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Commentary

NRH:quinone reductase 2: An enzyme of surprises and mysteries

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QR1, quinone reductase 1 (EC 1.6.99.2)
QR2, NRH:quinone oxidoreductase 2 (EC 1.10.99.2)
MT3, melatonin binding site

ABSTRACT

Quinone reductase 2 has been discovered in 1961 and rediscovered in 1997. Because of its sequence homology with quinone reductase 1, it has been suspected to detoxify quinones. Ten years later, evidences begin to point to a versatile role of this enzyme. Indeed, QR2 is strongly suspected to be the molecular target of anti-malarian drugs such as chloroquin or paraquine, and of red wine-derived resveratrol that might be responsible for the so-called *French paradox*. It also is identical to the melatonin binding site MT_3, and might therefore be a rationale explanation for the antioxidant role of melatonin. Finally QR2 might be implicated in the toxicity, in vivo, of quinones such as menadione. The present commentary attempts to summarize this information and discusses a series of hypotheses.

1. Introduction

Quinones, and particularly *para*-quinones, constitute an important class of ubiquitous and naturally occurring compounds. Human exposure to these molecules is abundant and achieved through food intake and air breathing, as automobile exhaust and cigarette smoke, and all burned organic materials in general, contain quantities of quinones [1–4]. Quinones are highly reactive compounds undergoing either one- or two-electron reductions. Enzymatic one-electron reduction, for instance by cytochrome P450 reductase, generates unstable semiquinone radicals which readily undergo redox cycling, in the presence of molecular oxygen, leading to the production of highly reactive oxygen species (ROS). These reactive compounds induce oxidative damages and consequently tissue degeneration, apoptotic cell death, premature aging, cellular transformation, and neoplasia [4,5].

Two-electron reductions of quinones and their derivatives by the enzymes of the drug metabolism phase 2 prevent these deleterious effects to occur. They produce more stable

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QR2 Man   1 MAGKKVLIVYAHQEPKSFNGSLKNVAVDELSRQGCTVTVSDLYAMNFEPRATDKDITGTLSNPEVFNYGVETHEAYKQRSLASDITDEQKKVREADLVIF 100
QR1 Man   1 MVGRRALIVLAHSERTSFNYAMKEAAAAALKKKGWEVVESDLYAMNFNPIISRKDITGKLKDPANFQYPAESVLAYKEGHLSPDIVAEQKKLEAADLVIF 100
            *  .. *** ** *  *** ..*  *   * ..*  *  ******** *  .  ****       *.*  *.  ***.  *. **  ****.  ******

QR2 Man 101 QFPLYWFSVPAILKGWMDRVLCQGFAFDIPGFYDSGLLQGKLALLSVTTGGTAEMYTKTGVNGDSRYFLWPLQHGTLHFCGFKVLAPQISFAPEIASEEE 200
QR1 Man 101 QFPLQWFGVPAILKGWFERVFIGEFAYTYAAMYDKGPFRSKKAVLSITTGGSGSMYSLQGIHGDMNVILWPIQSGILHFCGFQVLEPQLTYSIGHTPADA 200
            **** ** ******** .**    **.     ** *  . * ..**.****.  **.  *..**    ***.* * *.****.** **. ..   .  .

QR2 Man 201 RKGMVAAWSQRLQTIWKEEPIPCTAHWHFGQ                                                              231
QR1 Man 201 RIQILEGWKKRLENIWDETPLYFAPSSLFDLNFQAGFLMKKEVQDEEKNKKFGLSVGHHLGKSIPTDNQIKARK 274
                *      *  *    * * *        *
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Fig. 1 – Comparison of the human sequences of Q1 and QR2. The sequences have the following accession numbers: P15559, for QR1 and P16083 for QR2. Note that the homology between hQR2 and hQR1 is ca. 49%.

chemicals, hydroquinones, which can be conjugated with glutathione or glucuronic acid and then rapidly excreted.

There are two main quinone reductases reported in the literature. QR1 was first isolated in Ernster's laboratory [6,7] and has been widely studied ever since. QR2 was described for the first time in 1961 as an unknown mammalian cytosolic FAD-dependent flavoprotein catalysing the oxidation of reduced *N*-ribosyl- and *N*-alkyldihydronicotinamides by menadione and other quinones but not the oxidation of either NADH and NADPH, or NMNH (reduced nicotinamide mononucleotide), whatever the substrate [8–10]. This new enzyme, highly purified from bovine kidney, was extensively characterized, as its substrate and co-substrate specificities, its inhibitor profile, and its tissue distribution were determined. Surprisingly, QR2 has been then completely forgotten for 36 years. In the early 1990s, Jaiswal and co-workers, in the course of cloning human QR1 (a.k.a. NOQ1), isolated and described a second NAD(P)H:quinone oxidoreductase, which they called NQO2 [11,12]. It is only in 1997 that Zhao et al. [13] demonstrated that NQO2 was indeed the flavoprotein discovered by Liao and Williams-Ashman [8,9] more than 30 years before that they named quinone reductase 2 (QR2). Both QR1 and QR2 received the same EC number (1.6.99.2), a fact that enhances the confusion about the quinone reductase 2 role. This EC number for QR2 was changed recently to E.C. 1.10.99.2 (http://www.chem.qmul.ac.uk/iubmb/enzyme/morenz.html). Furthermore, the NQO2 nomenclature adopted since 1999, seems to be more confusing, since another enzyme shares the same name: the 24 kDa subunit of NADH:ubiquinone oxidoreductase (complex I) (see, for example, Zu et al. [14]). To avoid futher confusion, the use of the name NQO2 should be limited to complex I.

If NAD(P)H:quinone oxidoreductase (NQO1 or QR1) clearly catalyses a detoxification process, the NRH:quinone oxidoreductase (NQO2 or QR2) role is much more difficult to state. The aim of this revue is to revisit the various characteristics of QR2 and to suggest several hypotheses on the possible roles of this mysterious enzyme.

2. QR1/QR2 comparison

2.1. Nucleotide and amino acid sequences

The human QR2 gene is located on chromosome 6p25 (QR1 is located to 16q22) and encodes a physiological homodimer of 230 amino acids (25,821 Da) per monomer, that is 44 residues less than QR1 [4,15]. QR2 was therefore considered as a truncated homologue of QR1, containing the complete N-terminal catalytic domain of QR1 (up to residue 220) but lacking its C-terminal part (44 residues). However, despite these chain length differences, human QR1 and QR2 cDNAs and proteins share 54 and 49% sequence identity [4,11]. QR1 and QR2 sequences are presented on Fig. 1. The primary sequence analysis of mouse QR2 enzyme revealed the presence of glycosylation, (cryptic) myristylation, protein kinase C and casein kinase II sites, highly conserved in the human protein but absent in the mouse and human QR1 ones [3]. These differences, though, seem to bear little information.

2.2. Tissue distribution

hQR1 is ubiquitously expressed, but its expression is widely more variable (between organs, individuals, physiological, and pathological states). QR1 gene expression has been detected in various solid tumors including liver, thyroid, adrenal, breast, ovarian, colon, cornea, and non-small cell lungs cancers [16–21]. Its activity was also reported to be up-regulated in malignant tissues of the colon, breast, lung, and liver and down-regulated in solid tumors of the stomach and kidney (see Strassburg et al. [22], for references).

It is difficult to irrevocably establish in which tissues the QR2 gene is expressed. Indeed, many studies dealt with QR2 tissue distribution, but the results seemed to depend on the species studied. For example, QR2 gene expression in skeletal muscle has been described as high in human [4,12] but undetectable in mouse [3]. In normal human liver, QR2 is expressed at relatively high levels while only traces of QR1 can be found, a situation reversed in malignant tissues where QR1 $\gg$ QR2 [19,22]. In rodent, QR1 and QR2 are expressed at a similar level in normal conditions. In human, it has been shown that QR2 gene is also expressed in kidney, liver, and heart, that minimal expression is observed in brain and pancreas and that no expression is detected in placenta. It is important also to notice that QR2 is expressed at modest levels in human blood cells, particularly red blood cells (while QR1 is absent) [23].

2.3. Induction

QR2 and QR1 present differential regulation profiles. QR1 gene expression is induced in response to xenobiotics, antioxidants, oxidants, heavy metals, UV, light, and ionising radiation. This enzyme is part of an electrophilic- and/or oxidative stress-induced cellular defence mechanism that includes the induction of more than two dozen defensive

genes, such as gluthatione S-transferases and UPD-glucuronosyl transferases [4,24,25]. This coordinated induction may provide the required protection for cells against free radical damage, oxidative stress, neoplasia, and carcinogenesis caused by quinones and other chemicals [26–28]. There are two types of anticarcinogenic enzyme inducers: (i) monofunctional inducers that elevate phase 2 enzymes without significantly rising phase 1 enzymes, and (ii) bifunctional inducers that induce both phases 1 and 2 enzymes [29]. Several studies have been focused on the identification of the various inducers of the phase 2 enzymes, including QR1. Thus, many dietary compounds, including sulforaphane – isolated from *Saga broccoli*, baicalin – an extract of the scutellariae radix herb, chlorophyll, and phytoestrogens, appear to induce QR1 and so represent a theoretical way to modulate the risk of cancer [30–36]. The QR2 gene promoter (1336 bp) contains two copies of CCAAT box and four copies of the SP1 motif [3]. Both of these elements are found in many eukaryotic promoters and their activation leads to an increase transcription of the gene in which they are present or cooperate with other functionally related factors for their optimal effect. The QR2 promoter also contains one copy of the antioxidant response element (ARE), which is found in the promoter of the various detoxifying enzyme genes and appeared to be required for basal expression and induction of these genes in response to xenobiotics and antioxidants. The ARE mediates the induction of QR1 gene expression in response to polycyclic aromatic compounds and phenolic antioxidants [16,17,20]. Three copies of the sequence GCGTG can also be observed in the QR2 promoter. This sequence corresponds to five invariant bases of the xenobiotic region element (XRE). XRE is implicated in QR1 gene induction in response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [37].

Unlike for QR1, only a few studies dealt with the QR2 gene expression induction, and these studies gave contradictory conclusions. Indeed, because of the presence, in the QR2 promoter, of the various elements described above, it has been proposed that QR2 was coordinately induced with QR1 and the other phase 2 enzymes. It has been shown that, like QR1, QR2 expression could be induced by β-naphtoflavone (β-NF) and TCDD [38,39]. Moreover, Jaiswal [12] showed that the presence of QR2 promoter upstream a CAT gene induced an increase in CAT activity upon treatment with β-NF and TCDD. However, 4 years before, Jaiswal et al. [11] observed that TCDD treatment of the human hepatoblastoma Hep-G2 cells increased QR1 RNA but not QR2 RNA, suggesting that QR2 was not induced coordinately with QR1 and other phase 2 enzymes. Thus, the debate is still open.

2.4. *Enzymatic mechanism*

The close similarity between the QR1 and QR2 catalytic sites suggests that both enzymes present identical catalytic mechanisms. QR1 and QR2 reduction mechanisms have been described as ping-pong mechanisms, as the two enzymes use a unique catalytic site for both substrate and co-substrate binding: first, the electron donor (co-substrate) occupies the site, and only after its release, the electron acceptor (substrate) can enter the site. It has been shown that, in QR1, three residues—Gly^{149}, Tyr^{155} and His^{161} are directly involved in the electron transfer (from NAD(P)H to FAD and from $FADH_2$ to the quinone), and that only one of these is mutated in QR2, His/Asn^{161}, mutation that may be compensated by a water molecule [40,41].

Moreover, in QR2, the metal may also participate in the electron transfer mechanism, but its role is still unclear, essentially because the presence of the metal in vivo has not been established yet. And yet, Kwiek et al. showed that EDTA does not affect notably QR2 activity, which suggests that perhaps the metal is not implicated in the enzymatic mechanism [42].

Surprisingly, it is remarkable that both enzymes have been described as being able to catalyse various reactions involving different numbers of electrons transferred: 1, 2, or 4. This seems to be a function of the nature of the substrate (see Table 1), and of the type of reaction: CB1954 undergoes a nitroreduction (four electron reduction), menadione, an oxidoreduction (two electron reduction), and DCIP, a quinone-imine, an apparent irreversible reduction (one electron reduction). QR1 seems to be able to catalyse all these reactions as well, but by using only NAD(P)H, a feature that is different for QR2.

Despite their similarities, QR1 and QR2 present several differences. Most importantly, although QR1 can use NADH or NADPH as reducing agents (co-substrates) with almost equal kinetics, QR2 is unable to employ such reductants and uses

Table 1 – Substrate/co-substrate pairs used for quinone reductase 2 activity measurements

N-(*n*-propyl)-dihydronicotinamide	Menadione	Liao et al. [10]
N-ribosyldihydronicotinamide	Menadione	Liao et al. [10]
NADH[a]	CB10-200	Jaiswal [12]
N-ribosyldihydronicotinamide	CB 1954, DCIP, menadione	Wu et al. [38]
N-methyldihydronicotinamide	Menadione	Zhao et al. [13], Kwiek et al. [42], Graves et al. [23]
N-ribosyldihydronicotinamide	DCIP	Long and Jaiswal [4]
N-benzyldihydronicotinamide	Menadione	Nosjean et al. [58], Boutin et al. [43], Mailliet et al. [59]
N-(2-hydroxyethyl)dihydronicotinamide	Menadione	Buryanovskyy et al. [57]
Tetrahydrofolic acid	Menadione	Boutin et al. [43]
N-benzyldihydronicotinamide	Coenzyme Q0	Boutin et al. [43]

Note: Knox et al. [45], made a particularly complete survey of nicotinamide derivatives co-substrates of QR2 using CB1954 as substrate.
[a] A surprising result, since NADH is not recognized by QR2.

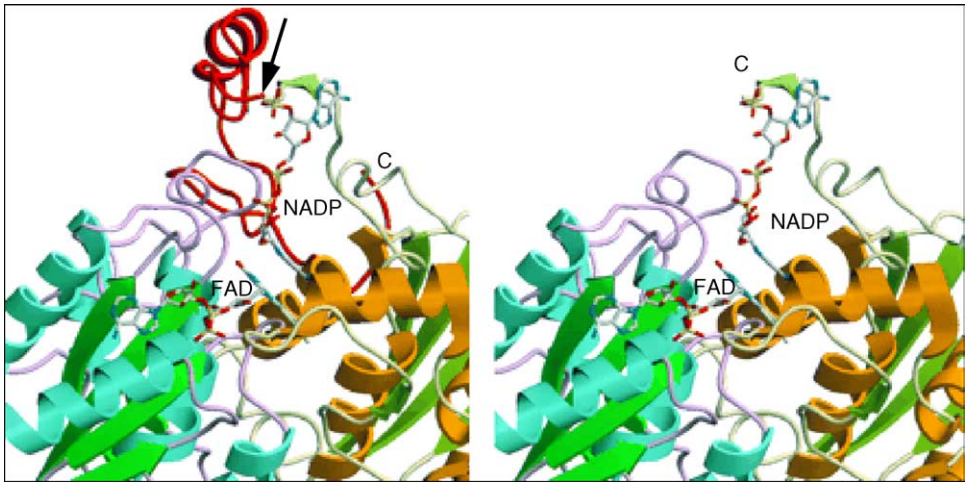

Fig. 2 – Comparison of mQR1 and hQR2 (Zhao et al. [13], printed with permission).

reduced *N*-ribosyl- and *N*-alkyl-dihydronicotinamide as a source of reducing equivalents. Another difference between the two enzymes lies on their inhibitor specificities. Indeed, QR2 is resistant to typical QR1 inhibitors, such as dicumarol, cibacron blue and phenindone [3] while flavones, and in particular quercetin and chrysoeriol, are potent QR2 inhibitors [38,43] and not QR1's (G. Ferry, S. Hecht, J.A. Boutin, unpublished).

2.5. *QR2 structure*

The determination of the crystallographic structure of human QR2, at a 2.1 Å resolution by Forster et al. [40], confirmed the close relationship previously observed between QR1 and QR2. The structure of the first 220 amino acids of QR2 is characterized by a α/β folding (five central, twisted, and parallel β-strands, surrounded on both sides by helices), a feature similar to the QR1 catalytic domain and to other flavoproteins. The C-terminal domain of QR2 (residues 221–230) forms a well-defined loop, whereas the corresponding region of QR1 corresponds to a loop and the first strand of a C-terminal anti-parallel hairpin (see Fig. 2 from Zhao et al. [13]). QR2, as QR1, contains one FAD molecule per monomer, firmly bound to the enzyme through multiple interactions. The amino acids involved in these interactions are very similar to those observed in QR1, as, among all the QR1 residues implicated in FAD binding, only one (Gln^{66}) is different in QR2 (Asn^{66}) [40,41]. The QR2 dimer also contains two identical catalytic sites, located far from each other at opposite ends of the dimer interface. Each site is a large cavity, lined by residues from both monomers and whose bottoms are formed by the FAD isoalloxazine ring. The QR1 catalytic sites are very similar, with the exception of three residues Tyr^{126}, Tyr^{128}, and Met^{131}, that are replaced in QR2 by Phe^{126}, Ile^{128}, and Phe^{131}, then making the QR2 cavity slightly larger and more hydrophobic than that of QR1. The residues involved in NADPH (co-substrate)/QR1 interactions through hydrogen bonds are all but one mutated in QR2 (Tyr/Phe^{126}, Tyr/Ile^{128}, Gly/Gly^{150}, His/Asn^{161}, His/Ile^{194} in QR1/QR2, respectively), or lost by truncation of the C-terminal domain of QR1 (lack of Phe^{232} in QR2) [41]. This may explain the incapacity of QR2 to use classical hydride donors (co-substrates) such as NAD(P)H. In QR1, additional stabilization of NADPH is achieved through aromatic staking of the nicotinamide ring and the FAD isoalloxazine ring [41]. This kind of interaction may essentially be responsible for the stabilization of the non-phosphorylated co-substrates in the QR2 catalytic site as the enzyme uses both polar and non-polar N-substituted dihydronicotinamide co-substrates [40]. With regard to the substrate, and more particularly to menadione, Foster et al. [40] showed, even if they could not determine precisely the orientation of the substrate molecule in the QR2 catalytic site, that there was no specific contact between QR2 and menadione, although they were linked by water molecules. Menadione binding did not induce significant conformational changes in the conformation of the enzyme, as the two QR2 structures (with and without menadione) were very similar. Unlike QR1, QR2 contains one metal binding site per monomer. This site is tetracoordinated, with two histidine ligands (His^{173} and His^{177}) and one cysteine residue (Cys^{222}) implicated twice in the coordination. Foster et al. [40] proposed that QR2 is a copper enzyme in which the metal binding site may be involved in the electron transfer, as its location on the protein surface excludes any structure stabilization participation, which however has been recently challenged [42] (see also Section 2.4).

3. QR2 characteristics and tools

Because QR2 has been identified as an analogue of QR1, on the basis of their sequence homology, one has often stated that QR2 is, as QR1, involved in detoxification processes, despite reports showing that QR1 can sometimes activate quinones, therefore rendering them more toxic [23,44]. As it will be more accurately discussed below (Section 4), it seems that QR2 is not a detoxification enzyme, but rather that the inhibition of its cellular activity prevents the cell from toxicity of, for instance, menadione. One way to know more about this catalytic role and mechanism, was to try to establish the partner(s) of the

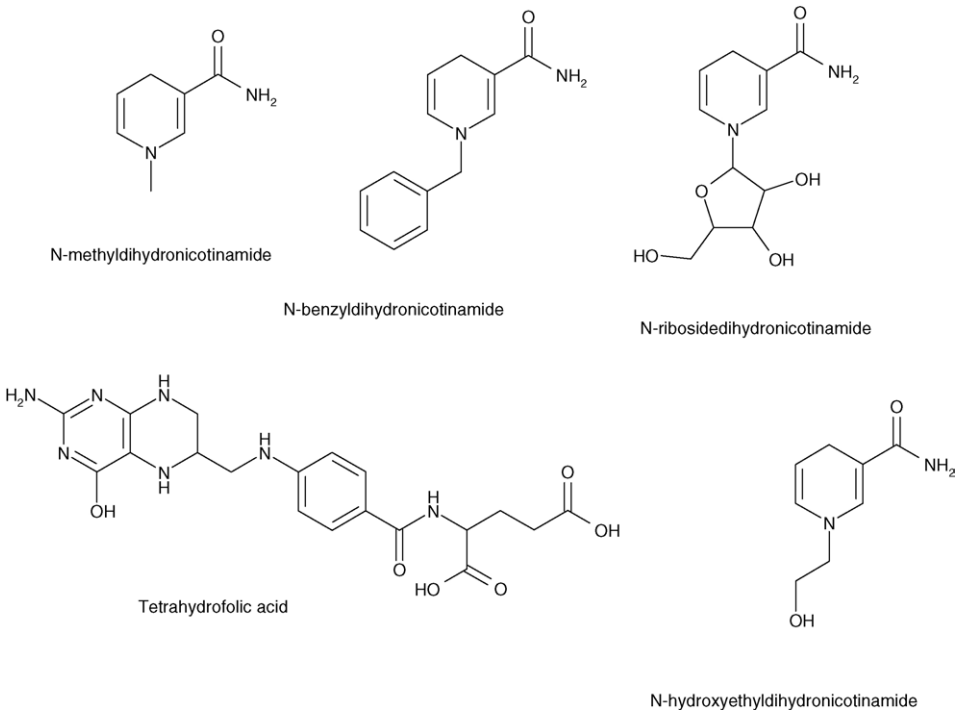

Fig. 3 – Reported co-substrates of quinone reductase 2.

enzyme in its activity. If little or nothing is known about the 'natural' protein partners of QR2, a little more is known about its substrate(s) and co-substrate(s). Furthermore, potent, selective inhibitors would also be extremely useful to study the enzyme activity in a natural cellular or tissue environment.

3.1. *Co-substrate(s) and substrate(s)*

QR2 does not recognise NAD(P)H as a co-substrate. The only contradictory report about this was a measure of NADH-dependent reduction of menadione using a recombinant QR2 expressed in COS cells. The observed reaction was probably due to a faint QR1 level in these cells [12]. A report exists on the construction and characterization of an hybrid protein constituted by the first 220 residues of hQR2 and the 43 C-terminal amino acids of hQR1. This chimeric QR2/QR1 is not able to recognize NAD(P)H as co-substrate neither [38]. The initial papers clearly stated that QR2 recognized only derivatives of the standard nucleotides [8,10]. The next question is then what are, amongst these, the naturally occurring ones?

3.1.1. *Natural co-substrate(s)?*

If there is no doubt that BNAH – *N*-benzyldihydronicotinamide, the co-substrate routinely used in our laboratory – is not a naturally occurring compound, the main co-substrates are NRH, the *N*-ribosyl derivative of dihydronicotinamide and NMH, the *N*-methyl one [10,45]. The former has been reported as a product of the degradation of NMNH (NMNH, reduced nicotinamide mononucleotide) by phosphatases [46–48] that is present at various concentrations in tissues [10]. Baum et al. [46] also reported that, by incubating NAD with brush border membranes of rat intestine, almost all the possible breakdown products were observed at physiological pH. The nicotinamide *N*-methyl derivative is believed to exist in urine coming from the NAD degradation process by several pathways, including nicotinamide nucleotide hydrolases and ADP ribosyltransferases (see Zhao et al. [13], for discussion). Indeed, the main reports suggested that NAD cleavage is catalysed by NADases (see Oppenheimer, for review [49]), an early step in the poly ADP-ribosylation of various protein targets. This step is an important metabolic regulating pathway. Finally, NMH has also been described in brain, as a catabolite of NADH [50]. Furthermore, we added to this list of unconventional nicotinamide derivatives, the fact that, although with a poorer affinity, QR2 could use tetrahydrofolic acid as an hydrure donor [43] (see Fig. 3, for structures).

3.1.2. *Coenzyme Q0 and more: natural substrate(s)?*

The technical complexity of finding naturally occurring substrates of enzymes is dramatic. Indeed, for enzymes involved in the detoxification steps of the drug metabolism pathway, such as cytochromes P450 or UGTs, it has been for a long time stated that most of these enzymes had no endobiotic substrate(s), but had evolved to accommodate xenobiotics. In a parallel manner, by using a survey of the naturally occurring quinones, it turns out that rather few has been described, apart from those issued from plants. The main reported quinones are Vitamin K (menaquinone and phylloquinone), coenzymes Q (ubiquinones) and dopaquinone, an orthoquinone. In a systematic search for quinone substrates of QR2, we screened more than 100 different compounds, all from commercial sources. One single hit was found, it was coenzyme Q0 [43]. This observation was extended to other members of the ubiquinone family, namely those with an

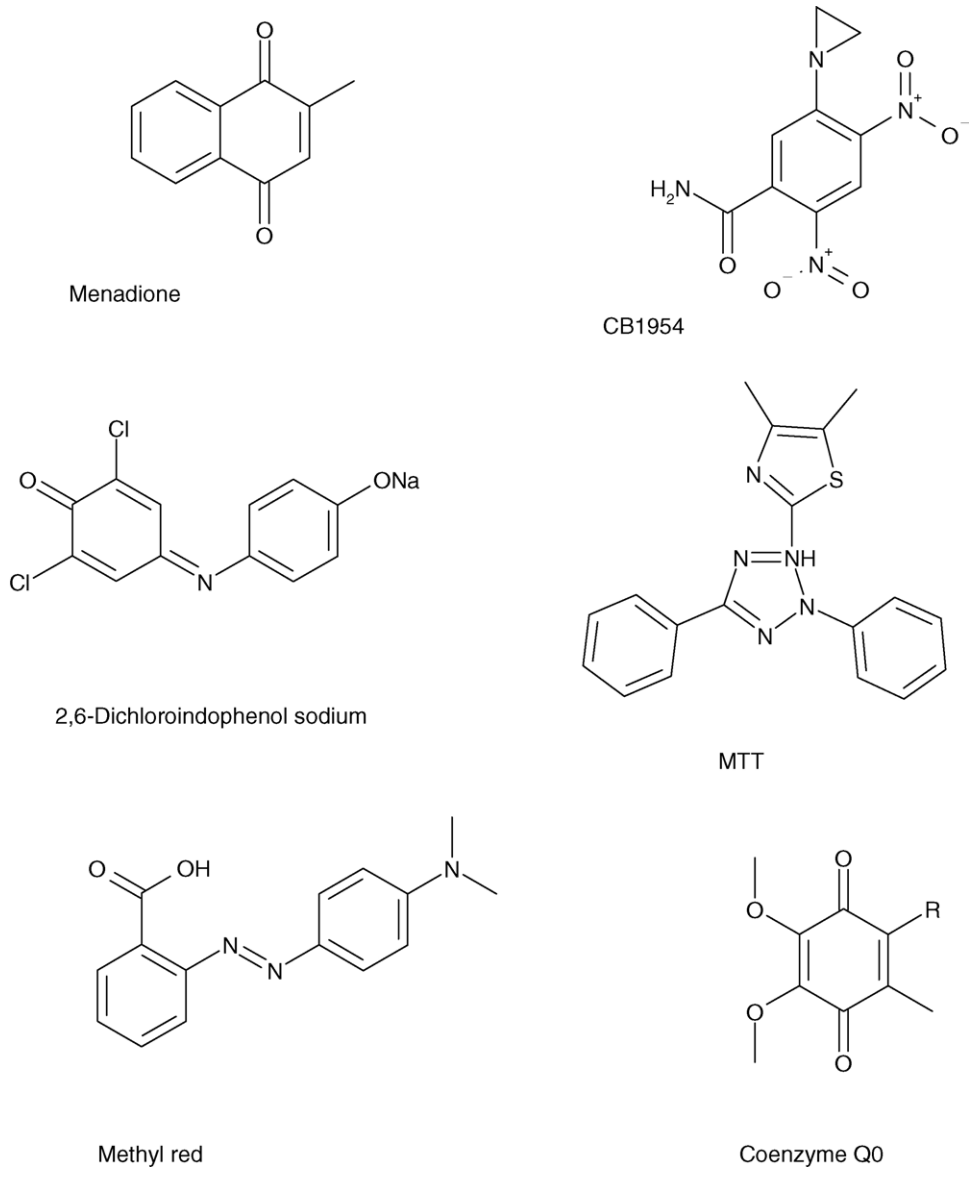

Fig. 4 – Reported substrates of quinone reductase 2.

isoprenyl side chain of various length (1–10 moieties), with the major limitation that the higher members of this family of compounds are insoluble in aqueous mixtures. Therefore, the actual capacity of these higher analogues to act as substrates of QR2 has not been documented yet. This property points to the location of these compounds at the membrane, and particularly at the mitochondrial membrane, where they are important, non-enzymatic members of the respiratory chain. Rat contains almost exclusively in this process the coenzyme Q9 derivative, while human, coenzyme Q10. It therefore seems that a link might exist between QR2 and the redox status of the mitochondrial respiratory chain, maybe for reducing back ubiquinol, and to maintain a homeostasis in this crucial mitochondrial process (see further discussions [51–53]). The available substrates of QR2 are drawn on Fig. 4. It is worth mentioning that phylloquinone (a.k.a. Vitamin K1), is a molecule distantly related to ubiquinone. It would not be surprising if QR2 was able to recognize this compound as a substrate, opening a route for a relationship between QR2 and coagulation, as QR2 could be implicated in the control of this quinone homeostasis.

3.2. *Inhibitors*

There are a few QR2 inhibitors reported in the literature (Fig. 5; see also [54], for review). Zhao et al. [13], confirming previous results from Liao et al. [10] described benzo(*a*)pyrene as a potent QR2 inhibitor (IC50 in the 100 nM range and below). Quercetin, a flavonoid once described as a tyrosine protein kinase inhibitor (see Barret et al. [55], for review), is a potent QR2 competitive inhibitor – K_i = 21 nM – with respect to NRH (the co-substrate) and shows a clear uncompetitive behaviour vis-à-vis the substrate, DCIP [38]. Interestingly, we showed a far less potency of this compound, IC_{50} = 300 nM, when QR2 activity was measured with BNAH as co-substrate, and menadione as substrate. Other flavone-like compounds have

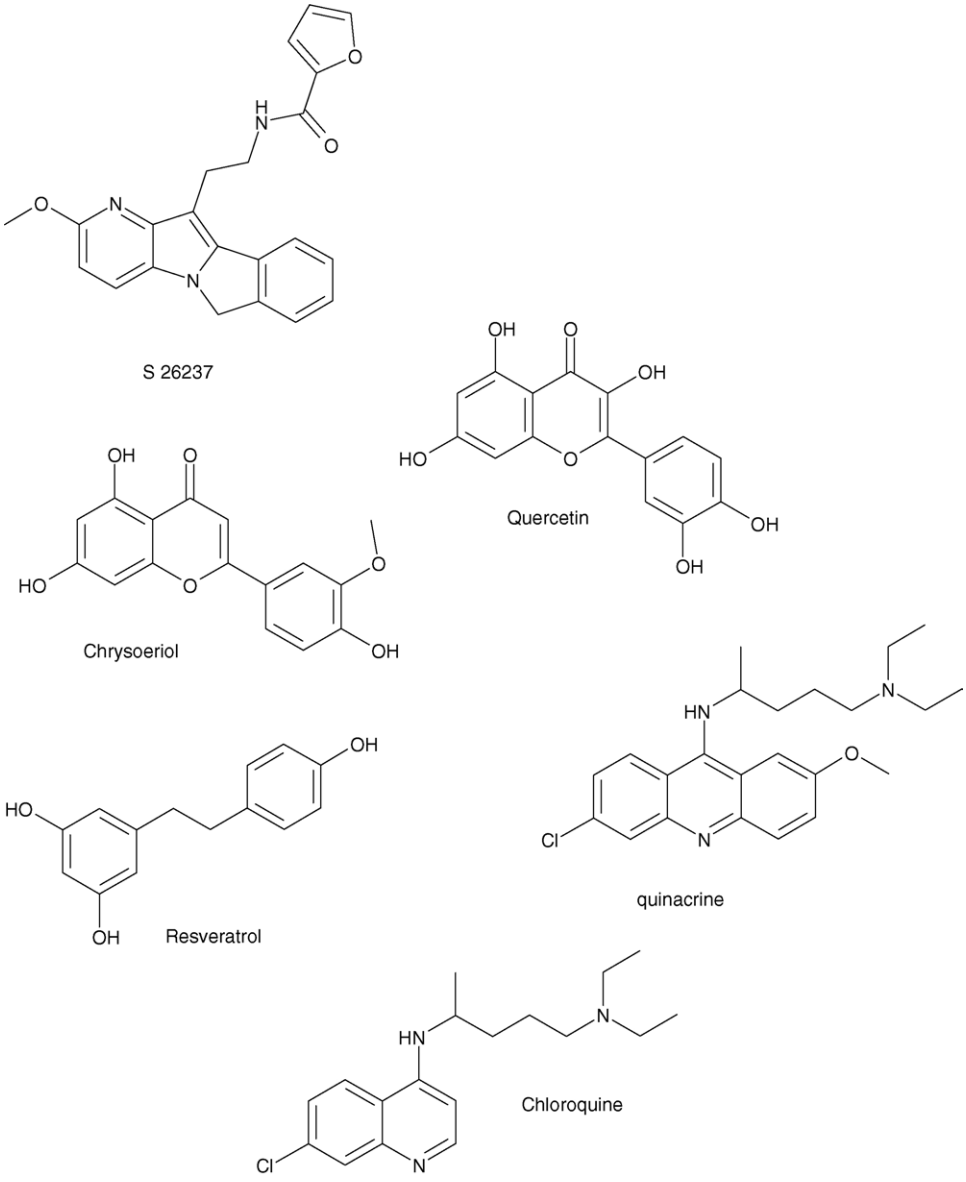

Fig. 5 – Reported inhibitors of quinone reductase 2.

been reported as potent inhibitor, although with not the same potency, as chrysoeriol with an IC50 of ca. 300 nM in the presence of BNAH and menadione [43]. Synthetic compounds also appeared to be potent QR2 inhibitors, such as S 26237 shown in Fig. 5 [56]. Furthermore, the efficiency of resveratrol as QR2 inhibitor was recently reported, with a K_d of 35 nM [57]. Although this compound has been described as an inhibitor of several different enzymes, such a nanomolar inhibitory potency towards QR2 seemed to be unique. Finally, Graves et al. [23] reported the anti-malarian quinacrine and chloroquine as potent inhibitors of QR2 in the 500 nM and 1 μM ranges, respectively (see Section 4.3).

3.3. *Limits of experimental determination*

The actual specificity of QR2, in terms of both substrate, co-substrate and inhibitors might be complicated by experimental considerations. While searching for inhibitors, we started by confirming data obtained by others, and particularly, on anti-malarian drugs. Using our own system (with BNAH as co-substrate), we found a dramatically poor inhibitory potency for chloroquine, as opposed to what was described in the original report where it was measured with another, more natural, co-substrate (*N*-methyldihydronicotinamide). Similarly, using quercetin, we found a right shift of the IC50 curve [43], as compared with what was originally reported with an other pair of substrate/co-substrate (namely, CB1954/*N*-hydroxyethyldihydronicotinamide). These few observations strongly suggest that the nature of the actual reaction catalysed by QR2 and therefore, the nature of the pair (substrate/co-substrate) has a dramatic impact on the measured potency of candidate inhibitors. What has been reported in this regard in the literature is summarized in Table 1. More systematic studies will be necessary to address these issues and some are currently ongoing in our laboratory. Furthermore, the physical association between the inhibitor and the

protein can be measured directly by use of the tryptophan fluorescence quenching by resveratrol [57] and of the FAD fluorescence quenching by chloroquin [42]. A similar observation was done for melatonin, as well as for a series of melatoninergic ligands, which are potent ligands at the third melatonin biding site, MT_3, while relatively poor inhibitors of QR2 [56].

3.4. Other tools

Other tools are becoming available: an anti-QR2 polyclonal antibody (Abnova Corp.), a cell line (CHO) stably expressing hQR2 [43,56,58], a cell line in which QR2 has been knocked down and that shows features similar to those of cells treated with resveratrol [57], as well as mouse strains in which QR2 has been permanently deleted [24,59]. The first strain was described for its decreased sensitivity to menadione as compared to the wild type, and the second is deprived of the melatonin binding site MT_3. A cross-characterization between these two strains has not been reported in the literature.

4. A role for QR2

QR1 has a clear role in detoxification. For QR2, though, a similar role would be difficult to established on the basis of the currently available experiments.

4.1. Human genetic studies

Two studies [60,61] reported an association between polymorphisms of QR2 and Parkinson's disease and schizophrenia. The polymorphism was in the form of an insertion/deletion of 29 nucleotide base pairs (bp) in the promoter region of QR2. According to the sequence involved, the authors speculated that this polymorphism would result in decreased production of the enzyme and may lead to an excess of the cathecholamine-derived *o*-quinones in the brain. Nevertheless, another group recently considerably modulated these conclusions [62]. It has been also reported that the promoter containing the 29-bp insertion polymorphism presented lower QR2 expression than that with the deletion [63]. Indeed, the insertion introduced a binding site to the transcription factor Sp3, which in this case acts as a repressor. Consequently, the deletion associated with either Parkinson's disease or schizophrenia leads to an excess in the production of QR2. This hypothesis has been confirmed in human fibroblasts from individuals with or without the deletion. The QR2 activity was statistically increased in the fibroblasts with the deletion. The authors concluded that higher QR2 activity might make individuals more susceptible to Parkinson's disease.

Similarly, Strassburg et al. [22] reported that in hepatocellular and biliary tissues, QR2 was down regulated, while QR1 was up-regulated, suggesting not only a differential regulation, but also, potentially a different role in carcinogenesis. QR2 might generate an harmful signal in cells, rendering its activity an activation/toxifying process. The impact of this activation process might become key on some disease developments. The availability of cellular models in which QR2 can be modulated will decipher the nature of the role of this enzyme, in particular in menadione-induced toxicity [57].

4.2. Mice deleted for QR2

Our initial KO mouse strain is currently transferred onto stable genetic backgrounds C57Bl/6 and also C3H/He, according to the fact that C57Bl/6 synthesize very low levels of melatonin. Meanwhile, we confirmed on the mixed genetic background (129Sv/C57BL/6) that organs from these $QR2^{-/-}$ mice were deprived of the melatonin binding site MT_3 [59]. Long et al. [24] reported a major experiment for the field on their own KO mouse strain (on an unspecified genetic background). These mice, once challenged by toxic doses of menadione, survived, while treated under the same conditions, their wild-type littermates died. The difference was even more significant when the mice were co-treated with the co-substrate of QR2, NRH. The same type of challenge had been previously done by the same group on their own $QR1^{-/-}$ mice, and they reported the expected and opposed result, that menadione was far less toxic to wild-type mice than to QR1 KO ones [64]. These data clearly indicated that the earlier hypothesis according to which QR2 – by analogy to QR1 – was a detoxification enzyme can be challenged. A discrepancy remains in the literature, though. It is the remarkable gain of sensitivity to benzo(*a*)pyrene-induced skin carcinogenesis of $QR2^{-/-}$ mice [65]. These KO mice seem to respond with an increased development of skin tumors when benzo(*a*)pyrene or benz(*a*)anthracene were topically applied to their skin, as compared with the wild-type animals. Hence, the QR2 deletion conferred a greater susceptibility to carcinogenesis induced by these compounds. In wild-type mice, it is difficult to understand how to link the QR2 activity with the carcinogenesis of the compound, since benzo(*a*)pyrene inhibits the enzyme [10,13]. In $QR2^{-/-}$ knock-out mice, the apparent enhanced activity of the carcinogen cannot be linked to the enzyme, since it is not present. Nevertheless, it has been hypothesized that the deletion of the enzyme in mice would induce other antioxidant mechanisms of defence [65].

4.3. QR2 = MT_3?

While attempting to characterize the third melatonin binding site, MT_3, Nosjean et al. purified QR2 by affinity chromatography and characterized some of its functions [58,66]. Because of the plasticity of phase 2 enzymes, particularly UGTs [67], we wondered if the apparent affinity of QR2 for melatonin was the reflect of an actual role of melatonin in the QR2 function. Using a series of different approaches, including the development of inhibitors [43,56] and $QR2^{-/-}$ mice [59], we attempted to understand this relationship. We demonstrated that the binding of melatonin in QR2 was possible at two distinct sites of the enzyme, including the binding site. As stated above (Section 4.2), all tissues from $QR2^{-/-}$ mice are depleted of melatonin MT_3 binding sites [59]. Altogether, these data demonstrate that QR2 is indeed MT_3. More questions, though, remain to be answered, among which the key one: is the specific interaction of melatonin with QR2 could explain the antioxidant properties of this hormone?

4.4. QR2 and malaria

In two outstanding publications from the same group, Kwiek et al. [23] and Graves et al. [42] reported on the search of the molecular target(s) of the anti-malarian quinolines, chloroquine and quinacrine. Two enzymes were identified by both proteomic studies, and by affinity purification onto a quinoline-immobilized column. In a manner similar to resveratrol, recently (see below), or to melatonin, some time ago (see above), these authors identified QR2 as the major (and in some case unique) target of these compounds. Furthermore, the authors tested in several different ways the capacity of these compounds to inhibit the catalytic activity of QR2. In particular, using the naturally occurring co-substrate, N-methyldihydronicotinamide, they found chloroquine to have a K_i in the micromolar range, and quinacrine, in the 0.5 micromolar range, either with a recombinant or a blood cell-purified QR2. Furthermore, using an assay based on the quenching of the natural FAD-absorbance at 450 nm, they also reported these compounds as being very potent inhibitors. These authors suggested that inhibiting QR2 activity may affect the redox status of red cells. By enhancing the production of reactive oxygen species derived from endogenous quinones – normally detoxified by QR2 – the inhibitor would create an inhospitable environment for the parasite, as it is sensitive to reactive oxygen species [68]. This particular hypothesis was proposed in the context of QR2 being a detoxifying enzyme. This might be a major breakthrough in the search of anti-malarian medicines.

4.5. QR2 = resveratrol main target?

A recent publication showed that using resveratrol [70] as bait in an affinity chromatography step, it was QR2 that was fished out from biological sources. As for quinolines or for melatonin, whether these observations are specific or linked to the function(s) of QR2 remains to be clarified. These reports [57,70] on resveratrol, were extremely convincing that this compound, believed to be at least in part responsible of the so-called '*French paradox*' [71] might, by its inhibition of QR2, protect mammals from over-exposure to activated reactive oxygen species. These reports were based on a series of observations, particularly the co-crystallisation of QR2 with resveratrol as well as the construction of a K562 cell line stably expressing a specific RNAi against QR2. These cells were resistant to menadione stress, as were the wild-type cells when treated by resveratrol while the naïve cells were killed by this menadione treatment. All this information convincingly pointed out to a central role of QR2 in the action of resveratrol. Even more recent data from the same group [72] showed that resveratrol might be an inducer of QR2, a feature that would complicate further the picture, since the same compound would be an inducer (enhancing the expression of the protein) and an inhibitor (impairing the enzyme catalytic activity).

4.6. Is QR2 a nitroreductase?

Amongst the surprising compounds specifically substrates of QR2, is the cytotoxic pro-drug, CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide]. This compound seemed to be specifically activated by QR2 into a potent cytotoxic compound [45,73]. The level of specificity is so high, that only added co-substrates of QR2 rendered CB1954 cytotoxic. This compound entered phase 1 study for cancer therapy [74]. In addition, several lines of evidence suggest a strong relationship between menadione, ubiquinone and nitroreductase, as can be extrapolated from Grimaldi et al. [75].

5. Hypotheses

Historically, QR2 has been considered to be a detoxifying enzyme, by analogy with QR1, and some initial experimental data supported this fact.

5.1. Pro (QR2 protects)

Several experimental reports argued for a protective role for QR2:

By inhibiting QR2, it seems that anti-malarian compounds such as quinacrine favour the red blood cell oxidative stress leading to the death of the parasite, which is very sensitive to it.

By deleting QR2 it seems that mice become increasingly susceptible to polycyclic aromatic hydrocarbon-induced skin carcinogenesis.

By inducing QR2, resveratrol seems to stabilize p53, which in turn would contribute to the diminution of growth in melanoma cells [72].

5.2. Con (QR2 activates)

Recently, data obtained from the KO mice ($QR2^{-/-}$) seemed to indicate that without QR2 – but in a very complex system (a whole animal) – menadione was not toxic anymore. This fact is stressed when a co-substrate of QR2 was co injected to these mice. These data seemed to argue against a detoxifying role of QR2.

These data were further backed up by data on resveratrol since this compound inhibits the enzyme, rendering treated cells as poorly sensitive to menadione as cells in which QR2 has been deleted by RNAi [57]. The decreased sensitivity to menadione of cells depleted of QR2 has been confirmed in our lab [69]. A parallel might be established with melatonin to some extent, since the inhibiting capacity of this neurohormone is about 10–100 times less potent than resveratrol and seems to be co-substrate dependent.

5.3. Coda: what does QR2?

In view of these data, it is hard to reconcile this increase susceptibility to skin carcinogenesis with a decrease sensitivity to menadione toxicity in the same QR2-deleted mice. Several factors might interfere with interpretation of the hard data: for instance, the substrate actually recognized in some situation by the enzyme (when it is still there). Not only it might not be the same, but its nature is still unknown. Even more perturbing is the exact nature of the co-substrate. It might be that NRH is mainly produced during NADH break-

down, in which case, the oxidative stress might be responsible of the awake of QR2 activity, if it helps cleaving NADH into NRH, while in standard situations, no co-substrate is available for QR2 to function.

A provocative hypothesis might be, in the red cells exposed to parasite, that QR2 renders the oxygen species produced during an oxidative stress, more aggressive and lethal for the cells. By limiting this process, QR2 inhibitors would limit the toxicity of the oxidative stress induced by parasite or other events leading to oxidative stresses. Obviously, this hypothesis needs to be confirmed by further hard data coming from KO animals, potent, and selective QR2 inhibitors [57], etc. For instance, it would be interesting to compare the capacity of these various QR2 inhibitors (such as S 26237, chrysoeriol, resveratrol, melatonin, quercetin, and quinacrine and more are becoming available), to limit or decrease the menadione-dependent toxicity in a unique cellular system. However, we should keep in mind that other explanations of the anti-malarial properties of quinolines, unrelated to the QR2 enzyme, have also been proposed [76].

Finally, and maybe most importantly, one should solve the co-substrate question. We can further tentatively hypothesise that QR2 behaviour differs as a function of its available co-substrate(s). We could question whether these co-substrate are similarly available in various cell types or/and in various physio-pathological conditions – ageing, oxidative stress, etc. By being present in a given condition, a given co-substrate would favor the formation, by QR2, of variously active and/or toxic molecular species.

6. Conclusion

It might be difficult to conclude in a few sentences the various points raised along the present commentary. The main question, to our view point, remains the nature of the activity QR2 actually catalyses. As long as this question will not be clearly answered, it will remain complicated to gather all the various observations inside a unique frame according to which the role of QR2 would be explained in such various physio-pathological situations, as in the melatoninergic system, the malaria aetiology and the quinone-induced toxicity. Furthermore, Knox et al. [43] stated that QR2 could be considered as a human NRH-dependent nitroreductase. To our knowledge, nitroreduction is not a function catalysed by mammals. The confirmation of this fact would be a complete change in perspective on how to study this enzyme. Despite a rather limited number of PubMed® entries, QR2 (unfortunately a.k.a. NQO2), remains a source of many questions: is QR2 a detoxification enzyme? As such, is its inhibition by either anti-malarian drugs or by resveratrol, a key explanation in the benefit of these compounds? Is melatonin binding a 'side feature', linked to the rather large size of the binding site (the catalytic site), or is melatonin acting like those compounds that provide some antioxidant protection? Obviously, the discovery of new chemical entities and the construction of new molecular tools (RNAi and KO cells, KO animals, directed mutagenesis, etc.) will help to answer those questions and build a comprehensible frame.

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