parameters were: scan rate, 1.2 G/s; scan number, 1; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 20 mW; sweep width, 100 G; sweep time, 83.88 s; time constant, 40.96 ms; magnetic field 3450–3580 G; the receiver gain has been given independently in each Figure caption. Areas under the EPR spectrum peaks were calculated by double integration of the peaks using WINEPR software. Errors on area-determination for experiments made at least in triplicate ranged between 10% and 15%. The nature of the primary radical ($O_2^{\bullet}-$, $^{\bullet}OH$) trapped by DMPO to give the secondary spin adducts was identified by the EPR spectrum pattern and by control experiments after adding SOD or catalase or both. First, when DMPO traps the superoxide anion $O_2^{\bullet}-$, it gives a $[DMPO-OOH]^{\bullet}$ spin adduct (12 lines). This adduct is not stable and is quickly transformed into $[DMPO-OH]^{\bullet}$ (four lines). In this case, both $[DMPO-OOH]^{\bullet}$ and $[DMPO-OH]^{\bullet}$ signals disappear when superoxide dismutase (SOD) is added to the mixture. Secondly, when the hydroxyl radical $^{\bullet}OH$ is trapped, it gives the $[DMPO-OH]^{\bullet}$ spin adduct, which disappears when catalase is added. Thirdly, in our experiments, DMSO was added to the assay mixture because of poor reactant solubility, depending on the experiments. With this solvent and a concomitant production of radical $^{\bullet}OH$, the $[DMPO-CH_3]^{\bullet}$ adduct is likely to arise. The primary radical $^{\bullet}OH$ attacks the solvent to give a secondary radical $^{\bullet}CH_3$, which is trapped by DMPO ($CH_3-SO-CH_3 + {}^{\bullet}OH \rightarrow CH_3-SO-OH + {}^{\bullet}CH_3$). Therefore, the detection of a $[DMPO-CH_3]^{\bullet}$ EPR signal (six lines) provides proof of the production of the primary radical $^{\bullet}OH$ by the biological system. This signal is suppressed when catalase is added. In conclusion, the observation of $[DMPO-CH_3]^{\bullet}$ and $[DMPO-OH]^{\bullet}$ EPR signals, both catalase-dependent, indicates the production of a hydroxyl radical and of H_2O_2, respectively. The observation of a $[DMPO-OH]^{\bullet}$ EPR signal that is SOD-dependent indicates superoxide anion production.

Final reactant and substrate concentrations for EPR experiments

All experiments were performed in 50 mM Tris-HCl, pH 8.5, with 1 mM octyl-GP, 50 μM EDTA and DMSO at 6%. The protein solution from cell homogenates (1.00 ± 0.05 mg/mL) or purified QR (15 μg/mL), DMPO (125 mM), menadione (125 μM), co-substrates (BNAH, NRH or NADPH, 1 mM), SOD-Mn (22.75 μg/mL), catalase (2 ku/mL), modulators (dicoumarol, resveratrol, melatonin and S29434 – 5 μM), co-enzymes Q0 and Q2 (100 μM) were added (or not, depending on experiments) to the flat quartz cell. The experimental mixtures were incubated for variable times at 37°C prior to the EPR experiments. All experiments were done in triplicate. Variations in concentrations were also studied to clarify certain biochemical reactions.

Controls

It was verified that none of the compounds or their combination were able to generate free radicals. Some compounds and buffer components may directly trap free radicals (chemical antioxidant properties) and modify the observed quantity of free radicals produced during the protein activity EPR analysis. For this reason, the free radical trapping capacities of isolated molecules (resveratrol and melatonin) were also tested and compared. The Fenton reaction was used to produce $^{\bullet}OH$ to test their radical scavenging capacities. $^{\bullet}OH$ was generated by mixing H_2O_2 (100 μM) and Fe^{2+} (100 μM) in a PBS buffer. Addition of resveratrol or melatonin (0.01 mM) to this solution gives rise respectively to a percentage decrease of the signal intensity equal to 5% or 20%.

Using DMPO (125 mM) as a spin trap, we also checked that adding H_2O_2 (100 μM) to QR2 (15 μg/mL) in 50 mM Tris-HCl, pH 8.5, with 1 mM octyl-GP or even in water did not produce any radical adducts.

Results

Quinone reductase activity (QR1 and QR2) and free radical production

Prior to the experiments, it was verified that none of the compounds (Figure 1) or their combination was able to generate ROS in the absence of enzyme, substrate or co-substrate (data not shown).

QR activities were initially studied in extracts of cells over-expressing QR1 [19], QR2 [22] or naïve cells, using menadione (Figure 1) as substrate. The results showed that increasing over-expression of QR1 or QR2 increases the production of radicals (not shown). We then chose to work with purified enzymes to be free from the basal production of free radicals in cell extracts and to avoid the presence of detoxifying proteins that could interfere with any radicals produced. Because a few studies have been published on QR1, we started our experiments with this enzyme in order to assess the experimental conditions and to be able to validate or compare them with the data in the available literature. In the case of human QR1, an incubation time of 15 min was required at 37°C to see the rise of significant peaks (Figure 2a). In these conditions, the reaction of menadione with QR1 in the presence of NADPH as co-substrate, using DMPO as spin trap, gave an EPR spectrum (Figure 2a), which mainly consisted of $[DMPO-CH_3]^{\bullet}$ adducts ($a_N = 16.28$ G, $a_H = 23.25$ G, six lines). The addition of SOD doubled the EPR signal intensity (Figure 2b),

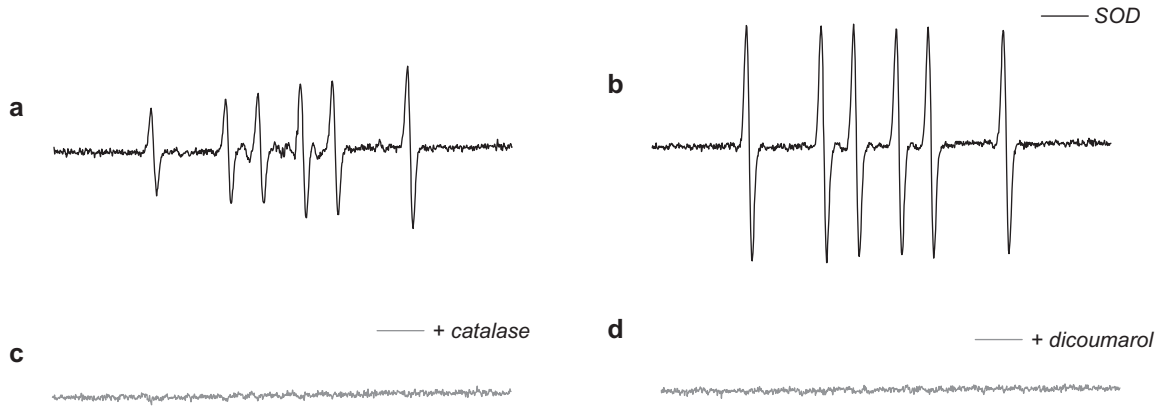

Figure 2. EPR spectra obtained after 15 min incubation at 37°C in the presence of DMPO (125 mM) in a reaction mixture containing purified QR1 (15 µg/mL), NAD(P)H (1 mM) and menadione (100 µM). (a): complete system; (b) addition of SOD-Mn (22.75 µg/L) to (a); (c) addition of catalase (2000 units/mL) to (a); (d) addition of dicoumarol (5 µM) to (a). Intensity range: ± 6000. Gain: 2.10^5

whereas the addition of catalase totally suppressed the $[DMPO-CH_3]^{\bullet}$ signal (Figure 2c) as did the addition of the specific QR1 inhibitor dicoumarol (Figure 2d).

In the case of QR2, two co-substrates were tested, BNAH and NRH. In all cases, the spectra also consisted of six lines characteristic of $[DMPO-CH_3]^{\bullet}$ adducts ($a_N = 16.28$ G, $a_H = 23.25$ G) as shown in Figures 3a and 3b obtained after 10 min incubation at 37°C. Addition of SOD gave an increase in the EPR signal intensity as shown in Figures 3c and 3d. Addition of catalase completely eliminated the six-line pattern (Figures 3e and 3f). With NRH as co-substrate addition of catalase reveals, after 10 min incubation at RT, a triplet of weak intensity (Figure 3g). The enlarged triplet signal and the corresponding simulation are shown as an insert in Figure 3g.

Influence of the substrate/co-substrate on QR2 redox cycle and free radical production

To obtain information on the influence of the substrate (natural or synthetic) on the radical production by the enzyme system, experiments were carried out with QR1 and QR2 and natural substrates such as co-enzymes Q0 and Q2 (Figure 1) instead of menadione. QR1 can activate both co-enzymes as shown in Figures 4a and 4b. The signal intensities obtained were less than those obtained under the same conditions with menadione (Figure 2). With QR2, the spectra were also less intense. For the system QR2/BNAH/CoQ0 (Figure 4c) and QR2/NRH/CoQ0 (Figure 4i), the feature of the spectrum is different with superimposed signals. As demonstrated by simulation, the spectrum presented in Figure 4c consists of DMPO-OOH ($a_N = 14.2$ G, $a_H^{\beta} = 11.34$ G, $a_H^{\gamma} = 1.25$ G) and $DMPO-CH_3$ ($a_N = 16.28$ G, $a_H = 23.25$ G) EPR signals. With both co-substrates, addition of SOD simplifies the signal, which becomes symmetrical (Figures 4e and 4k). Addition of catalase suppressed the sextuplet revealing a triplet ($a_H^{i} = 2.36$ G, $a_H^{j} = 1.99$ G; $g = 2.0046$) (Figures 4g and 4m). In the case of CoQ2, with BNAH or NRH, addition of SOD gave a clearer signal with increased intensity (Figures 4f and 4l), while the catalase totally suppressed the signal (except radical traces) (Figures 4h and 4n).

Radical production during the activation of the anti-cancer prodrug CB1954 by QR1 or QR2

The anti-cancer pro-drug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) is transformed into a potent cytotoxic drug upon reduction of its 4-nitro group to a 4-hydroxylamine by quinone reductases [23] or nitroreductase [24]. The transformation of the nitro group into hydroxylamine is a four-electron reduction reaction. The free radical production, if any, accompanying the reduction process of CB1954 by QR2 was studied and compared with QR1. The results are presented in Figure 5. No peak appeared for the system QR1/NADPH/CB1954 (Figure 5a), whereas spectra with high intensity were obtained for QR2 with both co-substrates. With BNAH, the spectral pattern consists of several signals as seen in Figure 5b. Addition of SOD restores the typical sextuplet but increases the signal intensity as shown in Figure 5d. Addition of catalase totally suppressed the signal (Figure 5f). With NRH as co-substrate (Figure 5c), under the same conditions, the spectrum is made more complex by the superimposition of different signals. As demonstrated by simulation, the spectrum consists of the DMPO-OOH ($a_N = 14.2$ G, $a_H^{\beta} = 11.34$ G, $a_H^{\gamma} = 1.25$ G) and $DMPO-CH_3$ ($a_N = 16.28$ G, $a_H = 23.25$ G) EPR signals. As in the case of BNAH, addition of SOD restored the classical sextuplet (Figure 5e), whereas addition of the catalase totally suppressed the signal (Figure 5g).

Influence of modulators on the radical production during the redox cycle of QR2

While dicoumarol is a reference inhibitor for QR1, inhibitors are not clearly identified for QR2. It is

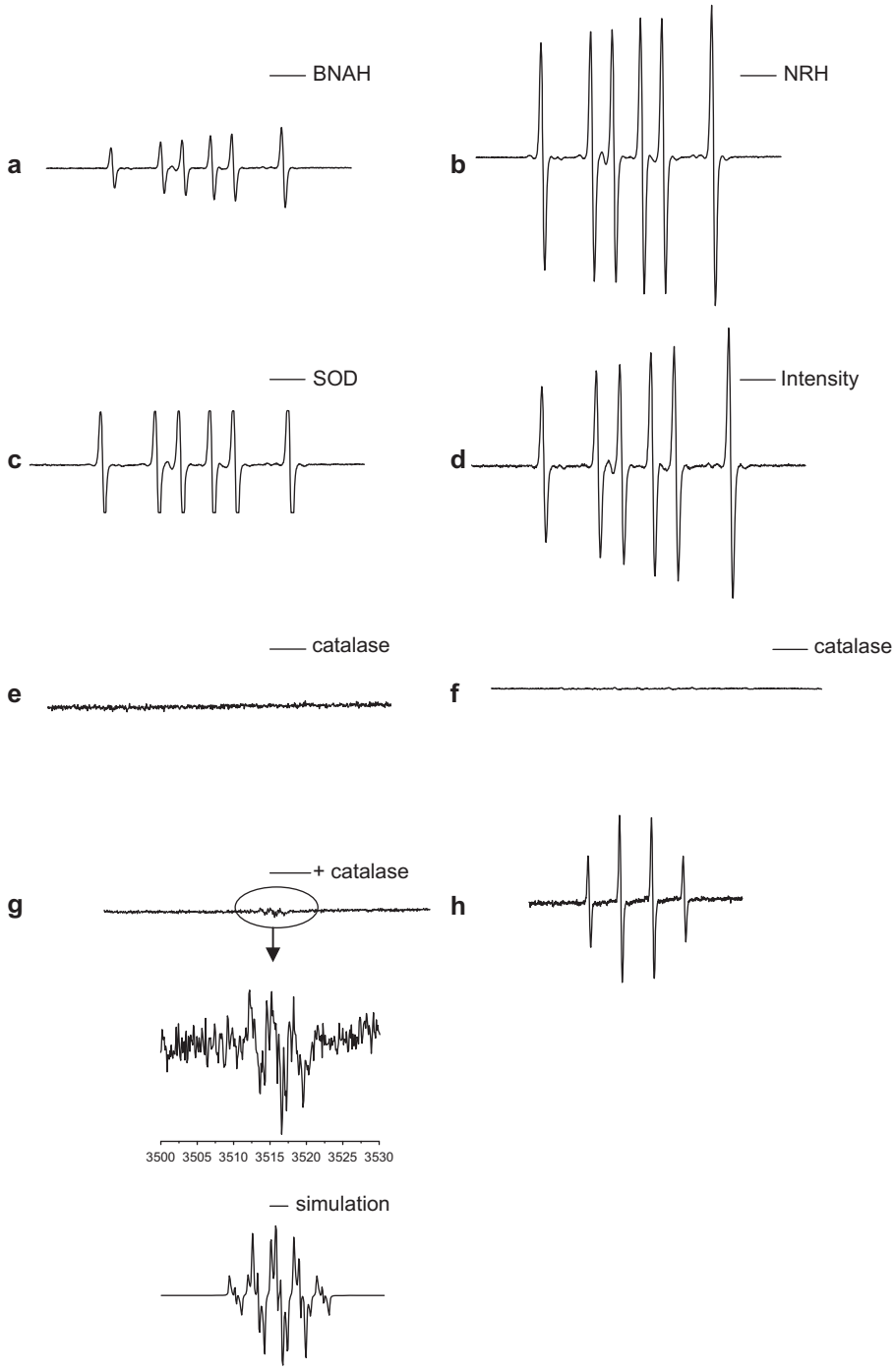

Figure 3. EPR spectra obtained after 10 min incubation at 37°C in the presence of DMPO (125 mM) in a reaction mixture containing purified QR2 (15 μg/mL) BNAH or NRH (1 mM) and menadione (100 μM). (a): with BNAH; (b) with NRH, (c) addition of SOD-Mn (73.5 units/mL) to (a); (d) addition of SOD-Mn (73.5 units/mL) to (b); (e) addition of catalase (2000 units/mL) to (a); (f) addition of catalase (2000 units/mL) to (b); (g) addition of catalase (2000 units/mL) to (b) after 10 min incubation at RT; (h) EPR spectra obtained with a reaction mixture containing DMPO (125 mM), menadione (100 μM) and H_2O_2 (100 μM) in 50 mM Tris-HCl, pH 8.5, with 1 mM octyl-GP, 50 μM EDTA (DMSO 1%). Intensity range: ± 30,000. Gain: 2.10^4

known that resveratrol and melatonin bind to the active-site cleft and modulate the activity of QR2, and the synthetic compound S29434 has been reported to be a potential inihibitor of QR2 by Ferry *et al* [19]. The effects of these modulators/inhibitors on the free radical production during the redox cycle of QR2 were studied. Resveratrol, melatonin and the synthetic compound S29434 were added to the system QR2/BNAH or NRH/menadione. Free radical production (•OH), deduced from the double integration of EPR spectra recorded in each case, is given as histograms in Figure 6 for BNAH and NRH. In both cases, the addition of melatonin at 10 μM significantly increased •OH production. Resveratrol at 10 μM also produced a large increase in •OH generation (almost twice as much) with BNAH, and a decrease around 30% with

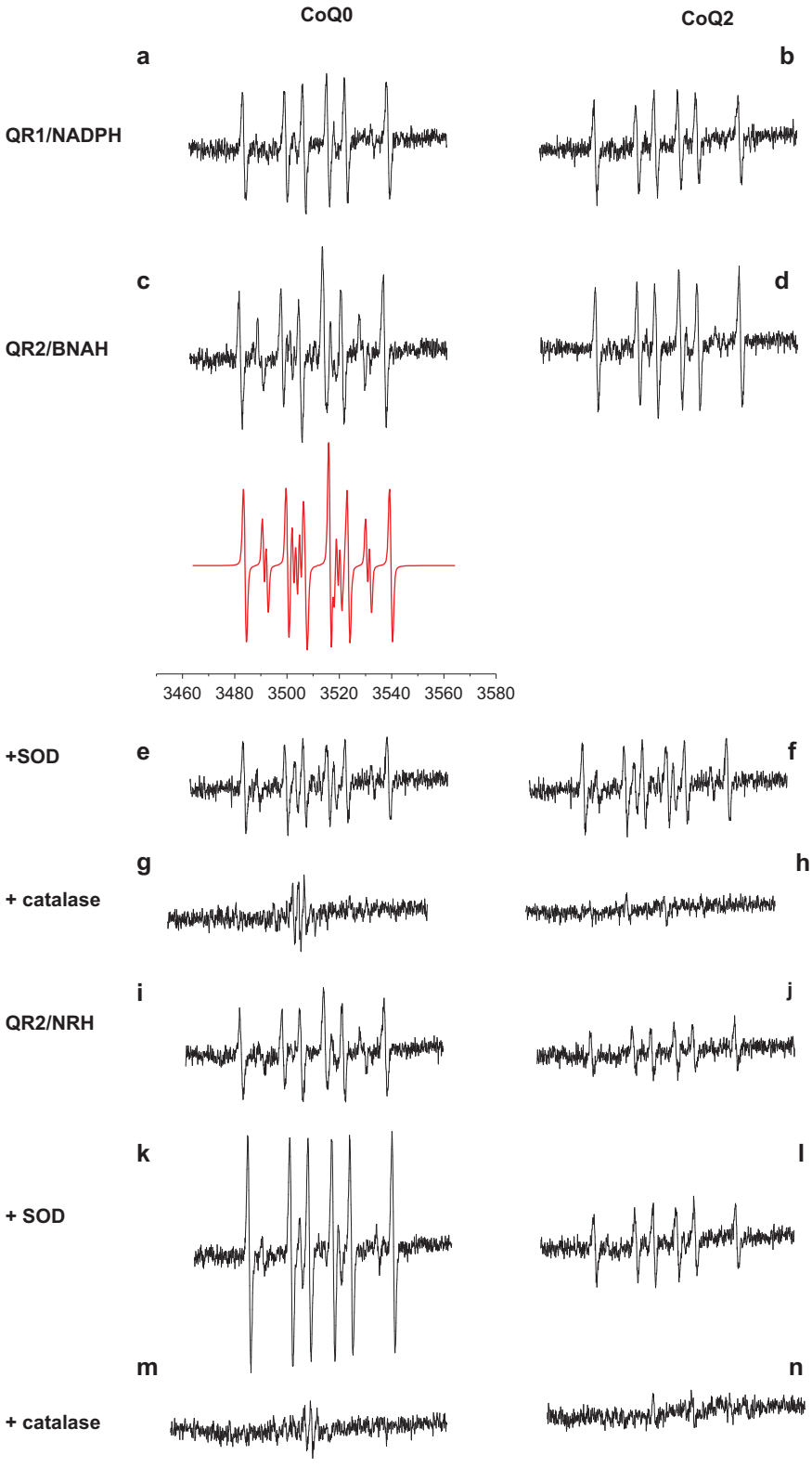

Figure 4. EPR spectra obtained after 10 min incubation at 37°C in the presence of DMPO (125 mM) in a reaction mixture containing (a) QR1 (15 µg/mL), NADPH (1 mM) and CoQ0 (100 µM); (b) QR1 (15 µg/mL), NADPH (1 mM) and CoQ2 (100 µM); (c) QR2 (15 µg/mL), BNAH (1 mM) and CoQ0 (100 µM) and the corresponding simulated spectra; (d) QR2 (15 µg/mL), BNAH (1 mM) and CoQ2 (100 µM); (e) addition of SOD (73.5 units/mL) to (c); (f) addition of SOD (73.5 units/mL) to (d); (g) addition of catalase (2000 units/mL) to (c); (h) addition of catalase (2000 units/mL) to (d); (i) QR2 (15 µg/mL), NRH (1 mM) and CoQ0 (100 µM); (j) QR2 (15 µg/mL), NRH (1 mM) and CoQ2 (100 µM); (k) addition of SOD (73.5 units/mL) to (i); (l) addition of SOD (73.5 units/mL) to (j); (m) addition of catalase (2000 units/mL) to (i); (n) addition of catalase (2000 units/mL) to (j). Intensity range: ± 12,000. Gain: 2.10^5

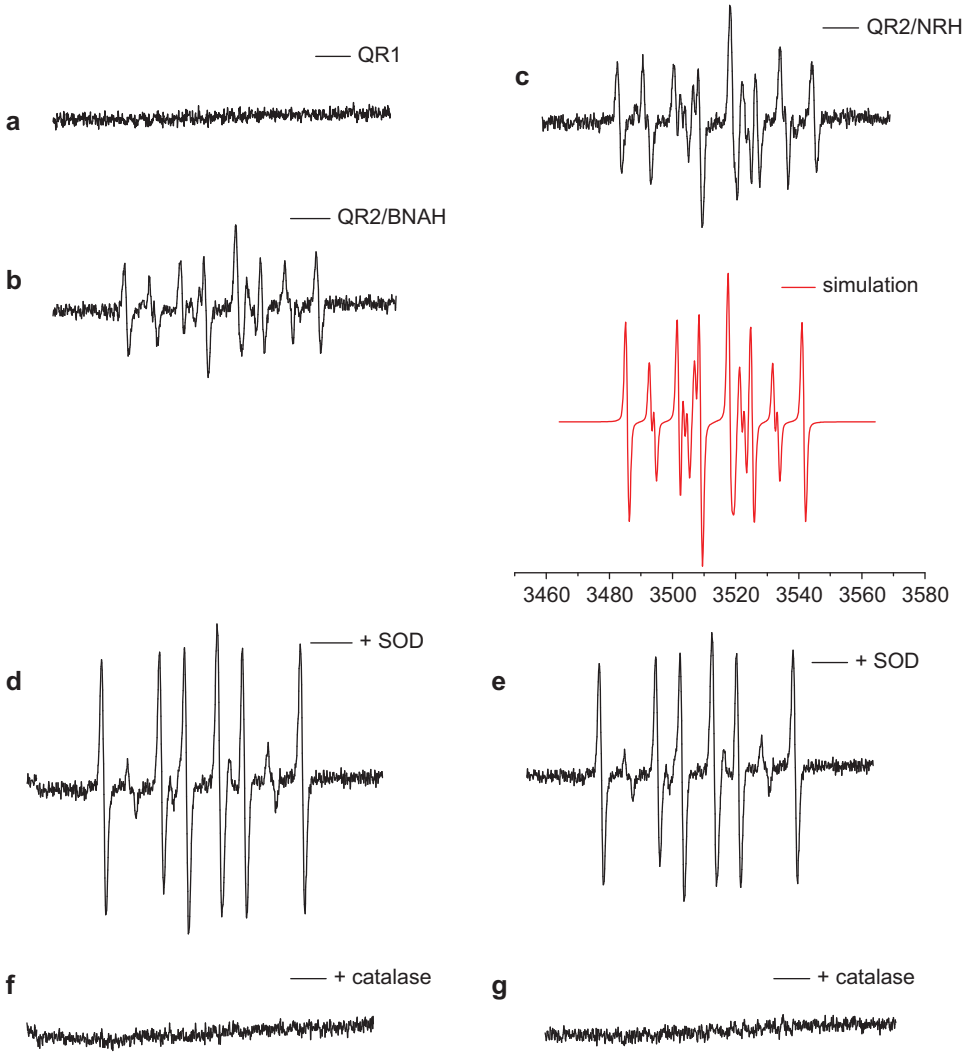

Figure 5. EPR spectra obtained in the presence of DMPO (125 mM) in a reaction mixture containing CB1954 (100 µM) and (a) QR1 (15 µg/mL), NADPH (1 mM) after 15 min incubation at 37°C; (b) QR2 (15 µg/mL), BNAH (1 mM) after 10 min incubation at 37°C; (c) QR2 (15 µg/mL), NRH (1 mM) after 10 min incubation at 37°C and the corresponding simulation; (d) addition of SOD-Mn (73.5 units/mL) to (b); (e) addition of SOD-Mn (73.5 units/mL) to (c); (f) addition of catalase (2000 units/mL) to (b); (g) addition of catalase (2000 units/mL) to (c). Intensity range: ± 20,000. Gain: 2.10^5

NRH. The synthetic compound S29434 completely eliminated •OH production with both co-substrates. Some components of our systems have the capacity to reduce free radicals (antioxidant molecular properties) in a QR2-independent mode. Therefore, we tested in parallel, the capacity of resveratrol and melatonin to reduce free radicals chemically produced by the Fenton reaction (H_2O_2/Fe^{2+}). The compounds tested are able to reduce the hydroxyl radical, suggesting that in our experimental conditions, the inherent antioxidant properties of the compounds may minimize the quantity of radicals produced during the QR2 redox cycle.

Discussion

Whatever the quinone reductase (QR1 or QR2) and co-substrate used, the enzyme reaction with the pure enzymes gives rise to the formation of radicals characterized by a sextuplet in the presence of DMSO and DMPO. The EPR study of this ROS production has been described for QR1 [25,26] from cellular extracts, while no information has been published on QR2-related ROS production. The main reason for this lack of information is primarily due to the recent re-discovery of QR2, on the one hand [27], and the close-sequence relationship of QR2 to QR1 on the other [28], suggesting that both enzymes should fall in the same category.

The production of hydroxyl radicals in the presence of QR1 was ascribed to the auto-oxidation of the unstable hydroquinone produced. This re-oxidation includes the oxidation of the hydroquinone with molecular oxygen (Eq. 1 & 2) and the subsequent reduction of molecular oxygen into the superoxide anion, and then H_2O_2 (Eq. 3). The radical •OH is produced from H_2O_2 by the Fenton reaction (Eq. 4). This hypothesis is supported by the fact that addition

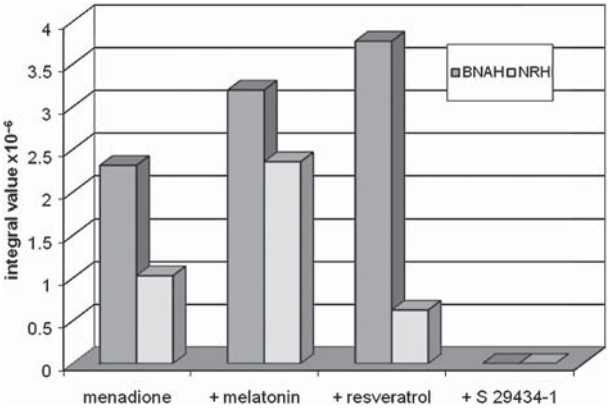

Figure 6. Comparison of the production of hydroxyl (OH•) radicals with different modulators. These comparisons were arrived at by calculating the double area integration ensuing from menadione reduction by purified QR2 in the presence of various modulators, from EPR spectra after 10 min incubation at 37°C. EPR spectra were obtained in the presence of DMPO (80 mM) in a reaction mixture containing purified QR2 (4 µg/mL), BNAH or NRH (1 mM), menadione (100 µM) and the tested modulator (100 µM); variation in areas ranged between 10% and 15%.

of catalase completely suppressed the signals, whereas addition of superoxide dismutase did not change the signal and even increase its intensity. Formation of hydroxyl radicals through a Fenton reaction raises the question of the metal's origin. In the case of QR2, Foster *et al.* [13] described the crystal structure and found a specific metal binding site, most likely a copper(I) site, which is near the protein surface, solvent accessible and separated from the FAD cofactor by a distance of about 13 Å. The studies of Buryanovskyy *et al.* [18], and those of Kwiek *et al.* [29] are also consistent with the presence of a metal ion site. While the first electron transfer between the substrate and co-substrate occurs in the deep hydrophobic pocket of the protein, the QR2 metal site may be involved in a second electron-transfer reaction, assuming that it is a redox-active site [copper(I)]. The possible presence of this redox active site is supported by the X-ray structural analysis of the nature of the ligands at the metal coordination site. Predicted pathways with computer simulations reported by Foster *et al.* show a possible electron-transfer route between the active site and the metal site. In the case of the hypothesis of a redox-active site to explain the formation of hydroxyl radicals, it is to be noted that when exogenous H_2O_2 was added to the protein, hydroxyl radicals were not detected with DMPO as spin trap. Therefore, a second hypothesis has to be proposed. A metal-independent formation of hydroxyl radicals by reaction of hydrogen peroxide with a quinone has been suggested by Zhu *et al.* [30] in the case of halogenated quinones. In our experimental conditions, the reaction of H_2O_2 with menadione (Figure 3h) also gave rise, in absence of DMSO (1%), to the production of hydroxyl radicals, which confirms that at least a part of the hydroxyl radicals is generated independently of a Fenton-like reaction [31]. This hypothesis is also in agreement with the hydroxyl radical production observed for QR1, which does not contain a redox-active site.

$$QH_2 + O_2 \longrightarrow {}^{\bullet}QH + O_2^{-\bullet} + H^+ \quad (1)$$

$${}^{\bullet}QH + O_2 \longrightarrow Q + O_2^{-\bullet} + H + \quad (2)$$

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \longrightarrow + H_2O_2 + O_2 \quad (3)$$

$$H_2O_2 + M^{(n)+} \longrightarrow {}^{\bullet}OH + M^{(n+1)+} + {}^{-}OH \quad (4)$$

$$QH_2 + Q \longrightarrow 2Q^{\bullet -} + 2H^+ \quad (5)$$

$$QH_2 + O_2^{-\bullet} \longrightarrow Q^{\bullet -} + H_2O_2 \quad (6)$$

These results may explain the catalytic capability and roles of QR2 in various patho-physiological situations. By-products of the QR2 redox cycle, such as H_2O_2, and subsequent hydroxyl radical generation, may be responsible for the propagation of the quinone toxicity. The re-oxidation also includes the comproportionation of the oxidized and reduced quinone (Eq. 5) [23,32] and in some cases, the reaction of the hydroquinone with superoxide leading to the production of semiquinone and hydrogen peroxide (Eq. 6). In the case of menadione, the absence of a semiquinone radical signal on the EPR spectrum indicates the highly unstable nature of menadiol, and is not in favour of this latter route (Eq. 6). This hypothesis is reinforced by the fact that addition of SOD did not decrease the intensity of the peaks. On the contrary, the addition of SOD enhanced the formation of hydroxyl radicals as shown by comparing Figures 2a and 2b. This phenomenon is easily explained by Eq. 3 since SOD produces H_2O_2 [23]. Indeed, superoxide dismutase increases the rate of superoxide dismutation from 5.10^5 M/s (spontaneous rate) to 2.10^9 M/s [33].

The similarity between EPR spectra recorded with QR2 with those obtained with QR1 confirms that QR2 can reduce menadione by transferring two electrons to produce hydroquinone, exactly in the same manner as QR1. As described in the case of QR1, catalase totally suppressed the sextuplet, whereas SOD slightly increased the quantity of radicals produced, confirming that the production of radicals follows Eqs. 1 to 4. In the case of QR2/NRH, addition of catalase revealed, after 10 min incubation at RT, a triplet (Figure 3g) that could be ascribed to the semiquinone radical. The hyperfine splitting constants recorded in this case ($a_H{}^2 = 3.1$ G^{3H}, $a_H{}^3 = 2.5$ G^{1H}, $a_H{}^6 = 0.75$ G^{1H}, $g = 2.0046$) are close to the values mentioned in the literature for menadione in water/EtOH ($a_H{}^2 = 2.97$ G^{3H}, $a_H{}^3 = 2.41$ G^{1H}, $a_H{}^6 = 0.73$ G^{1H}) [34]. These results might be an indication of a higher superoxide anion radical production with NRH (Eq. 6).

Many publications have ascribed an antioxidant character to QR1, thanks to its ability to prevent

the formation of deleterious semiquinone radicals [2,35]. However, in the case of hydroquinones, being potentially reactive metabolites, the toxification/detoxification character of quinone reductases will depend on the reactivity and properties of the hydroquinone that is generated as recently demonstrated by Nishiyama *et al* [10]. The difference between QR1 and QR2 does not rest on their toxification/detoxification character but rather on their different catalytic actions [11].

Another important point concerns the ability of QR1 and QR2 to reduce substrates other than menadione. Both co-enzyme Q0 and Q2 were activated by the three systems (QR1/NADPH, QR2/BNAH and QR2/NRH), but the EPR signals had weaker intensity compared to those obtained in the case of menadione. This difference may stem from the fact that the hydroquinones, produced from co-enzymes, are apparently more stable, and therefore, the electron transfer towards oxygen is more difficult. In the case of co-enzyme Q0, the reduction is accompanied by the production of two kinds of radicals giving rise to a 10-line spectrum. Simulation of the EPR spectrum (Figure 4c) reveals the presence of both DMPO-CH_3 ($a_N = 16.28$ G, $a_H = 23.25$ G) and DMPO-OOH ($a_N = 14.2$ G, $a_H^{\beta} = 11.34$ G, $a_H^{\gamma} = 1.25$ G) adducts. Addition of SOD suppressed the DMPO-OOH signal, indicating that this signal originated from the production of superoxide. Simulation of the corresponding spectra (Figure 4e) produces the appearance of a DMPO-OH spin adduct ($a_N = a_H = 14.71$ G). Addition of catalase totally suppressed the DMPO adduct signals, leading to the appearance of a triplet (Figures 4g and 4m), as in the case of the system QR2/NRH/menadione, which could be also ascribed to the semiquinone radical. The hyperfine splitting constants given by simulation ($a_H^6 = 2.36$ G^{3H}, $a_H^5 = 1.99$ G^{1H}; $g = 2.0046$) are close to values given in the literature for co-enzyme Q0 [36] ($a_H^6 = 2.33$ G^{3H}, $a_H^5 = 2.02$ G^{1H}) or other benzosemiquinones, for example *p*-benzosemiquinone [34]. The co-production of this radical explains the asymmetrical feature of the spectra. Co-addition of catalase and superoxide dismutase was required to totally suppress the signal, confirming, as shown in Eq. 6, that in this case the semiquinone radical originates from the reaction of hydroquinone with superoxide. These results underline the differences between substrates and highlight the importance of the nature of substrate/co-substrate pairs in the kinetics of the reduction of quinones, particularly in a 'natural' context. Differences in the chemical-structure rigidity of the substrate and co-substrate and steric hindrance have a very pronounced effect, bearing in mind that QR2 has a single catalytic site for both substrates and co-substrates. These results are important in view of the role of their naturally higher analogues (CoE Q's from Q0 to Q10) in cellular bio-energy generation and regulation [37,38].

In the case of the activation of the pro-drug CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] (Figure 1), also known as tretazicar, whereas no peak was obtained with QR1 (Figures 5a and 5b), strong signals were obtained in the case of QR2 particularly with the system QR2/NRH (Figure 5d). These results are in accordance with the literature that reports that human QR2 was 3000 times more effective than human QR1 in reducing CB1954 [39], supporting the role of residue 104 in the reduction of CB1954 [11]. It has been suggested that the quinone oxidoreductase QR2 can catalyze the four-electron nitroreduction of CB1954 to a potent, cytotoxic, bifunctional alkylating agent [23,40]. Our EPR study underlines the bioreductive power of QR2 compared with QR1. The metabolic products generated may undergo further enzymatic metabolism or self-re-arrangement to produce electrophiles and/or ROS that attack macromolecules, leading to cell cytotoxicity. The four-electron reducing properties of QR2 might be responsible for its differences with QR1 that prefers two-electron reduction of its substrates [39]. The concomitant production of ROS described herein, sparsely described in the literature, could take part in the cytotoxic effect of the pro-drug.

Another difference between QR1 and QR2 concerns their inhibitors/modulators. Molecular tools to study QR1 have been used for quite some time, particularly the relatively specific inhibitor, dicoumarol [41]. For QR2, some potent, but poorly specific inhibitors have been reported such as melatonin and casimiroin [22,42]. Several observations suggest that QR2 may recognize many substrates and inhibitors, on condition that there is sufficient planarity in their chemical structures [39]. This behaviour is a constant characteristic of the main enzyme families of Phase II drug metabolism, as discussed by Boutin [43] for UDP-glucuronosyltransferases and by Ma and Lu [44] for Cytochrome P450s. In the presence of S29434, no signal was detected, showing that S29434 is a competitive inhibitor of QR2 as dicoumarol is for QR1, as demonstrated by enzymology [19]. On the contrary, melatonin and resveratrol only modulate the radical production. Finally, these observations might explain the *in vivo* activity of S29434 in the memory processes, as recently reported [45]. Indeed, overactivity of QR2 in some pathological situations might lead to the overproduction of ROS, leading to the destruction of neurological cells [46].

The recognition of many different substrates as modulators by QR2 may explain its capacity to propagate the toxicity of quinones or pseudo-quinones more than QR1. It also shows that the evaluation of the 'cytotoxic' character has to be done with more than a limited number of substrates and co-substrates. In conclusion, this study demonstrates that there is a much higher cytotoxic balance for QR2 than for QR1.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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