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MINIREVIEW

Molecular Pharmacology of NRH:Quinone Oxidoreductase 2: A Detoxifying Enzyme Acting as an Undercover Toxifying Enzyme [S]

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ABSTRACT

N-ribosyldihydronicotinamide:quinone oxidoreductase 2 (NQO2/QR2, Enzyme Commission number 1.10.99.2) is a cytosolic enzyme, abundant in the liver and variably expressed in mammalian tissues. Cloned 30 years ago, it was characterized as a flavoenzyme catalyzing the reduction of quinones and pseudoquinones. To do so, it uses exclusively *N*-alkyl nicotinamide derivatives, without being able to recognize NADH, the reference hydrure donor compound, in contrast to its next of a kind, NAD(P)H:quinone oxidoreductase 1 (NQO1). For a long time both enzymes have been considered as key detoxifying enzymes in quinone metabolism, but more recent findings point to a more toxifying function of NQO2, particularly with respect to *ortho*-quinones. In fact, during the reduction of substrates, NQO2 generates fairly unstable intermediates that reoxidize immediately back to the original quinone, creating a futile cycle, the byproducts of which are deleterious reactive oxygen species. Beside this peculiarity, it is a target for numerous drugs and natural compounds such as melatonin, chloroquine, imiquimod, resveratrol, piceatannol, quercetin, and other flavonoids. Most of these enzyme-ligand interactions have been documented by numerous crystallographic studies, and now NQO2 is one of the best represented proteins in the structural biology database. Despite evidence for a causative role in several important diseases, the functional role of NQO2 remains poorly explored. In the present review, we aimed at detailing the main characteristics of NQO2 from a molecular pharmacology perspective. By drawing a clear border between facts and speculations, we hope to stimulate the future research toward a better understanding of this intriguing drug target.

SIGNIFICANCE STATEMENT

Evidence is reviewed on the prevalent toxifying function of *N*-ribosyldihydronicotinamide:quinone oxidoreductase 2 while catalyzing the reduction of *ortho*-quinones such as dopamine quinone. The product of this reaction is unstable and generates a futile but harmful cycle (substrate/product/substrate) associated with reactive oxygen species generation.

History

In the early 1960s, the group of Williams-Ashman discovered some quinone reductase activities supported by yet uncharacterized protein(s) that can reduce quinone to quinol in the presence of a derivative of nicotinamide (Liao and Williams-Ashman, 1961; Liao et al., 1962). These cosubstrates were and still are suspected to be intermediates in the synthesis of NADH or more probably catabolites of NADH. In 1990, Jaiswal in his seminal paper cloned what he believed to be an isoform of the DT-diaphorase (Jaiswal et al., 1990). DT-diaphorase was described initially by Wosilait and Nason (1954) and subsequently renamed to quinone reductase (QR) 1 and later NAD(P)H:quinone oxidoreductase 1 (NQO1). This ancient enzyme recognizes NAD(P)H as an electron donor and was described for decades as the main quinone reductase. Several years later, the Talalay group finally cloned and characterized a homolog of NQO1 that was named QR2 [now *N*-ribosyldihydronicotinamide (NRH):quinone oxidoreductase 2 (NQO2)] (Zhao et al., 1997). For the record, NQO2 should not be confused with the subunits of the proton-translocating

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ABBREVIATIONS: aa, amino acid(s); BNAH, 1-benzyl-1,4-dihydro-nicotinamide; CB1954, 5-(1-aziridinyl)-2,4-dinitrobenzamide; ClQ, chloroquine; GTEx, Genotype-Tissue Expression; NifL, nitrogen fixation regulatory protein; NMNH, *N*-methyldihydronicotinamide; NQO1, NAD(P)H:quinone oxidoreductase 1; NQO2, NRH:quinone oxidoreductase 2 (Enzyme Commission number 1.10.99.2); NRH, *N*-ribosyldihydronicotinamide; QR, quinone reductase; ROS, reactive oxygen species; S29434, [2-(2-methoxy-5*H*-1,4b,9-triaza(indeno[2,1-a]inden-10-yl)ethyl]-2-furamide; SNP, single nucleotide polymorphism; TF, transcription factor; UGT, UDP-glucuronosultransferase.

NADH-quinone oxidoreductase of the thermophilic bacterium *Thermus thermophilus* HD-8, as described in the 1990s by Yano et al. (1994, 1995, 1996, 1997). Further characterization clearly demonstrated the particular nature of QR2/NQO2, namely, its quite unique specificity toward electron donors. It was confirmed that the enzyme did not recognize NAD(P)H and that it had remarkable characteristics distinct from those of NQO1.

In the next few years, several characterization reports were published, particularly focusing on its possible role in melatonin pharmacology (Nosjean et al., 2000; Mailliet et al., 2005), its affinity for resveratrol (Buryanovskyy et al., 2004), its possible function characteristics as a catecholamine reductase (Fu et al., 2008), and its possible role in antimalarial properties of the chloroquine (ClQ) mode of action (Kwiek et al., 2004), among many other features. The purpose of the present review is to discuss the current state of knowledge from molecular pharmacology point of view.

Molecular Biology

Sequence. The human NQO2 gene, located on chromosome 6p25.2, comprises seven exons (the first is noncoding) spanning 19.8 kb. The *NQO2* gene locus is highly polymorphic and encodes a protein of 231 amino acids (aa) with a molecular weight of 25,956 Da. Nucleotide sequence analysis of the *NQO2* gene promoter/enhancer region, starting at −1.9 kb upstream of the transcription start site, reveals the presence of several *cis*-elements, including SP1 binding sites, CCAAT box, xenobiotic response element, and an antioxidant response element (see Fig. 1) and other DNA motifs that can bind as many as 488 different transcription factors (TFs). They include SP1, AP-1, ATF-2, NFE2L1/NRF1, NFE2, eight FOX-family members, four STATs, three C/EBPs, three JUNs, three SOXs, two SREBF TFs, and many others, as predicted by bioinformatics (www.genecards.org). Interestingly, the classic antioxidant response element–binding antioxidant TF NFE2L2/NRF2 does not interact directly with NQO2 promoter, but it may bind via three different musculoaponeurotic fibrosarcoma proteins that show high-affinity-to-DNA motifs present in this promoter. The transcriptional machinery potentially binding to NQO2 gene regulatory sequences regulates tissue specific expression and induction of the *NQO2* gene in response to xenobiotics and antioxidants (Vella et al., 2005), and presumably to proinflammatory factors, since many inflammation-related TFs such as STATs, C/EBPs, RELA, and JUNs are predicted binders of NQO2 promoter/enhancer.

Considering both the sequence and the biochemical function, the protein encoded by *NQO2* can be classified as one of the members of the quinone oxidoreductase subgroup of the flavodoxin-2 gene family, which is one of four members of the flavoprotein class (Vasiliou et al., 2006). The quinone oxidoreductase subgroup contains five homologous enzymes, but only NQO1 and NQO2 are expressed in mammals. An ancient NQO3 subfamily exists in eubacteria, with NQO4 and NQO5 subfamilies in fungi and archaebacteria, respectively. Interestingly, no NQO genes could be identified in the worm, fly, sea squirt, or plants because these taxa carry other quinone reductases capable of one- and two-electron reductions (Vasiliou et al., 2006). NQO2, with its 231 aa, is 43 aa shorter than NQO1 at its carboxy terminus. The human *NQO2* cDNA and protein have 54% and 49% homology to the human liver cytosolic NQO1 cDNA and protein, respectively (see Fig. 2). The respective aa differences give rise to several important differences in cofactor requirements, substrate and cosubstrate specificities, and inhibitor/ligand affinities, as discussed later. The analysis of the crystal structure of NQO2 revealed that NQO2 contains specific metal binding sites, which are not present in NQO1. NQO1 also lacks a melatonin binding site (Ferry et al., 2010), which is present in NQO2 (Boutin and Ferry, 2019). Finally, NQO2 can be acetylated, ubiquitinylated, and phosphorylated on several serine, threonine, and tyrosine residues as reported by the PhosphoSitePlus database (www.phosphosite.org). The presence of these modifications has been documented by experimental data from numerous high-throughput proteome screening projects. In particular, the ubiquitination at K23 has been independently confirmed (Mertins et al., 2013; Akimov et al., 2018), but the functional meaning of this and other posttranslational modifications has never been addressed.

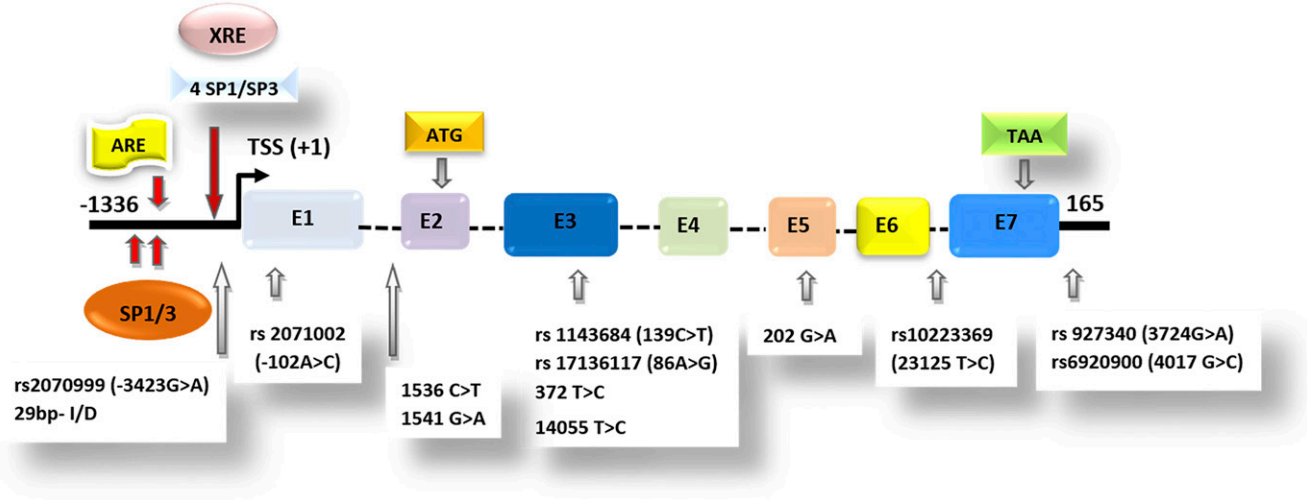

Fig. 1. Genomic structure of NQO2 with selected SNPs. Disease-associated SNPs and polymorphic variants are reported in boxes. Colored blocks indicate exons 1–7 (E1–E7); broken lines indicate introns; bold lines indicate 5′-flanking and 3′-flanking region. ARE, antioxidant response element; 29bp-I/D, 29-base-pair insertion/deletion; SP1/3, SP1/SP3 binding sites; TSS, transcription start site; XRE, xenobiotic response element.

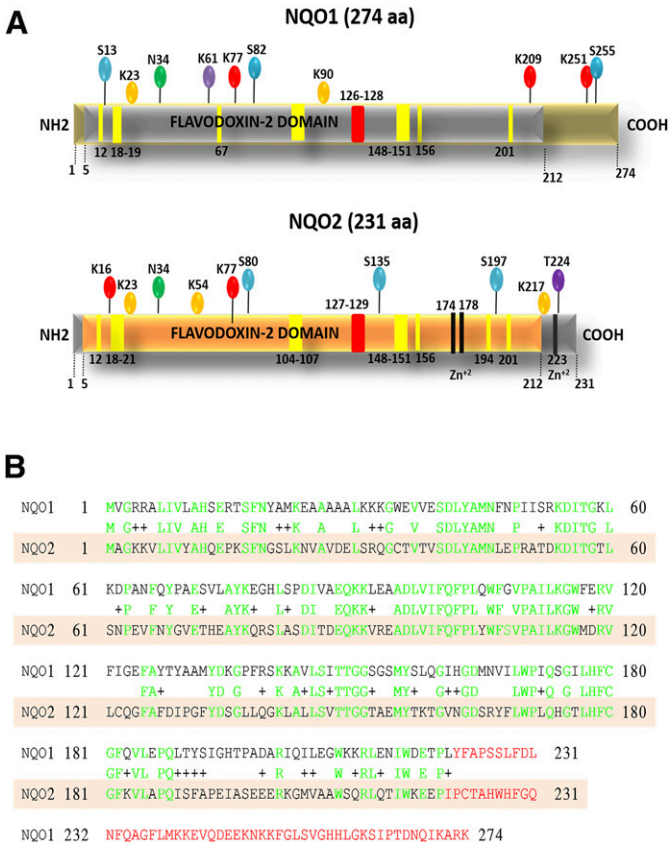

Fig. 2. Comparison of human NQO1 and NQO2 proteins. (A) Schematic protein structures of human NQO1 and NQO2. Colored bars indicate the following: red, substrate binding residues; yellow, FAD binding region; black, Zn^{2+} binding residues. Posttranslational modifications of NQO1 and NQO2 and their relative locations are indicated by the following code: blue dot, Ser phosphorylation sites; red dot, Lys acetylation sites; orange dot, Lys ubiquitylation sites; green dot, *N*-myristoylation sites; violet dot, Thr phosphorylation sites. (B) Alignment of amino acid sequences of human NQO1 and NQO2. The regions of sequence identity between human NQO1 and human NQO2 are in green (48.42%); different amino acids are in black; conservative replacements (amino acids with similar biochemical properties) are indicated by a "+" symbol; C-terminal misalignments with no sequence identity are in red.

Tissue Distribution. The NQO1 and NQO2 genes are expressed in numerous tissues but have different tissue distribution patterns. Both genes can be induced in response to antioxidants and xenobiotics, but NQO2 is less sensitive to the majority of antioxidants and generally more responsive to xenobiotics (Vella et al., 2005). Furthermore, NQO2 is characterized by larger expression between different tissues and species (Vella et al., 2005), which are complicated by interindividual variability (Riches et al., 2017). The initial Northern blot findings from human specimens indicated that the highest expression was in skeletal muscle followed by kidney, liver, lung, and heart. Expression of NQO2 mRNA in pancreas, brain, and blood cells remained minimal (Long and Jaiswal, 2000b). These old data are partially confirmed by modern high-throughput "omics" projects. For example, the Human Gene Atlas (www.biogps.org), based on microarray analysis from four or more pooled male and female individuals, confirms a high NQO2 mRNA expression in kidney, liver, and lung but not in skeletal muscle and heart, where it is 12 times lower than in kidney (Su et al., 2004). In addition, this database shows a high expression of NQO2 in the whole blood, myeloid cells, adipose tissue, and adrenal glands but very low expression in pancreas, ovaries, skin, and testis. The Genotype-Tissue Expression (GTEx) data collection from an RNA sequencing project of the GTEx Consortium (2013) confirms the high expressions of NQO2 gene (exons 8–13) in kidneys and livers, as well as in whole blood and adrenal glands (http://www.gtexportal.org). The same source reports high expression in skeletal muscles and moderate to high transcript levels in adipose tissue and brain (frontal cortex and cerebellum), when lung and heart NQO2 expressions are moderate. Interestingly, the GTEx database indicates expression of exon 1 in majority of brain regions, but not in other human organs and tissue sources, and no expression of exons 3, 6, and 9 in all tissues. In adult mice, the tissue expression pattern is similar, except for a low or undetectable expression in skeletal muscles (Long and Jaiswal, 2000a; Smith et al., 2019).

The Human Protein Atlas database, comparing the immunohistochemistry expression data from 44 human tissue sources from different individuals (Uhlén et al., 2015) reports the highest expression in kidney cortex, followed by liver, adrenal gland, skeletal muscle, and cerebellum. Surprisingly, a relatively high protein expression can be detected in other human organs with very low mRNA expression, such as colon, duodenum, testis, and thyroid gland. This database does not analyze NQO2 protein levels in blood cells, adipose, and lung tissue; however, the Human Proteome Map, collecting mass spectrometry data for 17 adult organ and blood cell samples, suggests moderate protein expressions in monocytes and T CD8+ cells, and low expression in lung (Kim et al., 2014). Human Proteome Map also confirms the highest NQO2 levels in kidney, followed by adrenal and liver samples. There are few differences between human and mouse NQO2 protein tissue distributions. Based on stable isotope labeling with amino acids (SILAC) in cell culture quantification proteomics from 28 tissue and organs of C57BL/6 mice (Geiger et al., 2013), the highest levels of NQO2 are present in liver, followed by kidney cortex, ileum, lung, spleen, and cerebellum, but lowest in midbrain, cerebral cortex, duodenum, and skeletal muscles, where it is high in humans.

Subcellular Expression. NQO2 was described for the first time in 1961 as a prevalent cytosolic protein (Liao and Williams-Ashman, 1961; Liao et al., 1962), but more recent data indicate the presence of NQO2 in nucleoplasm. For example, NQO2 is highly expressed in mouse oocytes during meiotic progression, where it colocalizes with nuclear membrane, chromosomes, and microtubules present in meiotic spindles (Chen et al., 2017). In addition, a database known as "a subcellular map of the human proteome," based on immunofluorescence microscopy data (Thul et al., 2017), shows comparable expression levels of cytoplasmic and nuclear NQO2 in three tumor cell lines [A-431 (epidermoid carcinoma), U-251 MG (glioblastoma), and U-2 OS (osteosarcoma cells)]. NQO2 expression pattern appears inexplicably granular or dot-like in all three cell lines and more intense at the cell membrane in glioblastoma cells. Interestingly, older studies also suggested a presence of NQO2 in membrane fractions. When NQO2 was initially discovered as the melatonin binding site MT3 (Nosjean et al., 2000; Boutin and Ferry, 2019), there was a harsh debate about its subcellular localization. The binding site was supposed to be membrane associated, whereas NQO2 was mainly described as cytosolic.

Nevertheless, as developed in Boutin and Ferry (2019), the sequence of NQO2 bears a cryptic myristoylation site that could be revealed upon caspase catalytic cleavage, leading to an N-terminal glycine that can be myristoylated as described for other proteins such as Bid (Degli Esposti et al., 2003) (for further discussion on myristoylation, see Boutin, 1997). Other authors also suggested that NQO2 is recruited to lipid membranes, since they found a portion of NQO2 both in detergent-soluble and -insoluble membrane fractions, containing mainly of lipid rafts. The interaction of NQO2 with lipid rafts might be mediated by caveolin-1, which is a marker component of lipid rafts (Dorai et al., 2018), or by AKT1, also recruited to the membrane by myristoylation (Hsieh et al., 2014), but these observations have not yet been consolidated.

Genetic Polymorphism. The *NQO2* gene locus is highly polymorphic, and according to the GeneCards database it contains several polymorphisms (Ps), including 11 coding variants (insertions and deletions) and 5362 single nucleotide polymorphisms (SNPs), mainly in introns (4385 SNPs) and within 10 kb of the 5′-end of the first exon and 2 kb of the 3′-end of the last exon. It is more polymorphic than its paralog NQO1 (4465 SNPs) and an average gene of this size. Many Ps are present in the promoter region (286 from GeneCards database), and several SNPs are found in exons (244), as shown in Figure 1. So far only a few of these Ps have been shown to be functional by modulating NQO2 mRNA levels or its enzymatic activity, and for most of them the evidence is rather fragmentary. The best studied NQO2 gene variant is a 29-base-pair insertion (I29) or deletion (D29) located in the gene promoter. Molecular studies suggest that I29 sequence is a recognition site for a transcriptional repressor Sp3, which binds to *NQO2* promoter causing a decrease in gene expression. In fact, when compared with the D29 promoter or a promoter containing an alternative 16-base-pair insertion sequence, the I29 variant demonstrated significantly lower *NQO2* gene expression, leading to lower enzyme activity and thus a partial loss of function (Wang and Jaiswal, 2004; Wang et al., 2008). These findings have been confirmed in an independent study that compared NQO2 mRNA levels in breast cancer tissue with I29 or D29 homozygosity and showed lower expression for the I29 variant (Yu et al., 2009). A similar differential *NQO2* expression due to SNPs in noncoding regions has been described for rs2071002 SNP located in the 5′ untranslated region of *NQO2* gene. In this case, +237C sequence variant demonstrated significantly higher *NQO2* expression compared with +237A-containing counterpart (Yu et al., 2009). Other SNPs of the *NQO2* 5′-UTR region do not seem to influence NQO2 expression at mRNA level, but they were associated with lower NQO2 activity when compared with wild-type homozygotes. This is the case of the 3423G (rs2070999) and 3777G alleles, analyzed in bladder tumor samples (Jamieson et al., 2007). A reduced activity associated with these SNPs suggests a posttranscriptional effect, probably at the level of translation, but no further evidence is available about the functional role of these variants.

SNPs resulting in aa substitutions may impact the enzymatic activity. However, only a few analyzed exon SNPs have been reported to influence NQO2 activity; one example is the exon 3 14055C allele, associated with a lower relative activity in human ovarian and bladder samples (Jamieson et al., 2007).

Biochemistry

Cosubstrates and Substrates. The most remarkable feature of this enzyme is certainly its inability to recognize as cosubstrates (hydrogen donor) either NADH nor NAD(P)H, unlike NQO1. This led to several incomprehensible publications that nevertheless reported measurements of its activity using NADH. An alternative claim was that at a low pH (slightly acidic: 5.8), NQO2 is able to recognize and use NADH (Jamieson et al., 2007). In our hands, even with a direct assay (enzyme + NADH + menadione at pH5.8) we were not able to observe any sign of NQO2 activity (Boutin et al., 2019). Instead, NQO2 recognizes a series of hydride donors that are derived from NADH, such as *N*-methyldihydronicotinamide (NMNH), NRH, or their synthetic counterpart, 1-benzyl-1,4-dihydro-nicotinamide (BNAH).

The current hypothesis is that these compounds are either in the metabolic (biosynthesis) pathway or more probably in the catabolic pathway of NADH, via enzymes such as nicotinamide *N*-methyltransferase (Enzyme Commission number 2.1.1.1) that would break the NADH molecule at the phosphate bridge level, leading to an AMP and NRH, the dihydro analog of which is the cosubstrate of NQO2. Alternatively, Toll/interleukin-1 receptor resistance domains have been proposed to be able to catalyze an NADase activity, at least starting from NAD^+ (as opposed to NADH), leading to some forms of nicotinamide derivatives such as *N*-methyl or *N*-ribosyl analogs (Horsefield et al., 2019). Furthermore, it has been claimed that these Toll/interleukin-1 receptor resistance domains-bearing proteins promote cell death and axonal degeneration (Wan et al., 2019). It is tempting to conclude that in case of higher concentrations of NRH, coming from NADH catabolism, NQO2 may get enough cosubstrate to become active, leading to the production of bursts of reactive oxygen species (ROS) by way of the futile cycle between quinone and quinol, as described elsewhere (Reybier et al., 2011). This might be a factor in starting or accelerating the cell death process, implicating a key role of this enzyme at the crossroads of fundamental catabolism pathways and cell death or neurodegeneration.

It is interesting to note that the chemical nature of the substrates of NQO2 has never been really investigated. Surprisingly, there are also no recent reviews about the specificity for the long-studied NQO1, and the closest work on this topic is certainly the paper of Lind et al. (1990). The structures are shown in Figure 3. It is clear, as stated elsewhere, that one can segregate the substrates in three categories: *para*-quinones, *ortho*-quinones, and pseudoquinones such as 5-(1-aziridinyl)-2,4-dinitrobenzamide (CB1954) for which the chemical nature of the reducible moiety is not obvious.

Concerning the substrates, NQO2 shares with NQO1 a similar specificity for *para*-quinones. Among the most used is menadione, which has been used for ages as an experimental substrate to measure NQO2 catalytic activity in many different situations. Alternative substrates have been described, particularly a catechol quinone such as adrenochrome, a close analog of dopamine quinones (Fu et al., 2008) that shed a completely new light on this enzyme, making it for the first time a key player on the metabolism of dopamine and other key compounds of the neuronal biology and thus of neurologic diseases. Not only did these

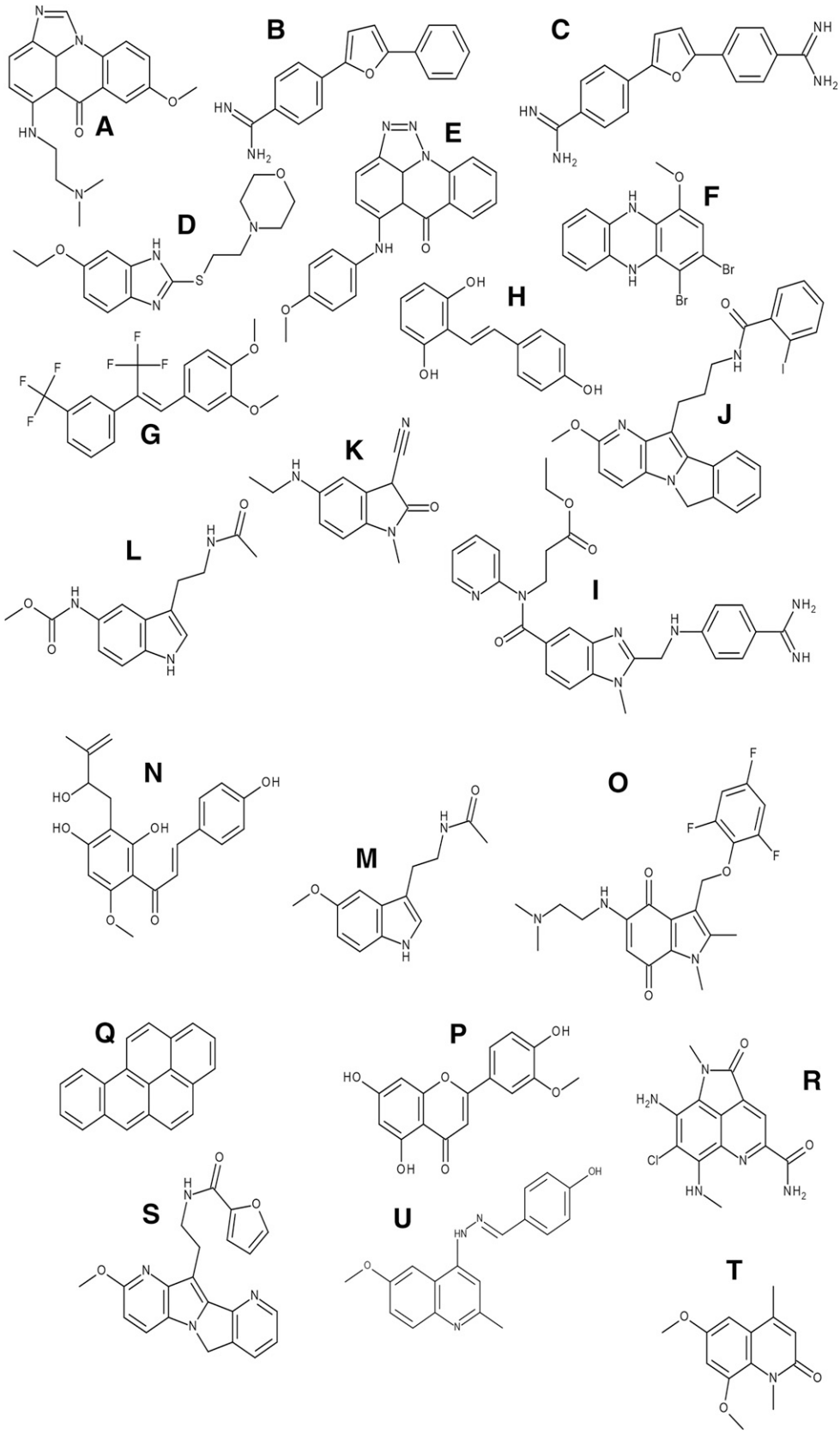

Fig. 3. Some reference inhibitors of NQO2. **Compound A**: Imidazoacridin-6-one 6a1 (Dunstan et al., 2011); **Compound B**: Furan-amidine 1 (Alnabulsi et al., 2018); **Compound C**: DB75 (Purfield et al., 2008); **Compound D**: Afobazole (Kadnikov et al., 2014); **Compound E**: Triazoloacridin-6-one 7c (Nolan et al., 2010b); **Compound F**: S28128 (Mailliet et al., 2005); **Compound G**: Resveratrol analog 1v (St. John et al., 2013); Compound **H**: Resveratrol (Buryanovskyy et al., 2004); **Compound I**: Dabigatran ethyl ester (Michaelis et al., 2012); **Compound J**: 1-Hydroxyphenazine 16 (Conda-Sheridan et al., 2010); **Compound K**: Indolone 12 (Volkova et al., 2012); **Compound L:** MCA-NAT (Pegan et al., 2011); **Compound M**: Melatonin (Calamini et al., 2008); **Compound N**: Xanthohumol D (Choi et al., 2011); **Compound O**: Indolequinone 2g (Dufour et al., 2011); **Compound P**: Chrysoeriol (Boutin et al., 2005); **Compound Q**: Benzo(a)pyrene (Zhao et al., 1997); **Compound R**: Ammosamide analog 38 (Reddy et al., 2012); **Compound S**: S29434 (Boutin et al., 2019); **Compound T**: Casimiroin analog 1j (Maiti et al., 2009); **Compound U**: 4-Aminoquinoline hydrazone 7d (Hussein et al., 2019).

authors demonstrate the unusual specificity of NQO2 toward this compound, but they also cocrystalized dopamine, as well as adrenochrome, with the enzyme. They also explained why, due to minute sequence and structural differences between NQO1 and NQO2, NQO1 is not able to accommodate these compounds (see below for further

details), leading to a possible major breakthrough in NQO2 molecular pharmacology.

Further studies were performed, essentially by Nepveu and coworkers (Reybier et al., 2011; Cassagnes et al., 2015, 2017, 2018), coupled with the measurements of the stability of the product of NQO2 catalytic reaction by electron paramagnetic resonance spectroscopy (see below).

In our studies of NQO2 specificity of substrates, we often noticed that *ortho*-quinones, like compounds derived from dopamine or other precursors, are better substrates for NQO2 than for NQO1, leading to our belief that in global terms, NQO2 would be more of an *ortho*-quinone reductase, whereas NQO1 would be a *para*-quinone one.

The literature claims many compounds to be substrates of NQO2, too often without experimental determinations, but based on indirect evidence, often of a toxicological nature. Among them, Miettinen and Björklund (2014) included paracetamol (acetaminophen), but our own experiments with pure enzymes did not lead to a similar substrate behavior of the drug.

More surprisingly, compounds that are only distantly related to quinone, such as the anticancer compound CB1954, are substrates of NQO2 (Wu et al., 1997). The initial finding trends were that only NQO2 could activate this therapeutic compound to an active drug (Wu et al., 1997).

Redox Reactions. NQO2 catalyzes a two-electron reduction of quinones. A quinone is an organic compound consisting of a benzene ring substituted by two oxo groups in the 1,2 or 1,4 position (*ortho*- or *para*-quinones, respectively) and most often by other cyclic or aliphatic groups. These groups have an important role in the redox behavior of quinones because they modify the substrate/enzyme interactions on the one hand and their spatial electron densities, thus their reduction potentials, on the other hand. The two-electron reduction of quinones produces more or less stable hydroquinones that can then be eliminated after conjugation by different conjugating enzymes [see, for example, the cooperation between NQO1 and UDP-glucuronosultransferase (UGT) A6 and A10 (Nishiyama et al., 2010)]. Quinone reductase two catalyzes the two-electron reduction of quinones, pseudoquinones, and other electron acceptors by using nonphosphorylated nicotinamide derivatives as electron donors, including NRH, NMNH, or synthetic cosubstrate BNAH in a ping-pong mechanism (Zhao et al., 1997; Reinhardt et al., 2018). Substrate and cosubstrate are found in the same catalytic site leading to the following final reaction:

$$\mathbf{NRH} + \mathbf{Q} + \mathbf{H}^{+} \Rightarrow \mathbf{NR}^{+} + \mathbf{QH2}.$$

This NQO2-catalyzed reaction is largely driven rightward with a kcat in the 2600 minute^{-1} range (Wu et al., 1997). It should be noted that NQO2 cosubstrates are much smaller in size in terms of steric bulk than NAD. This might be the reason why the catalytic activity of NQO2 cannot be measured in the presence of NAD(P)H, which is a cofactor of NQO1 (Wu et al., 1997). NQO2 can reduce the substrates of NQO1 and many other quinones in a two-electron reduction reaction (Reybier et al., 2011; Cassagnes et al., 2015), it can also perform two-stage reduction reactions (two times two electrons, i.e., to four electrons) on other substrates. It can thus reduce dimethylamino-4-phenylazo-2-benzoic (or methyl red) acid (Wu et al., 1997) and CB1954 (Knox et al., 2000) in a four-electron reduction reaction (Chen et al., 2000; Knox et al., 2000) that NQO1 cannot do. Many quinones or pseudoquinones are thus substrates of NQO2. The reductive power of NQO2 compared with NQO1 is thus strongly related to the nature of the substrate. For example, NQO1 and NQO2 reduce *para*-quinones to quinols in a comparable way, whereas the reduction capacity of NQO2 with respect to *ortho*-quinones is always greater (adrenochrome, aminochrome, dopachrome). NQO2 is thus able to reduce quinones with very low reduction potential (Cassagnes et al., 2018).

Mechanism of Catalytic Activity. Very early in the investigative history of this enzyme, Zhao et al. (1997) clearly showed that NQO2 follows an ordered ping-pong mechanism of catalytic activity (Lind et al., 1990). In other words, there is a single catalytic site to which the cosubstrate binds, reduces the FAD, and leaves a reduced enzyme; this is followed by the binding of the substrate, which is reduced in turn by FAD, leading to both an oxidized enzyme and a reduced quinone (a quinol or hydroquinone). Quinone reductases (both 1 and 2) share a common capacity of indirectly generating ROS. In simple terms, they reduce quinones to their corresponding quinols that, under aerobic conditions and depending on their chemical nature, may have different stabilities. This is particularly remarkable for *ortho*-quinones. In fact, in the presence of oxygen, those compounds are immediately transformed in quinones with a concomitant production of ROS. Those ROS are extremely reactive chemical species that would react with almost anything within angstroms of the production site (the catalytic site of the enzyme). This auto-oxidation might have very negative consequences, as its products break C-C bonds, and therefore depending of the amount of ROS produced and thus depending on the concentration of the substrate, as well as the status of antioxidant defenses in the cell, could lead to cell death within minutes.

Redox Switch. In a series of particularly remarkable papers (Leung et al., 2012; Leung and Shilton, 2013, 2015a,b), Leung and Shilton laid the basis of an interesting hypothesis that NQO2 has a sensitive mechanism of detection of redox state of its surrounding. They analyzed the binding of ClQ and other compounds (Leung and Shilton, 2013) to NQO2 in oxidized and reduced form and noticed a change in the geometry of FAD, with a bending of 4° to 5° in its reduced form. This change led to a differential positioning—and maybe affinity—of the compound for the active site. Thus, by changing the geometry of FAD during a redox event, the enzyme changes the affinity toward various inhibitors (Leung and Shilton, 2015a,b). It remains to be understood how this signal is integrated in the cell homeostasis. Another possibility is that the redox switch might also generate a conformation change sufficient to modify NQO2 affinity to other proteins. Such protein-protein interactions have been described for NQO2 and may lead to an increased stability or activity (p53, C/EBPα) or reduced activity (AKT1) of the interacting partner. Although the functional meaning of these interactions has not been fully elucidated, it is tempting to speculate that the redox switch may affect the affinity of binding and transduce important cellular signals downstream of the interaction partners. This is a likely scenario, since a similar conformational change occurs in several FAD- or FMN-containing proteins in which the oxidation state of the cofactor regulates interactions with other proteins, nucleic acids, or membranes (Becker et al., 2011). One well described

example of redox switch, albeit a prokaryotic one, is the azotobacter nitrogen fixation regulatory (NifL) protein (Hill et al., 1996). NifL is regulated by a redox switch within the C terminus of the NifL Per-Arndt-Sim domain induced by a change in the oxidation of FAD (Hill et al., 1996; Slavny et al., 2010). Thus, a flavin switch dependent on the cellular redox state and the presence of an appropriate ligand provides an intriguing and metabolically regulated link between NQO2 and cellular effects of drugs and bioactive compounds that bind to NQO2.

Structural Biology

The first crystal structures of rat NQO1 and human NQO2 in their native forms were published by Amzel's group (Li et al., 1995; Foster et al., 2000). When compared, the overall topologies of NQO1 and NQO2 were shown to be similar. Both proteins crystallize into a $P2_12_12_1$ space group as homodimers. Each monomer subunit consists of two domains: a catalytic domain (residues 1–220), predominantly folded in an α/β structure, and a C-terminal domain (NQO1, 221–274 aa; NQO2, 221–230 aa—see Fig. 1 for sequences and Fig. 4 for NQO2 crystal), which is far shorter in NQO2 and forms a well defined loop that has no sequence homology or structure similarity to the corresponding residues of NQO1. At the core of the protein, the residues forming the catalytic sites of NQO1 and NQO2 are mostly conserved and form a deep hydrophobic cavity with one face exposed to the solvent. Each catalytic site contains an FAD molecule, which constitutes the "floor" of this site. The FAD binding site is well conserved between the two enzymes, and it is coordinated to more than a dozen amino acid side chains.

Several differences between the two enzymes were also described. First, three residues making up the NQO1 catalytic site, Tyr^{126}, Tyr^{128}, and Met^{131}, were found to be replaced in NQO2 by Phe^{126}, Ile^{128}, and Phe^{131}, making the NQO2 catalytic site somewhat larger and more hydrophobic than that of NQO1. This finding explains in part why the two enzymes display different substrate specificity. In addition, in contrast to NQO1, NQO2 possesses a metal binding site, which is located on the protein surface. It was suggested that, as the structure of the metal binding site is similar to that of copper enzymes, the metal in NQO2 could have a role in an electron transfer pathway together with the flavin ring and could be linked to the active site of the enzyme by an electron transfer pathway (Foster et al., 1999; Bianchet et al., 2004). Finally, as the residues at the C-terminal domain of NQO1 that are involved in the binding of NADH are missing in NQO2, this enzyme lacks the NADH binding site. Therefore, NQO2 utilizes different sources of reducing equivalents compared with NQO1. Despite this difference in reductant specificity, both enzymes still operate using the same catalytic mechanism.

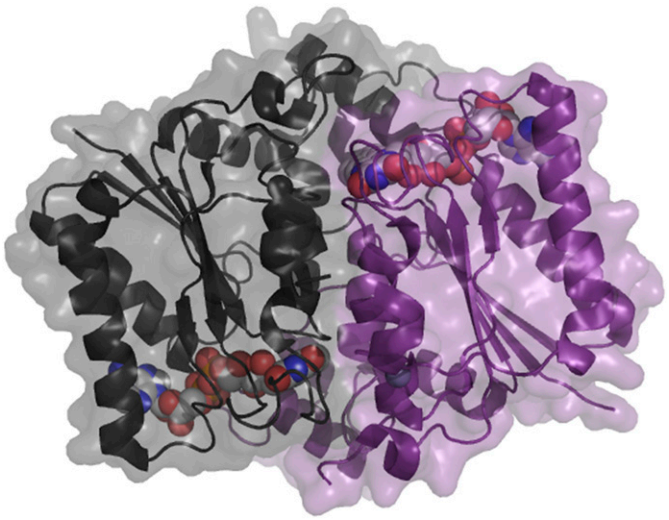

Fig. 4. Overall structure of NQO2 dimer obtained by X-ray crystallography. Ribbon diagram of the QR2 dimer in complex with the FAD cofactor. Each monomer is colored by subunit type (dark gray and purple). The catalytic domain is formed by five central parallel β-strands flanked on each side by α-helices. The FAD is shown as ball representation and is colored according to the subunit type. The structure is deposited in the Protein Data Bank under the code 1ZX1 (Ludwig et al., 2008). The figure was prepared using the Pymol program.

Structures with Substrates/Cosubstrates. To gain an insight into the physiologic function of NQO2, several groups, including ours, elucidated the X-ray structures of the enzyme in complex with its substrates and inhibitors. Those descriptions of the protein with some of its small molecule partners (inhibitors, substrates) were key in understanding the relationship between NQO2 and NQO1, but also in proving that NQO2 was MT3 [see review by Boutin and Ferry (2019) and also below]. An extract from the Protein Data Bank (www.rcsb.com) is presented in Supplemental Table 1 showing the main references in NQO2 crystallization data.

The crystal structure of the human NQO2 bound to one of its possible physiologic substrates, menadione (vitamin K_3), was published 20 years ago (Foster et al., 1999). The electron density of menadione was placed parallel to the plane of the FAD isoalloxazine ring. Menadione binding did not involve any direct contacts with the protein and, interestingly, did not lead to a significant conformational change in the protein structure when compared with the native structure, highlighting the structural rigidity of the enzyme. This work also clearly showed that the catalytic site handled both cosubstrate and then substrate in a serial, ordered fashion and provided information that both monomers were working independently from each other (Foster et al., 1999).

A few other structures of NQO2 in complex with different substrates have been subsequently reported. Two studies published the same year described the three-dimensional structure of NQO2 in complex with the aziridin-based anticancer prodrug CB1954 (AbuKhader et al., 2005). These two studies were of importance not only because they shed light on the mechanism of CB1954 activation by NQO2 but also because they identified two residues, Asn^{161} and Gly^{174}, that confer specificity of substrates toward NQO2. When compared with the NQO2-menadione complex, CB1954 was found to bind in a similar manner to the physiologic substrate. Analogously to menadione, the binding of CB1954 induced very little perturbation in the active site residues, thus reinforcing the preliminary observation that the oxidized form of NQO2 is rather rigid. Contrarily to menadione, CB1954 was able to bind directly to the enzyme by forming a direct hydrogen bond with Asn^{161}. Mutation of Asn^{161} residue to histidine, which is the corresponding amino acid in NQO1, abolished the enzymatic activity of NQO2 toward the reduction of the drug, thus explaining why CB1954 possesses a slightly better affinity toward NQO2

than NQO1 and why NQO2 can more efficiently activate the prodrug than NQO1.

Accordingly, NQO2 shows a clear preference for reducing catechol quinones, the oxidation products of catechol-amines such as dopamine and epinephrine, whereas NQO1 has no or very low activity toward these neurotransmitters. To understand the specificity of these substrates for NQO2, crystal structures of NQO2 bound to dopamine and adrenochrome were solved (Fu et al., 2005). Analogously to CB1954 and menadione-NQO2 complex structures, the aromatic planes of dopamine and adrenochrome were found to sit parallel to the plane of the isoalloxazine moiety of FAD. As observed for the cancer prodrug CB1954, the structures of these complexes formed a hydrogen bond network, which was critical to maintain the substrates in an optimal orientation and in proximity to the FAD hydride donor for reduction. In contrast, in the case of NQO1, the water molecule that is critical for substrate binding and orientation is not available. This residue difference, together with that observed for Asn^{161} in the NQO2-CB1954 complex, helped understanding the different catalytic activities and substrate selectivity for the two enzymes. Subtle structural differences in the NQO1 and NQO2 active sites account for the substrate preferences and for the difference in biologic functions of the two homologous enzymes. All this information permitted better understanding of the binding site geometry of NQO2 and the design of alternative substrates such as indolone antimalaria compounds (Cassagnes et al., 2017; Chhour et al., 2019).

Structures with Inhibitors. Due to the role of NQO2 in the etiology of different pathologies, including cancer, malaria, and neurodegenerative diseases, interest in developing inhibitors of this enzyme has gained considerably. Several types of inhibitors have been cocrystallized with the enzyme, including the polyphenol resveratrol (Buryanovskyy et al., 2004), the natural hormone melatonin (Calamini et al., 2008), the leukemia drug imatinib (Winger et al., 2009), casimiroin and its derivatives (Maiti et al., 2009), and the nanomolar tetracyclic inhibitor [2-(2-methoxy-5*H*-1,4b,9-triaza(indeno [2,1-a]inden-10-yl)ethyl]-2-furamide (S29434) (Pegan et al., 2011). A total of 44 crystal structures of NQO2 in complex with small molecule inhibitors have been deposited on the Protein Data Bank site and are summarized on Supplemental Table 1. Furthermore, a glimpse of the various compounds reported as inhibitors of NQO2, together with their IC_{50} values, is given in Table 1 and Figure 3.

As seen for the substrates, inhibitors bind to the NQO2 active site by adopting a flat conformation to sit parallel to the isoalloxazine ring of the FAD cofactor (Foster et al., 1999; Calamini et al., 2008; Maiti et al., 2009; Dunstan et al., 2011; Reddy et al., 2012; Cassagnes et al., 2018) (see also Supplemental Table 1 for complete listing). However, for optimal binding and inhibition, the inhibitors must also fully occupy the NQO2 cleft to form polar and hydrogen bond interactions. As the two extremities of the NQO2 cleft have several moieties accessible for hydrogen bond formation, they are often exploited by the small molecule inhibitors for binding to the NQO2 site. All NQO2 inhibitors take advantage of the same hydrogen bond network by binding to the same residues (Asn^{161}, Gly^{174}, Thr^{71}) within the NQO2 active site. As observed for the substrates, binding of the inhibitors to Asn^{161} seems to confer specificity toward NQO2. For example, resveratrol, by fitting deeply into the NQO2 catalytic site and by forming a direct hydrogen bond with Asn^{116}, potently inhibits NQO2 activity but displays only a weak inhibitory activity on NQO1.

TABLE 1
Inhibitors of quinone reductase 2 (NQO2)

Compound	IC_{50} on NQO2 (nM)	Type of assay	Reference
Ammosamide analog 38[a]	4	MTT reduction[e]	Reddy et al., 2012
Imidazoacridin-6-one 660841	6	DCPIP discoloration	Nolan et al., 2010c
Imidazoacridin-6-one 6a1	14	DCPIP discoloration	Dunstan et al., 2011
S29434	15	BNAH fluorescence decrease	Boutin et al., 2019
DB75	35	DCPIP discoloration	Purfield et al., 2008
Resveratrol	40	HEDHN fluorescence decrease[b]	Buryanovskyy et al., 2004
Furan-amidine 1	68	DCPIP discoloration	Alnabulsi et al., 2018
Triazoloacridin-6-one 7c	98	DCPIP discoloration	Nolan et al., 2010b
Indolequinone 2g[c]	<100	—	Dufour et al., 2011
1-Hydroxyphenazine 16	160	MTT reduction	Conda-Sheridan et al., 2010
Resveratrol analog Indolone 1v	180	NMeH fluorescence decrease	St. John et al., 2013
Chrysoeriol	300	BNAH fluorescence decrease	Boutin et al., 2005
4-aminoquinoline hydrazone 7d	500	—	Hussein et al., 2019
Benzo(a)pyrene	<1000	NMeH fluorescence decrease	Zhao et al., 1997
S28128	1000	BNAH fluorescence decrease	Mailliet et al., 2005
Indolone 12	7000	NMeH fluorescence decrease	Volkova et al., 2012
Dabigatran ethyl ester	800,000	NADH-dependent mitomycin C[d]	Michaelis et al., 2012
Casimiroin analog 1j	1900	NMeH fluorescence decrease	Maiti et al., 2009
Melatonin	11,000	BNAH fluorescence decrease	Calamini et al., 2008
MCA-NAT	37,000	BNAH fluorescence decrease	Pegan et al., 2011
Afobazole	254,000	BNAH fluorescence decrease	Kadnikov et al., 2014
Crenolanib	40[e]	Mass spectrometry	Klaeger et al., 2017
Pacritinib	4[e]	Mass spectrometry	Klaeger et al., 2017

DCPIP, 2,6-dichlorophenolindophenol.
[a]The bold characters refer to the name of the compound in the corresponding publication.
[b]1-(2-hydroxyethyl)dihydronicotinamide.
[c]This substrate is a suicide inhibitor that directly alkylates the enzyme.
[d]Despite the inactivity of NQO2 in the presence of NADH as cosubstrate. Those are Kd apparent as measured on immobilized enzyme.
[e]MTT stands for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Most of the crystal structures of NQO2 in complex with its substrate and inhibitors present the FAD in its oxidized form. However, NQO2 can exist as either oxidized or reduced forms, and inhibitors have been shown to have a higher affinity for one or the other. For example, ClQ and primaquine inhibit the enzyme with a similar potency (Leung and Shilton, 2013), but results of kinetics studies indicated that primaquine binds preferentially to the oxidized form of the enzyme, whereas ClQ binds to the reduced form. The comparison between the oxidized and reduced structures of the enzyme in complex with ClQ showed that the inhibitor binds in a completely different orientation in the two complexes, and this difference in binding is accompanied by the movement of an active site loop, a change in crystal packing, and a change in space group symmetry (Leung and Shilton, 2013). The conformational change adopted by NQO2 supports the proposed role for quinone reductases as flavin redox switches and raises the possibility that NQO2 might function as a signaling molecule in the cell (Leung and Shilton, 2013). As observed for ClQ, the crystal structures of other inhibitors bound to the reduced form of NQO2 show that these molecules bind less deeply in the catalytic site (Leung and Shilton, 2015a,b). The butterfly bend adopted by the FAD cofactor in the reduced NQO2 structures makes the region above the isoalloxazine ring less prone to aromatic stacking interactions, thereby pushing the inhibitors away from this region.

In summary, the crystal structures of NQO2 in complex with its substrates and inhibitors have helped elucidate the binding details necessary to obtain NQO2 specificity over NQO1 and to obtain a tight binding of the ligands. A requisite for substrates and inhibitors binding to NQO2 is the presence of a flat central moiety of the ligand that allows the formation of π-π hydrophobic interactions with the isoalloxazine ring of the FAD cofactor. In addition, strong binding and selectivity for NQO2 likely depend on both the size of the molecules and the subsequent interactions that result (Foster et al., 1999; Buryanovskyy et al., 2004; Calamini et al., 2008; Pegan et al., 2011). In addition, the crystal structures of NQO2 in complex with its inhibitors have led to the rational design and development of more potent and selective NQO2 mechanism-based inhibitors (Nolan et al., 2010a, 2012; Dunstan et al., 2011). Finally, the intriguing possibility that NQO2 might work as a flavin switch that is dependent on the redox state of the cells and on the presence of certain biologically active ligands opens the possibility of a nonenzymatic role for NQO2 and implicates NQO2 in regulation of cellular signaling (Khutornenko et al., 2010; Hsieh et al., 2012; Nolan et al., 2012).

Inhibitors

The first NQO2 inhibitors discovered and reported were Cibacron Blue and benzo(a)pyrene (Zhao et al., 1997). Both compounds were important laboratory tools, as Cibacron Blue has been used to purify the nucleotide pocket–bearing enzymes from complex medium, whereas benzo(a)pyrene was discussed more in a toxicity perspective than as an inhibitor (Zhao et al., 1997). Then, although wrongly claimed to be a substrate or a cosubstrate of NQO2 (Tan et al., 2007; Boutin et al., 2008), melatonin is indeed an inhibitor of the enzyme with a poor potency (in the 50 μM) range (Antoine et al., 2012). This feature is different from the affinity of the enzyme for the molecule that was reported to be in the nanomolar range (Calamini et al., 2008). The most interesting inhibitor is certainly the one we described recently, after several years of use in various laboratories: S29434 [see Boutin et al. (2019) and references therein for complete description]. This compound has an IC_{50} at NQO2 of about 15 nM, a good metabolic stability, and a fair penetration in the brain and has no other known targets than NQO2, including, of course, NQO1, for which it is not an inhibitor up to 100 μM. It is therefore a tool of choice for the understanding of the role of NQO2 in the cell and physiopathology. NQO2 might as well be responsible for many of the actions of melatonin at pharmacological concentration [see discussion in Boutin (2016)], because melatonin almost freely travels through biologic membranes and thus can reach extremely high concentrations inside the cells, without noticeable toxicity, despite warning on melatonin usage without limits (Yang et al., 2014; Claustrat and Leston, 2015).

Natural Inhibitors: Melatonin, Resveratrol, Flavonoids, Etc. We discovered that NQO2 was indeed the third melatonin binding site (Nosjean et al., 2000) [see also a summary of the controversy in Boutin and Ferry (2019)]. Although melatonin is a weak inhibitor of the enzyme, with an IC_{50} value in the 50 μM range, we have subsequently developed more potent and selective ligands at MT3/NQO2, which are briefly discussed in the next section. Another seminal work was the publication by Buryanovskyy et al. (2004) in which the cocrystallization of resveratrol with NQO2 was reported. This work described the ability of resveratrol to potently inhibit the catalytic activity (IC_{50} value of 35 nM) of NQO2, including in cellular settings. Interestingly, after this publication, the chemopreventive, cardioprotective, and antiaging properties of resveratrol were in part associated with its ability to inhibit the NQO2, thus suggesting that catalytic activity of NQO2 might have some "obscure" side effects. Resveratrol is a multitarget inhibitor suspected to be one of the key chemicals in wine more or less responsible for the French paradox (Yang et al., 2014; Pastor et al., 2019). This discovery opened or reinforced several routes of research on natural compounds such as flavonoids, stilbene, coumarin, and chalcone derivatives as well as casimiroin derivatives (Maiti et al., 2009) (casimiroin is derived from the fruit of the tree *Casimiroa edulis*). For instance, chrysoeriol was found to be an inhibitor of NQO2 (Boutin et al., 2005; Ferry et al., 2010), in the 300 nM range, among a series of flavonoids some of which, mainly flavones, are also submicromolar inhibitors of the enzyme (Boutin et al., 2005). Furthermore, some groups took these structures as pharmacophores and developed chemistry processes around some of those compounds to reach more specific, more potent, or more bioavailable compounds, including several broad melatonin derivatives from melatonin (Leclerc et al., 2002, 2011; Ettaoussi et al., 2008).

ClQ is another natural compound unexpectedly discovered as a ligand and an inhibitor of NQO2. As discussed in the previous sections, ClQ is a mild inhibitor of NQO2 activity, with a Ki in the 500 nM–1 μM range. It is interesting to note that the interaction with ClQ was discovered in the same way we discovered NQO2 was a target of melatonin. Indeed, in a remarkable work (Kwiek et al., 2004), ClQ was immobilized onto an affinity chromatography material, and mice

and human red blood cell lysates were chromatographed onto it. Two proteins were selectively retained on the column, aldehyde dehydrogenase 1 and NQO2 (Graves et al., 2002), and the interaction was further described and characterized (Kwiek et al., 2004). Interestingly, no protein was retained from *Plasmodium falciparum* homogenates treated in similar conditions. It was then believed that NQO2 might be a target for alternative treatment against malaria, an idea we developed in two previous papers (Cassagnes et al., 2017; Chhour et al., 2019), with mixed success. The main point remains not only that NQO2 inhibitors should be searched but that rather specific substrates such as dunnione derivatives should also be screened. A whole field of therapeutic exploration could be open in this regards, as standard antimalaria molecules—mainly ClQ (Ocan et al., 2018) and artemisinine (Oboh et al., 2018)—tend to fight against drug-resistant *P. falciparum* parasites. In the current context of the buzz around the effect of ClQ on viral infection (https://www.bbc.com/news/world-us-canada-52717161), the possibility that NQO2 might be implicated either in the controversial antiviral action of ClQ or in its related toxicity opens new avenues of research along those lines, although the probability of this being wishful thinking remains high.

Synthetic Inhibitors and Drugs. Table 1 lists some of the inhibitors reported in the literature. Most of the time, compounds are quite large, having at least three fused rings such as the imidazoacridin series (Dunstan et al., 2011), or various indeno(1,2-b)indol-10-one–based compounds (Boussard et al., 2006), the pyrriloquinoline series (Reddy et al., 2012), or the triaza(indeno[2,1-a]inden-based compound (S29434, see next section), or some other analogs (S32797) (Pegan et al., 2011).

The crystal structures of NQO2 in complex with inhibitors led to a better understanding of the structural requirements for potent NQO2 inhibition. For example, it was shown that the tetracylic compounds S32797 and S29434, due to their larger size and more hydrophobic structures, bury deeply in the enzyme active site and form a large area of favorable π-π interactions with the oxidized FAD structure (Calamini et al., 2008; Pegan et al., 2011; Boutin et al., 2019). In addition to the hydrophobic interactions, the hydrogen bond formation between the inhibitors and some of the polar residues of the NQO2 active site together with the displacement of water molecules are key determinants for binding. The displacement of water molecules from the catalytic site as a requisite for inhibitory potency has been supported by other studies (Dunstan et al., 2011; Reddy et al., 2012). Loss of water molecules in fact may provide entropic gain for the NQO2 inhibitor complex, thereby improving the inhibitor potency.

Of interest, an analysis of the different crystal structures of the NQO2-inhibitor complexes shows that in general there is a correlation between the compound inhibitory potency and the number of binding orientations adopted by the inhibitors in the NQO2 cleft (Maiti et al., 2009; Pegan et al., 2011). Potent NQO2 inhibitors in fact bind to the enzyme active site in one single orientation, whereas weaker inhibitors adopt two or more different orientations. For example, the casimiroin, with an IC_{50} value of 54 μM, binds to NQO2 in two different orientations, whereas its 10 times more potent analog, compound 1l from this publication, adopt the same orientation within each active site of the dimer (Maiti et al., 2009). Analogously, melatonin (IC_{50} of 11.3 μM) can bind in at least three different conformations, whereas iodomelatonin (IC_{50} of 1.1 μM) was shown to bind in the same orientation in independent crystal structures (Calamini et al., 2008). These results indicate that the binding of an inhibitor to NQO2 is a random process, which is dependent on the strength of interactions between the inhibitor and the enzyme active site residues. Other X-ray structures of NQO2-inhibitor complexes support this observation (e.g., prazosine vs. S32797 and S29434) (Maiti et al., 2009; Pegan et al., 2011).

The list of synthetic inhibitors of this enzyme in Table 1 shows about ten different types of synthetic compounds, some being analogs of natural products, such as resveratrol (Sun et al., 2010; St. John et al., 2013), dunnione (Chhour et al., 2019), curcumin (Meiyanto et al., 2019), casimiroin (Maiti et al., 2009), and ammosamide B (Reddy et al., 2012). The main series of structures leading to potent inhibitors can be reduced to six chemical families, as exemplified in Figure 3: quinolines (Kwiek et al., 2004), imidazoacridin-6-ones (Nolan et al., 2010c; Dunstan et al., 2011), benzimidazole derivatives (Kadnikov et al., 2014, 2015), furan-amidines (Alnabulsi et al., 2016, 2018), 4-aminoquinoline hydrazine (Hussein et al., 2019), and our indoyl-indol series (Boussard et al., 2006), as discussed thoroughly in the following section.

S29434. In our exploration of the melatonin pharmacological field and that relationship between the neurohormone and NQO2 [through the discovery that MT3, the third melatonin binding site, was indeed NQO2 (Nosjean et al., 2000)], we screened part of our chemical library on this enzyme. Among the hits identified in the screening, there were different compounds, including natural ones like flavonoids—vide supra—and synthetic ones such as S29434. This potent inhibitor has a central indol-like fused core. This compound allowed to further explore the NQO2 area as a pharmacological tool. S29434 is a 15 nM inhibitor of NQO2 that has no activity on NQO1. As such, it can be considered as a reference inhibitor of this enzyme. This compound has a special place in the list of NQO2 inhibitors because it has been thoroughly described in several independent publications, in cellulo and in vivo, in widely different physiopathologic situations. The most striking result concerns its memory enhancement property when injected in wild-type mice. This property disappeared in QR2−/− knockout mice, strongly suggesting that it is specific and selective for the enzyme, even in in vivo setting(s); see discussion and results in Boutin et al. (2019).

NQO2 and Drug Metabolism

Drug metabolism comprises roughly two main categories of enzymes: those hydroxylating enzymes, such as cytochrome P450s, and those conjugating enzymes, such as UGTs. The enzymes involved in these processes form a long chain of enzymatic reactions more or less in phase with one another, aiming first at functionalizing the compound and then to render it more water soluble. All details from these steps can be found in Testa and Krämer's series of chapters (Testa and Kramer, 2006, 2007a,b; Krämer and Testa, 2008, 2009; Testa and Krämer, 2008, 2009).

The metabolic pathway enzymes must deal with a complete lack of xenobiotic chemical similarity to biogenic compounds. In fact, these substances, which among others comprise

flavonoids, natural oils, all synthetic drugs, plant secondary metabolites, polycyclic aromatics, and pesticides, have no common features and even fewer common chemical structures. Evolution enhanced the capacities of endogenous metabolic enzymes to be able to cope with a large variety of chemical structures. Thus, most of the enzymes involved in these series of processes possess a large (or a lack of) specificity under the forms of a large number of isoforms (cytochrome P450, UGTs) or a largely plastic catalytic site of a restraint number of isoforms (glutathione *S*-transferases).

Among the less studied "families" of such enzymes lie the quinone reductases. Apparently, though, only NQO1 (formerly known as DT-diaphorase) has been thoroughly studied. It has been clear for several decades that the toxicity of quinones is reduced by the action of this enzyme. An extensive number of publications exist to document this fact. When NQO2 came into the picture, though, one assumed that it was yet another isoform with specificity overlapping that of NQO1. Indeed, it was believed that NQO2, like NQO1, was recognizing NAD(P)H as cosubstrate (Jaiswal, 1994). Talalay's group demonstrated that it was not the case and that NQO2 was not recognizing NADH, but a rarer form of hydride donors, such as NMNH and NRH (Zhao et al., 1997). These compounds were and still are poorly described in the literature, to the point that we still do not know how they are produced in the cell (intermediary step for the synthesis of NADH or catabolite of the same?). Thus, it becomes difficult to imagine that an enzyme playing a key role in detoxification does not have vast amounts of cosubstrates at disposal in cells.

In support of this, a core fact can be found in the experiment reporting the toxicity of menadione in genetic NQO1- or NQO2-knockout mice. Indeed, whereas the toxicity of menadione is clearly enhanced in NQO1-knockout mice, the toxicity of menadione is reduced in NQO2-knockout mice, strongly suggesting that NQO2 might be catalyzing a toxification step (Long et al., 2002).

Errors and Misconceptions in NQO2 Research

The purpose of this article is to provide a solid molecular pharmacology background for further research on NQO2. To this end it is important to highlight wrong concepts and errors that accumulated in 30 years of research on NQO2. Among those misleading concepts, it has been claimed that NQO2 was using NADH as a cosubstrate (Jaiswal et al., 1990; Jamieson et al., 2007; Riches et al., 2017), but that was not confirmed (Zhao et al., 1997; Ferry et al., 2010). It was proposed that NQO2 was using melatonin as a cosubstrate, but we showed it was not the case (Boutin et al., 2008). It was claimed that paracetamol (acetaminophen) was a substrate of NQO2—even if it not a quinone, like CB1954—but we could not find any evidence of that in our laboratories (no activity, no inhibition, no ROS production). It was claimed that the NQO2-knockout mice were prone to develop skin cancer while ageing (Shen et al., 2010 [retracted]). Our colonies never show this kind of particular property. Later, that paper was withdrawn (Shen et al., 2018).

Many scientists seem to believe that QR2 is a "bad guy" as its inhibition has been associated with toxic effects. For example, the toxicity of some kinase inhibitors has been thought to be due to their off-target effect as QR2 inhibitors (Leung and Shilton, 2015b). In contrast, we have shown that NQO2 is responsible for the toxicity of paraquat (Janda et al., 2013, 2015) and probably other Parkisonian toxins, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), since a specific inhibitor of NQO2, S29434 (Boutin et al., 2019), is able to diminish if not annihilate the toxicity of these compounds' toxicity in vivo. Thus, a key question remains: is NQO2 catalyzing a detoxification step like NQO1, or, alternatively, in certain particular conditions, does it catalyze metabolic transformation(s) that enhance the toxicity of the compound?

Another key finding about NQO2 that should be questioned is the interaction between p53 and NQO2. According to the proposed model, NQO2 was supposed to bind to p53 and thereby prevent 20S proteasome–mediated degradation of p53 (Gong et al., 2007 [retracted]). Unpublished data from our group did not confirm the physical association between human p53 and hNQO2, although we cannot exclude a possibility that NQO2 regulates p53 levels by other mechanisms.

Conclusions and Future Perspectives

The analysis of the available literature leads to an important conclusion that NQO2 is a target for hundreds of small-molecular-weight compounds subjected to oxidoreduction or modifying the enzyme activity. The structural details of the enzyme-ligand complex as well as the fine mechanisms of the redox reaction in the catalytic site of NQO2 have been studied thoroughly in the last 30 years. Although NQO2-substrate interaction is characterized by a structural rigidity, the binding of certain inhibitors, like ClQ to NQO2, may cause a small conformational change called "redox switch" and modify the structure of NQO2. However, nothing is known on how the redox switch may influence the enzyme environment and its interactions with other proteins. Currently, we can only speculate that this may impact on second messengers and signaling pathways that regulate important cellular processes. NQO2 may work as an interface between quinones, ligands, and the cell machinery and may transduce chemical information into biologic processes. In fact, NQO2 targets or is a target of important natural compounds known to regulate biologic systems such as melatonin, estrogen quinones, adrenochrome, catechol quinones, resveratrol, quercetin, and other polyphenols and drugs, such as ClQ, imatinib, mitomycin C, imiquimod, and others. Thus, it is unlikely that NQO2 does not mediate at least a part of the effects of these drugs.

In fact, NQO2 enzymology, ligands, and inhibitors have been well described, but little is known of its interaction partners and the biologic pathways regulated by NQO2. The association with p53 and C/EBPa and a role of NQO2 in the stability of these transcription factors are attractive mechanistic concepts, but they need to be reexamined with the ad hoc rigor. Similarly, the interaction with the oncogene AKT and caveolin-1 should also be confirmed by independent hands to gain the status of an established scientific fact. Thus, further solid evidence is urgently needed to understand the actual role of NQO2 in therapeutic effects of drugs.

It is somewhat extraordinary that this enzyme, briefly explored in the 1960s and rediscovered by serendipity in the 1990s, continues to be a fascinating mystery (Vella et al., 2005). From microarray and "omics"-generated data, one describes an enzyme that has exactly opposite roles to what would be expected for an enzyme implicated in protective

cellular processes such as detoxification and redox reactions. Furthermore, the question arises about the nature and the role of ROS produced due to NQO2 activities in some instances. Is it a desperate signal arising from a cell in which all alternatives have disappeared, leading to a "new" signal by which the cell embarks in a suicide-like process, or rather a signal transduction process that accompanies a normal cell physiology? Another important and yet unanswered question is if and how NQO2 redox status and ligands trigger conformational changes that may influence NQO2 binding to other proteins. Future studies should shed light on these fundamental questions.

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Wrote or contributed to the writing of the manuscript: Janda, Nepveu, Calamini, Ferry, Boutin.

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