X-ray structural studies of quinone reductase 2 nanomolar range inhibitors

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Abstract: Quinone reductase 2 (QR2) is one of two members comprising the mammalian quinone reductase family of enzymes responsible for performing FAD mediated reductions of quinone substrates. In contrast to quinone reductase 1 (QR1) which uses NAD(P)H as its co-substrate, QR2 utilizes a rare group of hydride donors, *N*-methyl or *N*-ribosyl nicotinamide. Several studies have linked QR2 to the generation of quinone free radicals, several neuronal degenerative diseases, and cancer. QR2 has been also identified as the third melatonin receptor (MT3) through *in cellulo* and *in vitro* inhibition of QR2 by traditional MT3 ligands, and through recent X-ray structures of human QR2 (hQR2) in complex with melatonin and 2-iodomelatonin. Several MT3 specific ligands have been developed that exhibit both potent *in cellulo* inhibition of hQR2 nanomolar, affinity for MT3. The potency of these ligands suggest their use as molecular probes for hQR2. However, no definitive correlation between traditionally obtained MT3 ligand affinity and hQR2 inhibition exists limiting our understanding of how these ligands are accommodated in the hQR2 active site. To obtain a clearer relationship between the structures of developed MT3 ligands and their inhibitory properties, *in cellulo* and *in vitro* IC₅₀ values were determined for a representative set of MT3 ligands (MCA-NAT, 2-I-MCANAT, prazosin, S26695, S32797, and S29434). Furthermore, X-ray structures for each of these ligands in complex with hQR2 were determined allowing for a structural evaluation of the binding modes of these ligands in relation to the potency of MT3 ligands.

Keywords: quinone reductase II; QR2; MCA-NAT; 2-I-MCA-NAT; prazosin; MT3; chemoprevention; inhibitors

Introduction

Mammalian quinone reductases form a small family of FAD-dependent enzymes that utilize Ping-Pong Bi Bi kinetic mechanisms. There are two main FAD-dependent quinone reductases in humans including quinone

Abbreviations: 2-I-MCA-NAT, 2-I-5-methoxycarbonylamino-*N*-acetyltryptamine; 2-I-MLT, 2-iodomelatonin; BNAH, dihydrobenzyl nicotamide; CHO cells, chinese hamster ovary; MCA-NAT, 5-methoxycarbonylamino-*N*-acetyltryptamine; MLT, melatonin; MT, melatonin binding site; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide; NMeH, *N*-methyl dihydronicotinamide; NRH, dihydronicotinamide riboside; QR1, quinone reductase 1 (NQO1); QR2, quinone reductase 2.

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reductase 1 (QR1, DT-diaphorase) and quinone reductase 2 (QR2). QR1 has been well characterized and implicated as playing a major role in the quinone detoxification pathway that leads to quinols being readily eliminated as glucuronides.¹ In contrast to QR1, QR2 was originally discovered in the 1960s but has not been as widely studied as QR1 due to its obscured identification as NQO2, the current name of another, mitochondrial membrane-bound enzyme also known as complex I.² However, interest in QR2 underwent a resurgence in the 1990's and has gained attention in a myriad of biological fields for its proposed roles in malaria, cancer, neurological degenerative diseases, memory and, more generally, oxidative stress.^{3–10} Initially, renewed interest in QR2 was strongly linked to its ability to utilize a rather rare form of hydride donors (*N*-methyl or *N*-ribosyl nicotinamide) instead of the classical NAD(P)H donors, a situation contrary to that of QR1.¹⁰ QR2 has also been proposed to generate free radical quinones since the QR2 genetic knock-outs in some living systems are less sensitive to quinone toxicity in contrast to QR1, which deactivates quinones through a two electron reduction.^{4,11,12} Beyond QR2 activation of quinones, it has also been implicated in the so-called French paradox due to its nanomolar affinity for resveratrol, a well known cancer preventive agent.¹¹ Finally, recent interest in QR2 stems from the fact that its overexpression in some cells also appears to be associated with several neuronal degenerative diseases.^{3,5}

Despite the numerous hypotheses that have been put forth to explain the role for QR2 in some of these biological systems, for the majority of these systems, the specific role of QR2 is yet to be determined. One obstacle for deciphering the role of QR2 has been the lack of potent and specific inhibitors of the enzyme that could be used to probe its function. Various compounds characterized as inhibitors and/or ligands of QR2 have been elucidated through enzymatic and X-ray crystallographic studies. Specifically, these studies have been reported for antimalarial quinolines, cancer prodrug CB1954, and resveratrol.^{8,11,13} However, most of these potential molecular probes lack the properties required to definitively assign the functional roles of QR2 in many of the systems in question.

The potent inhibition of QR2 by antimalarial quinolines, which were initially characterized as possessing IC₅₀ values near 300 nM, has recently been revised as possessing IC₅₀ values in the micromolar range.^{2,8} Moreover, inhibition of QR2 by resveratrol, a well known nanomolar inhibitor of QR2, may not happen *in vivo* since resveratrol is rapidly metabolized into resveratrol metabolites that no longer inhibit QR2.¹⁴ All of the aforementioned compounds have properties that make them non-ideal to serve as specific molecular probes of human QR2.

However, the search for effective molecular probes for QR2 may be close at hand. Indeed, melatonin recognizes at least 3 binding sites: the two seven transmembrane domain, G-protein coupled receptors, MT₁ and MT₂, and QR2 which was identified as the third melatonin receptor (MT3) with specific inhibitors with micromolar and nanomolar affinity.^{15–17} Initially, the melatonin pharmacology was described using a specific radioligand 2-[¹²⁵I]-iodo-melatonin (Fig. 1) agonist at MT₁ and MT₂. Two distinct pharmacological profiles were established: those with high-affinity (picomolar), MT₁ and MT₂, and one with a lower affinity (nanomolar), designated MT3.¹⁸ Furthermore, prazosin, an α_1 -adrenergic antagonist, was also observed to be a ligand at MT3, although with poor potency – in the micromolar range.¹⁹ To study MT3 in more details, specific MT3 melatonin (MLT) derivatives, 5-methoxycarbonylamino-*N*-acetyltryptamine (MCA-NAT) and 2-I-5-methoxycarbonylamino-*N*-acetyltryptamine (2-I-MCA-NAT) were developed (Fig. 1).²⁰ MCA-NAT and 2-I-MCA-NAT have 550-fold greater affinity for MT3 over MT₁ and MT₂, and these compounds inhibit QR2 thereby aiding in the identification of QR2 as MT3.^{17,19,21} Using the previously referenced radioligand assay and a human QR2 (hQR2) enzymatic assay, several bi-, tri-, and tetra-cyclic ligand/inhibitors for MT3/hQR2 were developed such as S26695, S32797, and S29434 (Fig. 1).²² These inhibitors exhibited low nanomolar K_i values in the 2-[¹²⁵I]-MLT and 2-[¹²⁵I]-MCA-NAT competitive binding assays. However, no linear or obvious correlations appear to exist between MT3 affinity, as obtained in binding studies, and hQR2 inhibition, using catalytic activity inhibition.²¹ Additionally, limited structural information is available for how these compounds (S26695, S32797, and S29434) might bind to the hQR2 active site.

Recently, X-ray structures of hQR2 in complex with MLT and 2-I-MLT were determined by our group and a model of the hQR2 active site bound with the more specific MT3 ligand, MCA-NAT, was suggested.²³ To better understand the structural details of the MCA-NAT/hQR2 interaction and the inhibition properties of the other QR2 ligands, a representative set of molecules (MCA-NAT, 2-I-MCA-NAT, prazosin, S26695, S32797, and S29434) was used to form inhibitor complexes with hQR2 and their X-ray structures were determined. Furthermore, IC₅₀ values, derived from the well-documented *in vitro* hQR2 assay, and the recently developed hQR2 formazan-based assay^{22,23} are presented. Utilizing the data from these assays and the structural studies performed with hQR2, we are able to identify several factors that lead to micromolar and nanomolar inhibitors. We also discuss discrepancies between these assays, the use of these ligands as molecular probes and propose paths to synthesize additional hQR2 inhibitors with increased potency.

Figure 1. Chemical structures of specific MT3 compounds used in the study.

Results

Comparison of compound affinity among different hQR2 assays

Previously, determining the localization of MT3, as well as identifying it as hQR2, generally relied on assaying hQR2 and MT3 activity through two assay types.²¹ The MT3 specific assay involved monitoring the competition between the radiolabeled 2-[¹²⁵I]-MLT and/or MT3 specific compound (2-[¹²⁵I]-MCA-NAT) against the potential ligand in mammalian cell lysates. Affinities calculated using this method for all compounds fall in the low nanomolar range (data not shown). The second assay also used cell lysates but monitored oxidation of BNAH by hQR2 while including 100 μ M dicoumarol to ensure specificity of the assay against hQR1.¹⁷ To gain an overall perspective of the potency of MLT, MCA-NAT, of their 2-iodo derivatives, and of the recently discov-

ered hQR2 inhibitors, the BNAH assay was used (Table I). Although the BNAH and radiolabeled assays both indirectly measured the affinity of ligands to MT3 in the more relevant cellular environment that contains other cellular components, these cellular components may also interfere with observing the direct interactions of the MT3 ligands with hQR2. Furthermore, these cellular components could also alter MT3 ligands by metabolism or modification. To elucidate the interaction of these MT3 ligands with hQR2 directly, two additional *in vitro* hQR2 assays were included. One of these assays monitors the oxidation of NMeH at saturating concentrations with menadione at concentrations near its K_m value, while the other assay monitors the reduction of MTT at saturating MTT concentrations while fixing NMeH concentrations near its K_m value.²³ Using the IC_{50} values for each of the compounds that were derived from each of these three

Table I. *In Cellulo and In Vitro* hQR2 IC₅₀s

	Average rank order ^a	Mammalian cell menadione and BNAH IC ₅₀ assay (nM)	Recombinant QR2 menadione and NMeH IC ₅₀ assay (nM)	Recombinant QR2 Formazan and NMeH IC ₅₀ Assay (nM)
MCA-NAT	7.5	295,000 ± 19,000 ^b	37,100 ± 6600	29,300 ± 800
MLT	7.5	130,000 ± 35000 ^b	30,200 ± 3400	72,000 ± 8000
Prazosin	5.33	4000 ± 250	17,600 ± 2000	22,000 ± 2900
S26695	5.00	37,600 ± 5000	7100 ± 500	7500 ± 100
2-I-MLT	4.66	16,000 ± 2000 ^b	2700 ± 390	14,000 ± 3000
2-I-MCA-NAT	3.00	650 ± 43	1900 ± 130	2900 ± 400
S32797	2.00	498.1 ± 2.4	704 ± 10	637 ± 80
S29434	1.00	14 ± 7	2.4 ± 0.7	11 ± 2

^a Average Rank Order determined by the mean of ligand's relative ranking across all three assays.

^b Data taken from Mailliet et al. [21]

assays, an average ranking of potency, indicated as the Average Rank Order in Table I, for hQR2/MT3 was established: S29434 > S32797 > 2-I-MCA-NAT > 2-I-MLT > S26695 > prazosin > MLT > MCA-NAT (Fig. 2 and Table I).

X-ray structures of hQR2 in Complex with MCA-NAT and 2-I-MCA-NAT

To probe the structural details of the interaction of MCA-NAT and 2-I-MCA-NAT with hQR2, hQR2 crystals were soaked in the presence of either MCA-NAT or 2-I-MCA-NAT at a final concentration of 1 mM. X-ray data sets were collected to 2.1 and 2.3 Å for MCA-NAT and 2-I-MCA-NAT, respectively, and the data collection, processing and refinement statistics are summarized in Table II. Difference electron density in $F_o - F_c$ omit maps with MCA-NAT omitted from the calculations was observed for MCA-NAT in the active sites of both monomers of the hQR2 dimer. Similar to hQR2 binding of MLT, hydrophobic interactions and specific hydrogen bonds are key in the interactions between of MCA-NAT and hQR2.²³ MCA-NAT stacks on top and parallel to the flat, oxidized isoalloxazine ring of the FAD cofactor, and additional hydrophobic interactions are formed between the inhibitor and residues Phe131, Phe178, and Phe126 of one chain, and Trp105 which is located in the second monomer of the dimer [Fig. 3(A)]. In contrast to the previously determined structures of MLT-hQR2 which showed that MLT binds in multiple conformations within each active site, MCA-NAT is orientated in essentially one conformation within each active site of the dimer similar to that observed in the 1.5 and 1.7 Å MLT-hQR2 structures [2QWX, 2QX4; Fig. 3(B)].²³ However, residual electron density near the pyrrole nitrogen of the inhibitor indicated the presence of a water molecule. This water molecule was too close, that is, 2.2 Å, for it to form a *bona fide* hydrogen bond with the nitrogen, indicating that the occupancy of the MCA-NAT inhibitor and water molecule may not be 100% or 1.0 within the active sites. Using values of 0.90 and 0.75 for the occupancy of MCA-NAT in active sites of

monomer 1 & 2, and attributing the remaining occupancy of 0.10 and 0.30 to the water in question, accounted for the overall density observed in both of the active sites. The geometry of MCA-NAT within both active sites is oriented so that the five-member ring of the indole moiety points towards the side chain of Asn161. Also, the hydrophilic side chain of MCA-NAT points toward the bulk solvent forming an H-bond with a water molecule, that is, bound to Asn161. The resulting orientation of the side chain is similar to that of the analogous hydrophilic side chain of MLT.

Although the binding conformation of MCA-NAT shares several similarities with one of the bound conformations of MLT, MCA-NAT is shifted ~1.7 Å closer towards Asn161 [Fig. 3(B)].²³ This shift is somewhat different from a computationally derived model of a binding pose of MCA-NAT recently proposed where MCA-NAT was expected to shift away from Asn161 allowing the MCA-NAT side chain to be accommodated over the FAD isoalloxazine ring.²³ Instead, the MCA-NAT-hQR2 structure illustrates that a shift in the opposite direction occurs. This shift toward Asn161 allows the methyl carbamate, which replaced the smaller ether group of MLT, to sit in the active site [Fig. 3(A)]. The placement of the methyl carbamate moiety of MCA-NAT over the FAD isoalloxazine also allows for additional stacking interactions. The shift also facilitates the formation of an H-bond between the nitrogen of the MCA-NAT side chain and an active site water molecule that also forms a H-bond with Asn161. Despite the presence of several nearby polar side chains and carboxyl oxygen moieties, such as Q122 and G174, these residues are located beyond the distance for formation of typical H-bonds and therefore appear not to be involved directly in binding of MCA-NAT. Additionally, the hydroxyl group of Tyr155, implicated in binding of MCA-NAT through docking studies, is located far enough away to allow the formation of direct interactions with MCA-NAT [Fig. 3(A)].²³

Interestingly, both MCA-NAT and MLT exhibit a 7–10-fold increase in affinity for hQR2 upon

Figure 2. *In vitro* IC₅₀ of hQR2 ligands. Formazan (A) or menadione (B) and *N*-methylidihydro-nicotinamide assay derived inhibition curves of selected hQR2 ligands. Assays were conducted in duplicate using a mixture of 100 μ M NMeH, 30 μ M menadione, 100 mM NaCl, 50 mM Tris HCl pH 8.0, 0.1% Triton-X 100 and 6 nM hQR2. Depending on the potency of the compound, different concentration ranges of compound were used; 2-I-MLT (300 μ M – 3 μ M); MLT (50 μ M – 0.5 μ M); S26695, MCA-NAT, and 2-I-MCA-NAT (299.8 μ M – 0.29 μ M); S29434, S32797 (100.00 μ M – 0.09 μ M); prazosin (149.9 μ M – 0.14 μ M). Assays were initiated by addition of hQR2 and monitored at 360 nm. For the formazan-using assay, kinetic activity of the hQR2 enzyme was monitored by measuring the absorbance of MTT-formazan at 612 nm formed by the conversion of MTT by the enzyme in the presence of NMeH, in the same buffer as above.

Table II. Data Collection and Refinement Statistics QR2 Inhibitor Complexes

	MCA-NAT	2-I-MCA-NAT	Prazosin	S26695	S32797	S29434
Data collection						
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell dimensions						
a, b, c (Å)	56.4, 84.1, 106.6	56.3, 83.7, 106.6	56.5, 83.6, 106.8	56.6, 83.7, 106.4	56.7, 83.9, 107.2	56.4, 83.6, 106.4
$\alpha = \beta = \gamma$ (°)	90.0	90.0	90.0	90.0	90.0	90.0
Resolution (Å)	66.08–2.09	65.8–2.3	65.8–1.8	65.8–2.0	66.08–2.4	65.8–1.8
No. reflections observed	165,976	262,786	153,173	162,180	146,446	231,617
No. unique reflections	24,894	23,562	43,715	33,965	20,400	47,239
R_{merge} (%)	9.8 (36.8) ^a	12.3 (40.3) ^a	7.6 (50.9) ^a	6.2 (31.1) ^a	12.5 (40.3) ^a	7.1 (42.3) ^a
$I/\sigma I$	18.4 (5.1) ^a	24.2 (5.5) ^a	13.0 (2.3) ^a	18.7 (4.8) ^a	17.8 (4.8) ^a	25.5 (3.4) ^a
% completeness	97.4 (86.1) ^a	99.9 (99.8) ^a	99.2 (99.0) ^a	97.1 (95.2) ^a	99.8 (99.0) ^a	99.5 (99.5) ^a
Refinement						
Resolution range	66.08–2.09	65.8–2.3	65.8–1.8	65.8–2.0	66.08–2.4	65.8–1.8
No. reflections in working set	28,357	22,298	41,518	32,206	19,310	44,764
No. reflections in test set	1504	1211	2197	1709	1043	2387
R_{work} (%)	18.7	16.3	19.2	17.5	16.3	18.5
R_{free} (%)	23.6	22.0	23.0	22.4	23.4	21.8
Average B-factor (Å ²)	26.3	29.6	23.3	20.8	31.3	21.3
Protein B-factor (Å ²)	25.3	29.1	21.6	18.7	30.4	19.6
Water B-factor (Å ²)	36.4	38.7	35	30.8	34.8	32.1
Ligand B-factor (Å ²)	32.2	26.7	32.5	36.5	33.3	32.6
FAD/ZN B-factor (Å ²)	24.3	28.9	22.9	22.1	27.5	23.3
Occupancy of ligand (site 1/2)	0.90/0.75	0.75/0.75	0.5:0.5/0.5:0.5	1/1	1/1	1/1
RMS deviation:						
Bond lengths (Å)	0.01	0.01	0.01	0.011	0.02	0.01
Bond angles (°)	1.41	1.53	1.27	1.31	1.95	1.63
Protein/water atoms	3626/340	3626/284	3626/384	3639/432	3684/226	3635 / 393
Monomers in asymmetric unit	2	2	2	2	2	2

^a The last resolution shell is shown in parentheses.

iodination at the second indole ring position (Table I). Previous studies using 2-I-MLT proposed that the increase in affinity for hQR2 is the result of a H-bond network through a water molecule to the carboxylic oxygen of Gly68 and the side chain of Gln22.²³ Surprisingly, the 10 σ signal in $F_o - F_c$ omit maps for iodine in 2-I-MCA-NAT was observed next to Asn161 [Fig. 4(A)]. Despite this, the presence of an active site water molecule, representing the unbound enzyme, similar to that found near the pyrrole nitrogen suggests that the occupancy of 2-I-MCA-NAT is ~ 0.75 . The remaining density, or occupancy of 0.25, is attributed to a water molecule in the unbound enzyme, that is, located between the pyrrole nitrogen and carbonyl oxygen of Gly174. Refinement of 2-I-MCA-NAT and the water molecule at these occupancies was able to account for all of the active site electron density. The resulting conformation of 2-I-MCA-NAT was comparable to that of MCA-NAT but, interestingly, it was flipped $\sim 180^\circ$ in comparison to 2-I-MLT in the 2-I-MLT-hQR2 structure [2QX9; Fig. 4(B)]. In the 2-I-MCA-NAT-hQR2 structure, the iodine atom replaces a previously observed water molecule in the MCA-NAT-hQR2 structure and forms polar interactions with Asn161 instead of H-bonds as observed with 2-I-MLT. In addition, an H-bond is seen between the secondary amide of the MLT homologous 2-I-MCA-NAT side

chain and backbone carboxylic oxygen of Gly149. The side chain ketone oxygen of 2-I-MCA-NAT is also ideally positioned to form H-bonds with the carboxylic oxygen of Gly149.

Mechanism of binding for micromolar range hQR2 IC₅₀ bicyclic inhibitors

Prazosin, originally developed as an α_1 -adrenergic antagonist, has been shown in many studies to bind to MT3 and to inhibit hQR2 activity (Table I).^{19,21} Additionally, it has been proposed that the bicyclic, indole-like, 6,7-dimethoxy attached moiety of prazosin is most likely the pharmacophore for MT3/hQR2. To gain clarity on the conformation of prazosin when bound to hQR2, and as well as on the conformations of other bicyclic-based inhibitors, prazosin and S26695 were soaked into hQR2 crystals and their X-ray structures determined to 1.9 and 2.0 Å, respectively. The resulting data collection and refinement statistics are summarized in Table II.

Within both active sites of the dimer of the prazosin-hQR2 complex structure, electron density was observed in $F_o - F_c$ omit maps [Fig. 5(A,B)]. In contrast to MCA-NAT and 2-I-MCA-NAT, two conformations of prazosin could be modeled into the active site since the density was too ambiguous for assignment of only one orientation. Manual refinement led us to assign an occupancy of 50% for both

Figure 3. Orientation of bound MCA-NAT in comparison to MLT. (A) Divergent-eyed stereo view of hQR2 site 1 bound with MCA-NAT. hQR2 is depicted in purple, and the FAD in gold. Carbons of MCA-NAT are shaded according to B-factor (minimum = 20, light blue; maximum = 50, dark orange) with nitrogen and oxygen of MCA-NAT represented in blue and red respectively. Interatomic distances (measured in Å) corresponding to hydrogen bonds are shown as orange dashed lines, and other reference distances are shown as black lines. Nearby water molecules are rendered as spheres in cyan. An $(|F_{\text{obs,MCA-NAT}}| - |F_{\text{calc,MCA-NAT}}|) \exp(2\pi i \phi_{\text{calc,MCA-NAT}}) F_o - F_c$ electron density difference map (gray) with MCA-NAT omitted from the calculations is contoured at the 3σ level and to a resolution of 2.1 Å. Labels are in black for QR2 chain A and reference distance lines while they are outlined in black for chain B and orange for hydrogen bond distances. (B) Divergent-eyed stereo view of hQR2 site 1 bound with MCA-NAT and overlaid with hQR2 bound to MLT (2QWX). hQR2 complex with MLT-hQR2 in gray. Interatomic distance lines are omitted for clarity. MCA-NAT in magenta with other colors, labels, electron density and rendering styles are as in (A).

Figure 4. Orientation of bound 2-I-MCA-NAT in comparison to 2-I-MLT. (A) Divergent-eyed stereo view of hQR2 site 1 bound with 2-I-MCA-NAT. hQR2 is depicted in purple and the FAD in gold. Carbons of 2-I-MCA-NAT are shaded according to B-factor (minimum = 20, light blue; maximum = 50, dark orange). Nitrogen, Oxygen, and Iodine atoms of 2-I-MCA-NAT are shaded, blue, red and purple respectively. Interatomic distances (measured in Å) corresponding to hydrogen bonds are shown as orange dashed lines, and other reference distances are shown as black lines. Nearby water molecules are rendered as spheres in cyan. An $(|F_{\text{obs,2-IMCA-NAT}}| - |F_{\text{calc,2-IMCA-NAT}}|) \exp(2\pi i \phi_{\text{calc,2-IMCA-NAT}})$ electron density difference map (gray) with 2-I-MCA-NAT omitted from the calculations is contoured at the 3σ level and to a resolution of 2.3 Å. Labels are in black for QR2 chain A and reference distance lines while they are outlined in black for chain B and orange for hydrogen bond distances. (B) Divergent-eyed stereo view of hQR2 site 1 bound with 2-I-MCA-NAT with hQR2 bound 2-I-MLT structure (2QX9) superimposed. The hQR2 of 2QX9 and waters are rendered in gray with 2-I-MLT in pink. 2-I-MCA-NAT is rendered in blue. Interatomic distances have been omitted for clarity. Other colors, labels, electron density and rendering styles are as in (A).

Figure 5. Conformations of prazosin within the active sites of the hQR2 dimer. (A) Divergent-eyed stereo view of hQR2 site 1 bound with prazosin. Carbons of prazosin are shaded according to B-factor (minimum = 20, light blue; maximum = 50, dark orange). Nitrogen and Oxygen atoms of prazosin are shaded, blue and red respectively. hQR2 is depicted in purple, nearby water molecules as spheres in cyan and FAD in gold. The interatomic distances formed between hQR2 and conformation 1 of prazosin are shown as orange lines for hydrogen bonds, and those formed between conformation 2 are shown as tan lines. Other interatomic distances are shown as black lines for reference. An $F_o - F_c$ omit map (gray) with Prazosin omitted is contoured at the 3σ level and to a resolution of 1.8 Å. Labels are in black for hQR2 chain A and reference distance lines (measured in Å), whereas labels that are outlined in black represent chain B residues. (B) Divergent-eyed stereo view of hQR2 site 2 bound with prazosin. Colors, labels, electron density and rendering styles are as in (A).

conformations per active site. Although all four orientations observed were divergent, they all shared a similar binding mode in the interaction of the bicyclic moiety of prazosin with the FAD cofactor. The remaining portions of the prazosin molecule lacked significant density to model the atomic positions accurately [Fig. 5(A,B)]. We performed $(|F_{obs, prazosin}| - |F_{obs, melatonin}|) \exp(2\pi i \phi_{calc, melatonin})$ difference electron density map calculations to provide further support for the assignments or prazosin orientations (Supporting Information Fig. 5S). Extra electron density in these maps was observed within the site supporting that the larger prazosin molecule is within the active site.

As with the other hQR2 inhibitors studied, hydrophobic interactions appear to be one of the major binding determinates. However, none of the orientations for prazosin are in an ideal binding orientation where the ring is parallel with the flat oxidized FAD isoalloxazine ring, nor is there enough space to accommodate favorably the prazosin molecule entirely. For these reasons, the bicyclic moiety of prazosin may adopt an orientation that balances the favorable hydrophobic forces with steric hindrance. In addition to the hydrophobic forces of the bicyclic ring moiety, the 6,7-dimethoxy entity contributes to the binding of prazosin to hQR2 by supporting three different orientations of the bicyclic

moiety of prazosin. In one orientation, the 6,7-dimethoxy component form H-bonds with Asn161. In another orientation, one of the methoxy groups interacts with N5 of the FAD isoalloxazine ring. In the last orientation, each methoxy group interacts with Asn161 and N5 of the FAD isoalloxazine ring, respectively. The multiple interactions that the dimethoxy entity forms within the hQR2 active site, coupled with a likely compromise in orientation of the bicyclic ring, suggest that the potency of prazosin for hQR2 is the result of either a modified prazosin, such as a bicyclic metabolite, for example, or the synergy of the multiple conformations.

The S26695-hQR2 complex structure provides further insight into how bicyclic inhibitors bind to hQR2. Within both hQR2 active sites, the S26695 bicyclic ring adopts one orientation with well-defined density in $F_o - F_c$ omit maps, whereas the side chain of S26695 appears to adopt two conformations with weak density [Fig. 6(A,B)]. One conformation of the side chain suggests a possible H-bond with Gln122, while the other has the secondary amide forming an H-bond with an active site water molecule. In comparison to the prazosin bicyclic moiety, the rings of S26695 are almost in an ideal parallel position relative to that of the FAD isoalloxazine ring. Additionally, the two oxygen atoms of the sulfonyl group form favorable interactions with the main chain

Figure 6. Conformations of S26695 within the active sites of the hQR2 dimer. (A) Divergent-eyed stereo view of hQR2 site 1 bound with S26695. Carbons of S26695 are shaded according to B-factor (minimum = 20, light blue; maximum = 50, dark orange). Nitrogen, Sulfur and Oxygen atoms of S26695 are shaded, blue, gold, and red, respectively. hQR2 is depicted in purple, nearby water molecules as spheres in cyan and FAD in gold. An $(|F_{\text{obs},\text{S26695}}| - |F_{\text{calc},\text{S26695}}|)\exp(2\pi i\phi_{\text{calc},\text{S26695}})$ electron density difference map (gray) with S26695 omitted from the calculation is contoured at the 3σ level and to a resolution of 2.0 Å. Labels are in black for hQR2 chain A whereas labels that are outlined in black represent chain B residues. Hydrogen bonds and reference lines are same as for other figures. (B) Divergent-eyed stereo view of hQR2 site 2 bound with S26695. All colors and rendering styles are as in (A).

amide of Gly149 and side chain of Asn161. The nitrogen of the sulfonamide interacts with the sulfur of the nearby Met158 group. The methyl group points toward a hydrophobic rich surface formed of Phe131, Phe178, Val160, and Tyr132 [Fig. 6(A,B)]. The significant interactions that the sulfonamide forms, suggests its role in determining S26695 orientation within the parallel plane generated from the bicyclic stacking interactions with the FAD isoaloxazine ring.

Mechanism of binding for nanomolar hQR2 tetracyclic inhibitors

Although hQR2 has a relatively large active site, the prazosin-hQR2 complex structure illustrates the limitations in accommodating large inhibitors with flexible side chains. However, tetracyclic compounds originating from a fused dual indole ring system are ~100 times more potent inhibitors of hQR2 than prazosin, suggesting that they may be fully accommodated in the active site (Table I). To observe whether the hQR2 active site is large enough for these tetracyclic compounds, two potent tetracyclic compounds, S32797 and S29434, were soaked into hQR2 crystals and their X-ray structures determined at 2.4 Å and 1.8 Å, respectively. Table II summarizes the X-ray data collection and refinement statistics for these two new complexes.

Well-defined electron density in $F_o - F_c$ omit maps is observable in both active sites of the S32797-hQR2 complex structure, indicating that the entire tetracyclic ring, two-methoxy ring substitutes, and a

hydroxyl can be accommodated in the active sites of both hQR2 monomers within the dimer (Fig. 7). As expected from the findings on bicyclic inhibitor structures, hydrophobic and stacking interactions are the major driving forces for binding as the planar tetracyclic ring of S32797 fits directly over the FAD isoaloxazine ring. In order for S32797 to fit within the hQR2 active site, several water molecules found in previous hQR2 structures are displaced which may contribute to the increased affinity of S32797 as well. A single and identical orientation of S32797 is found within both active sites. The structural determinates for defining the orientation for S32797 within the active site are the methoxy ring substitutes and the ketone oxygen of the ring system. Electron density omit maps ($F_o - F_c$) for these groups places the dual methoxy substituted ring of S32797 towards Asn161. The oxygen molecules of the methoxy groups form H-bond interactions with the side chain of Asn161 and the backbone carboxylic oxygen of Gly174. This orientation allows the lone nitrogen in the tetracyclic ring to stack directly above the tertiary amide of the FAD isoaloxazine ring as well as prevent steric clash of the ketone oxygen with hQR2, as the oxygen points towards the solvent. The position of S32797 also allows its hydroxyl group to form H-bond interactions with an active site water molecule bound to the backbone carboxylic oxygen of Asp117 and the Thr71 side chain.

Interestingly, the tetracyclic compound S29434 does not adopt the same conformation as S32797 in

Figure 7. Bound conformations of S32797 within the active sites of the hQR2 dimer. Divergent-eyed stereo view of hQR2 site 1 bound with S32797. Carbons of S32797 are shaded according to B-factor (minimum = 20, light blue; maximum = 50, dark orange). Nitrogen and Oxygen atoms of S32797 are shaded, blue and red, respectively. hQR2 is in purple, nearby water molecules as spheres in cyan, and the FAD in gold. Hydrogen bonds and reference lines are same as for other figures. An $(|F_{\text{obs},\text{S32797}}| - |F_{\text{calc},\text{S32797}}|)\exp(2\pi i\phi_{\text{calc},\text{S32797}})$ electron density difference map (gray) with S32797 omitted from the calculation is contoured at the 3σ level and to a resolution of 2.4 Å. Labels are in black for hQR2 chain A, outlined in black for chain B, dark green for distances.

the hQR2 active site (Fig. 8). Although S29434 has a planar tetracyclic ring similar to S32797, it lacks the two-methoxy groups that S32797 used to interact with Asn161 possibly allowing for more space within the active site. Furthermore, two carbons in the ring system have been replaced with nitrogen. In contrast to the observations made with the S32797-hQR2 complex structure, the active site electron density supports the placement of S29434 not entirely over the FAD isoalloxazine ring (Fig. 8). Similar to S32797, the tetracyclic ring of S29434 is in a planar relationship with the FAD isoalloxazine ring but it is rotated in the plane that causes one six-member ring to sink back into the active site. This rotation provides for a more stable ring-stacking interaction with the side chain of Phe178, but also creates a void, that is, filled with the side chain. Although some entropic force may be lost, the rotation displaces the methoxy substituted ring from the FAD isoalloxazine ring, facilitating a network of H-bonds from the oxygen of the methoxy group through a water molecule to the side chain of Gln122 and the backbone carboxylic oxygen of Gly68. The side chain of the tetracyclic ring of S29434 is also involved in H-bonding to hQR2 through a water molecule. The S29434 side chain

ketone group along with the oxygen atom in the ring coordinates a water molecule to Asn161.

Discussion

The recent findings of the close relationship between melatonin pharmacology and oxidative stress as well as the ability of S26695 and S29434 to overcome QR2 linked learning deficiencies are bridged by new studies on the structure and mechanism of QR2 and presents a unique opportunity to evaluate a new array of potent QR2 molecular probes.^{17,24} Effective molecular probes designed to study melatonin pharmacology, and observed to have specific MT3 activity, have been documented for years without the identity of MT3 or the mode of binding for these molecular probes being known^{20,21} see also^{2,15} for reviews. Recently, our studies have elucidated the mode of binding for melatonin and 2-iodomelatonin thanks to X-ray structures of hQR2 co-crystals, initiating a discussion on the mode of binding of MCA-NAT to hQR2.²³ To further our understanding on this binding of MCA-NAT as well as the more potent bi- and tetracyclic compounds, we adopted a similar approach. In addition, we sought to determine and compare the inhibition of hQR2 by various MT3

Figure 8. Bound conformations of S29434 within the active sites of the hQR2 dimer. Divergent-eyed stereo view of hQR2 site 2 bound with S29434. Carbons of S32797 are shaded according to B-factor (minimum = 20, light blue; maximum = 50, dark orange). Nitrogen and oxygen atoms of S29434 are shaded, blue and red respectively. hQR2 is in purple, nearby water molecules as spheres in cyan, and the FAD in gold. Hydrogen bonds and reference lines are same as for other figures. An $(|F_{\text{obs},\text{S29434}}| - |F_{\text{calc},\text{S29434}}|)\exp(2\pi i\phi_{\text{calc},\text{S29434}})$ electron density difference map (gray) with S29434 omitted from the calculations is contoured at the 3σ level and to a resolution of 1.8 Å. Labels are in black for hQR2 chain A, outlined in black for chain B, dark green for distances.

ligands to determine the chemico- physical properties that could lead to potent QR2 molecular probes.

Previous reports have listed the order of MT3 ligand potency as 2-I-MLT > 6-chloromelatonin > methy-isobutyl-amiloride > acridine orange > MCA-NAT > prazosin > *N*-acetylserotonin > melatonin.^{16,18,20} The order stemming from our results, based on the average potency across various assays, is S29434 > S32797 > 2-I-MLT > 2-I-MCA-NAT > S26695 > prazosin > MLT = MCA-NAT, confirming the relationship between MLT, MCANAT, and their 2-iodo derivatives. However, a minor inconsistency is observed when comparing potency of MLT with that of MCA-NAT. Indeed, those compounds are above 10 μ M in potency, a feature that turn them into moderately interesting tools for further description of QR2. Nevertheless, since each of the three assays utilizes different co-substrates and different concentrations of these substrates relative to their K_m values, exact comparison from one assay to another is difficult even if each inhibitor was tested under the same conditions within each assay, the order of their respective IC₅₀'s still holds merit. In previous studies, MCA-NAT was observed to be more potent than MLT. However, we observe here that their IC₅₀ values are statistically identical when NMeH and menadione are used as substrates at saturating concentrations of NMeH and at a concentration of menadione, that is, near its K_m value.^{20,21} In comparison, hQR2 is observed to have a twofold increase in the IC₅₀ of MLT when using the *in cellulo* assay where both co-substrates are at saturating conditions and a twofold decrease in the IC₅₀ value when the formazan assay is used. As the formazan assay is carried out in saturating MTT concentrations, the assay is more sensitive to inhibition of the oxidized enzyme state. Despite the presence of reducing agents, all FAD isoalloxazine rings resolved in hQR2 co-crystals appear flat, suggesting they are in an oxidized state (Table I).^{9,11,23,25} The stronger inhibition of MCA-NAT over MLT in the formazan assay corresponds to MCA-NAT being found in a single conformation instead of multiple ones, as is the case for MLT. This observation matches our other recently reported observations that stronger inhibitors tend to adopt only one orientation in the hQR2 active site.²⁶ Furthermore, two other aspects of those comparisons should be pointed out: 1) Ferry *et al.*²⁷ addressed several of these questions by testing various inhibitors (including MLT, S29434 and S26695) using a set of conditions, particularly regarding substrates, co-substrates, their nature and concentrations; and 2) Initial observations on MCA-NAT/MT3 relationships were mainly conducted in binding assays, by displacement of 2-iodo-MLT. This system is a rather static one, as opposed to the dynamics of enzyme catalysis measured in all the other systems. These differences in nature might very well

precluded accurate comparisons between the data. Definitive comparisons are currently being explored further (Nosjean *et al.*, unpublished data).

Furthermore, the relative potency of prazosin also appears to vary across assays. In particular, the potency of prazosin in the *in vitro* assays ranks it only as possessing the sixth best IC₅₀, whereas *in cellulo* assays its IC₅₀ is the fourth best. This observation suggests that prazosin may be metabolized or modified within mammalian cells or that another mechanism is in place. Moreover, as electron density was observed only for the bicyclic portion of prazosin; if metabolism occurred, the resulting metabolite likely contains this portion of prazosin [Fig. 5(A,B)]. In contrast, S29434, S32797, and S26695 do not exhibit changes in ranking from the *in cellulo* assays to the *in vitro* assays suggesting that these inhibitors are less prone to alteration by cellular components.

Beyond the confirmatory aspects of our study, discussion on micromolar inhibitor inconsistencies, and insights into mechanism of prazosin binding, the analysis of the X-ray structures of hQR2 in complex with S29434, S32797, and S26695 provides further information for the development of more effective molecular probes of hQR2. Two major factors for increased affinity of inhibitors to hQR2 are observed. First, the QR2 active site is generally hydrophobic in nature with few polar side chains, and the oxidized FAD cofactor presents a flat molecular surface amiable to π - π interactions. Not surprising, the larger, more hydrophobic tetracyclic compounds, S29434 and S32797, exhibit improved binding and inhibition properties. The increase in binding affinity of these compounds likely results from the formation of a large area for favorable π - π interactions as well as from the exclusion of water molecules from the hQR2 active site that are not displaced by the smaller bicyclic compounds (Figs. 7 and 8). However, as illustrated from the structure of the prazosin-hQR2 complex, there is a fine limit to the size of a potential ligand (Fig. 5).

In conclusion, we provide a perspective on the physical-chemical properties required for the binding of a series of old and new MT3 ligands to hQR2, based on a series of X-ray structures of these ligands in complex with hQR2 and on a series of potency data derived from different assays. The outcome of our study depicts that the larger, tetracyclic compounds, such as S32797 and S29434, that form hydrophobic, π - π stacking interactions, as well as specific polar and H-bond interactions, result in nanomolar binding affinities and inhibition. As a result, S32797, S29434, and compounds derived from them²² are not likely to be recognized by other receptors/enzymes of indole scaffolds. Through observation of these compounds and the others probed here, future synthesis of compounds with chemistry

distant from an indolic core can be realized. Thus, a new array of potent hQR2 inhibitors can be utilized as effective molecular probes for deciphering the role of hQR2 in various physio-pathological models.

Materials and Methods

Sources for hQR2 inhibitors and cosubstrates

The *N*-methyl Dihydronicotinamide (NMeH) used for the study was synthesized using established protocols.²⁸ Menadione (Sigma M9429), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma M2128), melatonin (MLT; Sigma M5250), and Prazosin (Sigma P7791) were purchased from Sigma. Dihydrobenzyl nicotinamide (BNAH) was purchased from Maybridge Chemical. 2-Iodomelatonin (2-I-MLT) and 5-methoxycarbonylamino-*N*-acetyltryptamine (MCA-NAT) were purchased from Tocris. 2-I-5-methoxycarbonylamino-*N*-acetyltryptamine (2-I-MCA-NAT) was synthesized using a previously developed protocol.²² 2-[¹²⁵I]-MLT and 2-[¹²⁵I]-MCA-NAT were custom-synthesized by Amersham Pharmacia Biotech. Bi- and tetra-cyclic inhibitors were synthesized according to the following methods: *N*-(2-(7-(*N*-methylsulfamoyl)naphthalen-1-yl)ethyl)acetamide (S26695),²⁹ *N*-(2-(2-methoxy-6H-dipyrido [2,3-*a*:3',2'-*e*]pyrrolizin-11-yl)ethyl)furan-2-carboxamide (S29434),³⁰ 2,8,9-trimethoxy-6H-isoindolo[2, 1-*a*]indol-6-one (S32797).²²

Expression and purification of human QR2

The human QR2 enzyme used for crystallization and *in vitro* kinetic assays was expressed and purified from *E. coli* BL21 (DE3) cells harboring a pET-23d vector containing the hQR2 gene as described in our previous study.²³ In short, plasmid containing *E. coli* BL21 (DE3) was grown in LB to an OD₆₀₀ of 0.6 and induced for 12 hrs with 1 mM IPTG at 16°C. Cells were subsequently harvested and lysed. Pure protein was obtained using the previously described two step purification that employs a 30 mL XK26 DEAE-Sepharose (GE Healthcare) and a Hiload 26/60 Superdex 75 (Amersham Biosciences).²³ Final protein was concentrated to 16 mg/mL in a buffer containing 50 mM Tris HCl pH 8.0, 150 mM NaCl, and 10% glycerol using a Centricon Plus-20, assayed for activity, and frozen at −80°C.

In vitro hQR2 IC₅₀ assays

The hQR2 activity assays, using menadione and BNAH as co-substrates, were conducted using previously established protocols,^{21,23} while the assay using menadione and NMeH as co-substrates were conducted in duplicate using a mixture of 100 μM NMeH, 30 μM menadione, 100 mM NaCl, 50 mM Tris HCl pH 8.0, 0.1% Triton-X 100 (Fisher Biotech 93004), and 6 nM hQR2. Depending on the potency of the compound, different concentration ranges of

compound were used; 2-I-MLT (300 μM –3 μM); MLT (50 μM –0.5 μM); S26695, MCA-NAT, and 2-I-MCA-NAT (299.8 μM –0.29 μM); S29434, S32797 (100.00 μM –0.09 μM); prazosin (149.9 μM –0.14 μM). Assays were initiated by addition of hQR2 for a final enzyme concentration of 6 or 12 nM and monitored at 360 nm. IC₅₀ values for compounds binding to hQR2 were determined using NMeH and MTT as substrates. Kinetic activity of the hQR2 enzyme was monitored by measuring the absorbance of MTT-formazan at 612 nm formed by the conversion of MTT by the enzyme.²⁶

For both *in vitro* assays, the percent inhibition of hQR2 in the presence of the respective compounds was calculated using the following formula: (sample rate-negative control rate)/(positive control rate-negative control rate). Resulting data were plotted and fit via non-linear regression using the equation, %I = (%I_{max}/(1+(IC₅₀/[I]))).

QR2 crystallization and inhibitor soaking

Initial crystallization for hQR2 was achieved using conditions from previously published protocols.²³ Final crystallization of hQR2 was achieved by hanging-drop vapor diffusion using a gradient screen of NH₄SO₄ and pH at room temperature. The optimal conditions were a one-to-one mix of protein stock solution (24 mg/mL QR2 containing 50 mM Tris HCl pH 8.0, 100 mM NaCl) and well solution (1.3 M ammonium sulfate, 0.1 M Bis-Tris pH 6.7, 0.1 M NaCl, 5 mM DTT, and 12 μM FAD). Crystals grew within 3–7 days.

Crystals were soaked in a solution that consisted of 1 μL of a 10 mM stock of MCA-NAT, 2-I-MCA-NAT, prazosin, S26695, S32797, or S29434 (all dissolved in DMSO) mixed into 9 μL of soaking solution, which contained 1.3 M ammonium sulfate, 0.1 M Bis-Tris pH 6.7, 0.1 M NaCl, 5 mM DTT, and 12 μM FAD. Crystals were soaked for 24 hrs then mounted on nylon loops, swiped through 5 μL of well solution supplemented with 30% glycerol and flash-frozen.

Data collection, reduction, and refinement

X-ray diffraction data were collected on MCA-NAT, 2-I-MCA-NAT, Prazosin, and S32797 soaked hQR2 crystals at the SER-CAT beamline 22-ID using a MAR300 CCD detector. With the S26695 and S29434 soaked hQR2 crystals, X-ray data were collected at the BioCars 14-BM beamline using a ADSC Quantum 315 CCD detector. Both beamlines are located at the Advanced Photon Source (APS) at Argonne National Laboratories. The flash-frozen crystals were mounted on a goniostat (sample charger) under a stream of dry N₂ at 100 °K, and X-ray data were collected with a 1° oscillation. X-ray images were indexed, processed, integrated, and scaled in the P₂₁2₁2₁ space group using the program HKL2000.³¹

Initial phases for the hQR2 complexes were determined via molecular replacement using hQR2 (2qwx) as a search model and the program Phaser.³² COOT 0.6.1 was used for model building, and REFMAC 5.5 from the CCP4 suite was used for refinement.^{31,33} Coordinates and molecular library files for the ligands MCA-NAT, 2-I-MCA-NAT, Prazosin, S32797, S26695, and S29434 were built using Sketcher in the CCP4 suite program. Water molecules were added to $2F_o - 1F_c$ density peaks that were greater than 1σ using the "Solvate" program function in Coot. Final models were checked for structural quality using the CCP4 suite programs Procheck and Scheck. Structure factors and final coordinates have been deposited with the Protein Data Bank and assigned codes 3OVM (MCA-NAT), 3OWH (2-I-MCA-NAT), 3OWX (Prazosin), 3OX2 (S32797), 3OX1 (S26695), and 3OX3 (S29434).

Acknowledgments

X-ray data were collected at Southeast Regional Collaborative Access Team and BioCARs, Advanced Photon Source, Argonne National Laboratory. SER-CAT beamline 22-ID was used for data collection. Supporting institutions may be found at www.ser.aps.anl.gov.

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