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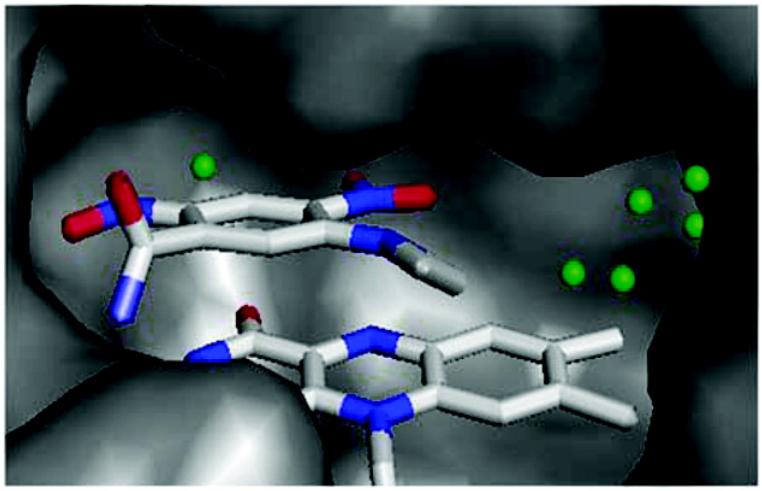

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Binding of the Anticancer Prodrug CB1954 to the Activating Enzyme NQO2 Revealed by the Crystal Structure of Their Complex

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CB1954 is an attractive prodrug for directed-enzyme prodrug therapy (DEPT) and a conventional prodrug against tumors in which the enzyme NQO2 is highly expressed. We have determined the crystal structure of the NQO2−CB1954 complex to 2.0 Å resolution. The binding of the prodrug is governed by hydrophobic forces, while two key electrostatic contacts determine the specific orientation of the ligand. The structure also reveals an unfavorable interaction, therefore suggesting possible avenues for DEPT-tailored engineering studies.

The use of prodrugs to treat cancer relies on the enzymatic activation of a relatively harmless compound into a cytotoxic molecule. The nitro-aromatic compound CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide, Figure 1) has been extensively studied as a prodrug since it was first synthesized over 30 years ago during a series of drug design studies.[1] CB1954 is relatively nontoxic to cells but is transformed to a potent cytotoxic agent upon reduction of its 4-nitro group to a 4-hydroxylamine (Figure 1). The latter acts as a bifunctional alkylating agent capable of forming DNA−DNA interstrand cross-links, via the hydroxylamine and aziridine groups.[2,3] Originally, it was demonstrated to inhibit in vivo tumor growth in rat Walker 256 carcinoma cell lines[1,4] following activation by the enzyme NQO1 (also known as NAD(P)H:quinone oxidoreductase 1 or QR1 or DT-diaphorase).[5]

NQO1, a mammalian detoxifying NAD(P)H-dependent flavoenzyme expressed in several tissues, catalyzes the two-electron reduction of a number of quinone metabolites[6] and also appears to function as a nitro-reducing enzyme (involving a four-electron reduction). NQO1 protects the body from oxidative damage of semiquinones produced in the one-electron reduction of quinones by several enzymes (e.g. cytochrome P450).[7] On the basis of the experiments in rat, CB1954 was tested on human cancer cell lines. The poor results obtained have been attributed to the presence of Gln in the human enzyme in place of Tyr104 in the rat enzyme. Site-directed mutagenesis of Gln104 to Tyr confers to the human enzyme similar CB1954-nitroreductase activity to the rat enzyme.[8,9]

Efficient turnover of CB1954 is also carried out by the *E.coli* nitroreductase (NTR), and this observation has been key to the development of treatments to target cells using directed enzyme prodrug therapy (DEPT) (e.g.: antibody-directed, gene/virus-directed and clostridia-directed enzyme prodrug therapy, ADEPT and GDEPT/VDEPT;[10,11] and CDEPT[12]). These two-step approaches involve the delivery of an enzyme to the cancer tissue, followed by the administration of a specific prodrug which is activated by the enzyme causing tumor-specific cytotoxicity. To this end, the *E.coli* NTR/CB1954 system has been implemented in clinical trials.[13,14]

Another enzyme with pronounced CB1954-nitroreductase activity is NQO2;[15−17] this is of special interest since it is expressed at high levels in several human cancer cell lines.[3] Expression of NQO2 in cancer tissue means that the use of CB1954 in a conventional prodrug therapy approach, without the complications of the DEPT strategies, can be reevaluated, and this is currently under clinical investigation.[1] NQO2 shares 49% sequence identity with NQO1 and was therefore thought to be an isozyme of NQO1, although it is 43 amino acids shorter. Recent work in mice suggests that NQO2 acts as an endogenous factor protecting against chemical carcinogenesis.[18] Interestingly, NQO2 is not active with NAD(P)H, but receives electrons from NRH (dihydronicotinamide riboside) and other reduced pyridinium derivatives.[1] Coadministration of an appropriate electron donor is essential for efficacy of any therapy using NQO2-mediated bioactivation of CB1954. Both NQO1 and NQO2 contain the redox cofactor FAD which receives electrons from a donor and then transfers these onto the substrate. These homodimeric proteins contain two FAD molecules bound at the extensive interface formed between the monomers.[17] The "head" of the FAD, the three-ring isoalloxazine system, is held in the active site by noncovalent interactions. A similar arrangement is observed in the bacterial NTR, which contains the cofactor FMN (FMN has an identical headgroup but a shorter tail with respect to FAD). It has been established that transfer of two electrons, in the form of a hydride anion, takes place from the reduced nitrogen atom at position 5 (N5) in the center of the isoalloxazine ring.[19,20]

Even though NTR catalyzes the reduction of the nitro group at either position 2 or 4 of the CB1954 aromatic ring, and therefore half of the product is the less cytotoxic 2-hydroxylamine, its rates of turnover are

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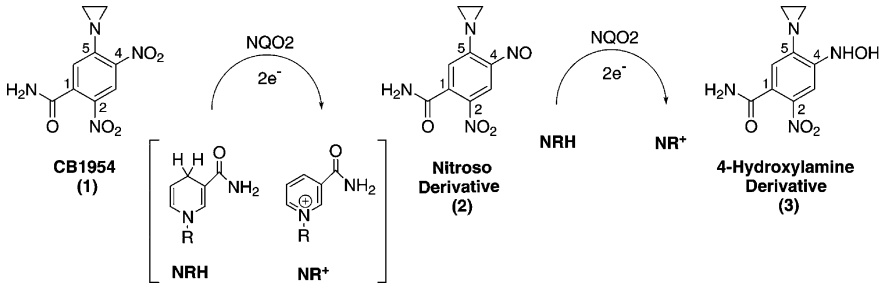

Figure 1. The enzyme NQO2 activates the prodrug CB1954 (1) through a four-electron reduction of the nitro group at the 4 position to the hydroxylamine derivative (3), presumably via the nitroso derivative (2). This will require two electron donor molecules with two successive two-electron reduction steps in which an intermediary product dissociates from the active site to allow access to the electron donor necessary to restore the cofactor FAD to its reduced state.

sufficiently high to yield a therapeutic effect. Human NQO2 catalyzes the reduction of CB1954 at the 4 position only, with rates comparable to NTR (K_{cat} 6 s^{-1}) and a better K_m (NQO2: 263 μM and NTR: 862 μM).[1] Both rat NQO1 and human NQO2 have a tyrosine that packs underneath the isoalloxazine ring, but NQO2 has better kinetic parameters for the activation of the prodrug. Efforts have been made to gain insights into the binding of the CB1954 compound to the active site of these enzymes. Molecular modeling studies based on the structures of rat[21] and human NQO1[22] suggested two possible binding modes for the association of the prodrug in the active site.[17,22,23] The modeling work on the rat NQO1, which also encompassed data from mutagenesis results, places the aziridine group near Tyr155 with carbon atoms 2 and 3 of the benzene ring positioned above N5 of the FAD. In the model based on the structure of the human enzyme, the aziridine is thought to engage in hydrophobic interactions with Trp105, thus putting the aziridine nitrogen and the benzene carbon 5 close to N5.

The only structural data reported in the literature to date on a CB1954−protein complex are those with the NTR enzyme.[24] The authors interpreted the electron density as indicating that the prodrug is bound in a given orientation in one monomer and a different orientation in the other. In one of these arrangements, the ligand has its 4-nitro oxygen atoms in proximity of the N5 of the FAD which implies reduction to the 4-hydroxylamine derivative, the most cytotoxic product, while in the other monomer the binding of the compound is such that reduction would occur at the 2 position.

Herein we report the crystal structure of the NQO2−CB1954 complex refined against data to 2.0 Å resolution (the cloning, expression, and purification procedures are presented in the Supporting Information). The structure was solved using crystals soaked in solutions of artificial mother liquor containing the ligand. The starting model was derived from the coordinates of the native (apo) enzyme (PDB code: 1QR2,[25]). The refined model of the NQO2−CB1954 complex has excellent crystallographic statistics and very good geometry; the only Ramachandran outlier is Tyr132, but its conformation is supported by an indisputable electron density map in both monomers, as also seen in the original structure of NQO2 (PDB code 1QR2[25]).

Initial maps showed large peaks of density in the active sites of both monomers, indicating the presence of a bound ligand; the size and shape of these peaks were consistent with the characteristics of CB1954. The pseudo-symmetry of the ligand's electron density however made it difficult to interpret the data unambiguously for the assignment of the prodrug's orientation. Visual inspection indicates that there are four possible orientations of CB1954 which might fit the data; in each of these the ligand is parallel to and stacked onto the isoalloxazine ring of the FAD, but with differing substituents facing Trp105, a residue that distinctively packs beside the cofactor perpendicular to it. To resolve this issue, our approach has been to carry out four separate model refinement procedures, with the ligand in each of the four possible orientations. In two orientations, both with the nitro groups both pointing away from Trp105, peaks of negative density (in $F_o - F_c$ difference maps) resulted over the atoms of the nitro groups, thus demonstrating that these models were incorrect. On the other hand, the arrangements with either the 4-NO_2 or the 2-NO_2 in front of Trp105 gave satisfactory and comparable refinement results. The former orientation puts the 4-NO_2 group in proximity to the flavin's N5, while the latter results in a nonproductive binding mode. As shown in Figure 2, the shape of the electron density map provides a good fit for both arrangements. At 2.0 Å resolution it is not possible to discriminate between the density of the aziridine and carboxamide groups and thus the possible coexistence of two distinct binding modes cannot be confirmed (see Experimental Section). Previously, it was shown in another flavoprotein that the averaging effect of alternate orientations in the active site could not be resolved at 1.5 Å but only with data to 0.9 Å.[26] In both our refined models, the B factors for the ligand atoms range between 50 and 54 $Å^2$ while those for the FAD and the protein atoms are in the 20−50 and 20−30 $Å^2$ range, respectively. It is possible that the densities shown in Figure 2 result from an average between a productive and a nonproductive binding mode of the prodrug. Nonproductive binding has been observed in the structure of the antibiotic nitrofurazone bound to *E.coli* NTR;[27] in addition, "flipped binding geometries" of the substrate have been observed in different redox states in the flavoenzyme PETN.[28] This would imply that the active site conformation of the oxidized form is somewhat different from that of the reduced form. However, at least in the case of the *E.coli* NTR, only slight atomic shifts in protein atoms were detected when the reduced structure was compared to the oxidized form.[24,29] It is known that the isoalloxazine ring changes from a very slightly bent "butterfly" conformation in the oxidized

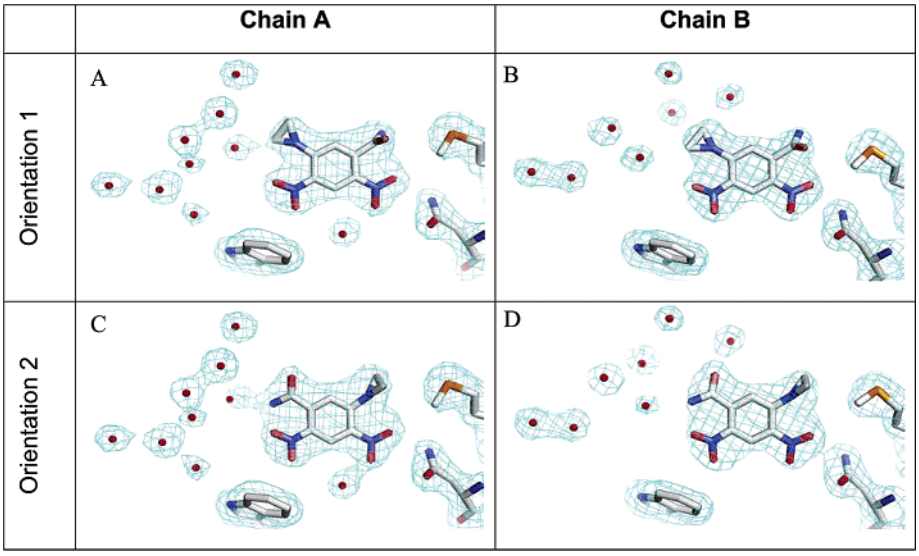

Figure 2. Electron density maps for the final $2F_o - F_c$ syntheses contoured at 0.8 σ are shown over two refined models of the CB1954 ligand, one with the 4-NO_2 above N5 (panels A and B for chains A and B, respectively) and the other with 2-NO_2 above N5 (panels C and D for chains A and B, respectively). Density for Trp105 and Asp161, as well as for the cluster of ordered water molecules in the active site, is also shown. Difference maps and omit maps were also carefully examined; the refinement gives good results with the ligand in either of these orientations.

state to a more bent form in the reduced state of many flavoenzymes.[20] The extent of this effect may differ in different flavoenzymes. In addition, in the oxidized structures of most flavoproteins, the N5 atom of the isoalloxazine ring is engaged in an interaction with a hydrogen bonding donor.[20] Upon reduction of the FAD N5, this interaction is likely to be affected, and this might be associated with structural changes. It is therefore conceivable that even small structural changes could, in concert, affect the binding of the ligand. In our crystallographic analysis, refinement of the ligand with its 2-NO_2 group placed toward Trp105 forces the carboxamide group in plane with the nitro group, resulting in a highly unfavorable steric clash (from the oxygen or nitrogen atom of the carboxamide to one of the oxygens of the nitro group: 2.0−2.1 Å). With the 4-NO_2 in front of Trp105, the electron density map clearly indicates that the carboxamide is not coplanar with the benzene ring and the nitro group. The latter conformation appears more consistent with the high-resolution X-ray crystallographic structure of CB1954[30] in which the carboxamide is offset at 64.7° from the plane of the aromatic ring, and the neighboring nitro group at position 2 is offset at 26.3° (compared to the 4-NO_2 at 13.6°); the authors comment on the role of steric hindrance in determining this conformation.[30] Semiempirical and ab initio molecular orbital calculations of the energies of these two conformations of isolated CB1954 showed substantial differences, with the conformation arising from the ligand with 2-NO_2 pointed toward Trp105 showing substantially higher energy, of the order of 300 to 400 kJ mol^{-1} (see Supporting Information). In light of the likely enhanced stability, together with the productivity of its binding orientation, the remainder of this discussion will focus on the model with the 4-NO_2 positioned toward Trp105.

The conformations of the two monomers are very similar, with an rms deviation of 0.8 Å for all well-ordered main chain atoms (excluding the first three and last three residues of the N- and C-terminal ends respectively, as well as residues 51−59 of a disordered loop in the B chain). All the large differences between the two monomers occur at surface residues and do not involve atoms within the active site. The position of the ligand and its interactions with the protein are the same, within experimental error, in both monomers. A minor difference, in the A chain but not in the B chain, is given by the presence of a water molecule that provides bridging contacts between the 2-NO_2 and the carbonyl oxygen of Gly174. With respect to the modeling work carried out on the binding of CB1954 to the NQO1 (DT-diaphorase) enzyme,[22,23] the orientation of the prodrug in the crystal structure in the NQO2 complex is different to what would perhaps have been expected based on the similarity between the active sites of the two enzymes. It is unclear whether the amino acid differences between rat NQO1, human NQO1 and human NQO2, might account for the different predicted and observed orientations of CB1954.

The prodrug fills at least half of the space in the binding pocket, aligning itself over the pyrimidine dione ring of the FAD headgroup (Figure 3); the remaining space, mainly contained within a pocket beyond the benzo-fused architecture of the FAD head, is occupied in both monomers by a cluster of well-ordered water molecules, clearly defined by the electron density map (Figure 2). It is striking to note that six of the eight water molecules in the NQO2−CB1954 complex are in the same position (within experimental error) as in the structure of the native (apo) enzyme (PDB code 1QR2;[25]); only two waters have moved (1.5−2.0 Å) upon arrival of the ligand. Binding of CB1954 induces little perturbation in this oxidized active site, the structure of which is presumably rather rigid, as indicated by the conserved positions of the solvent molecules. Hydrophobic side chains that engage in interactions with CB1954 show only very slight shifts but preserve their conformation, relative to the native NQO2 structure. Further evidence of structural rigidity is provided by the complex of NQO2 with the inhibitor resveratrol:[31] this larger

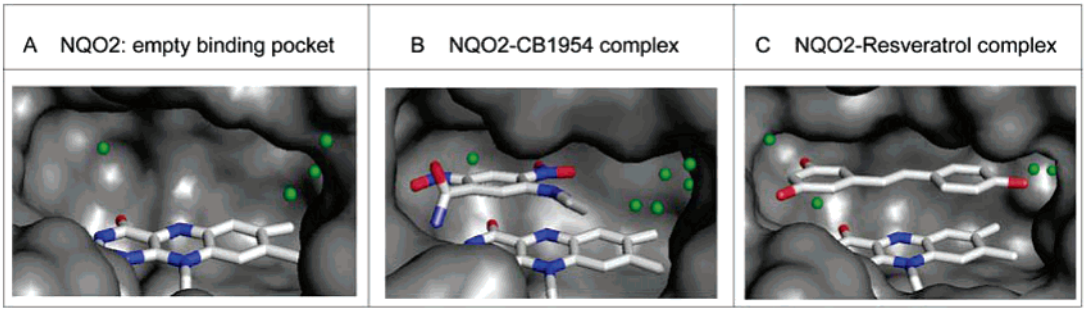

Figure 3. Surface representation of the active site of NQO2 in the apo-form (A), in complex with the prodrug CB1954 (B) and in complex with the inhibitor resveratrol (C). Both the FAD cofactor and the ligands are shown in a stick model while water molecules are drawn green in a cpk model.

ligand has to displace the waters in order to bind but does not cause any significant shifts in atomic positions within the active site. A comparison of the binding of resveratrol and CB1954 is highlighted in Figure 3.

CB1954 is bound at an angle of 35° in the A chain and 37° in the B chain (relative to the FAD); the plane of the benzene ring cannot adopt a perfectly parallel orientation with respect to the plane of the isoalloxazine ring, as in the case of menadione (PDB code 2QR2;[25]), since this would cause steric hindrance between the carboxamide of the prodrug and the atoms of the peptide segment 149−150. Despite this mode of binding, the association is clearly driven by extensive π-stacking and hydrophobic interactions. Over 40 contacts with interatomic distances less than 4 Å exist between the ligand and the FAD. In addition, the snug fit with the roof of the active site, lined with a hydrophobic triad formed by Phe126, Ile128, and Phe178, results in numerous nonpolar contacts, as illustrated in Figure 4a. In fact, formation of the complex is accompanied by the burial of a significant amount of solvent accessible surface area, 228 Å^2 in the A chain and 212 Å^2 in the B chain, of which about 80% is nonpolar. While the complex with *E. coli* NTR is characterized by a range of different

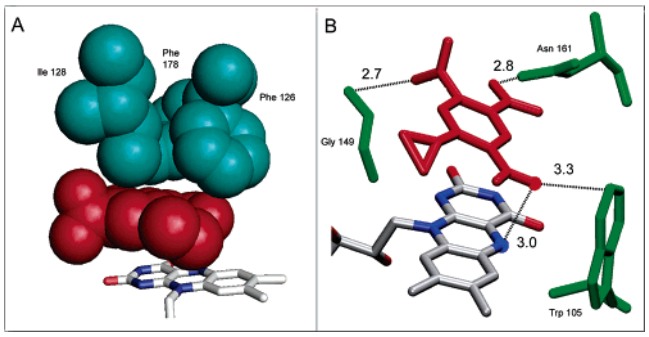

Figure 4. Interactions governing binding and molecular recognition of CB1954 in the NQO2 active site. (A) The roof of the active site is lined with a hydrophobic triad formed by Phe126, Ile128 and Phe178 that make extensive nonpolar contacts with the bound prodrug. Both the residues (cyan) and the ligand (red) are displayed in a space-filling model which highlights the extent of the interactions and the complementary fit of the atomic surfaces. (B) CB1954 (red) makes two electrostatic interactions with the protein (green), one with Asn161, the other with the main chain of Gly149. The latter is likely to be a key anchor to favor the binding of the prodrug in a productive orientation. There is, however, a set of unfavorable contacts between the nitro group and Trp105. Finally, the close approach of the nitro oxygen atom to the FAD N5 is emphasized.

interaction types (PDB code 1IDT[24]), the binding of CB1954 to NQO2 is governed by hydrophobic contacts. There are, however, two important hydrogen-bonding interactions (Figure 4b). The first involves one of the nitro groups and Asn161; either nitro group could in theory interact with Asn161, as is apparent from Figure 2. In the second interaction, the carboxamide group of the prodrug is engaged in contacts with the main chain carbonyl oxygen of Gly149 (Figure 4b). This interaction cannot take place if the 2-NO_2 group is placed above the N5 atom of the FAD; it is therefore likely to provide a key anchoring point for the binding of the prodrug in its correct orientation for catalysis and therefore determines the specificity of the enzyme for the hydroxylation at the 4 position.

In all flavoproteins the substrate atoms at the site of reduction are in proximity to the FAD N5 (within 3.8 Å) to facilitate direct hydride transfer.[20] In the structure of the NQO2−CB1954 complex, the 4-NO_2 group is placed with one oxygen atom at 3.0 Å (3.1 Å in the B chain) and the nitrogen at 3.2 Å (3.3 Å in the B chain) from the N5 (Figure 4b). These distances are closer than the average distance observed across a range of flavoenzymes[20] and are also shorter than in the *E.coli* NTR−CB1954 complex (oxygen at 3.8 Å and nitrogen at 3.9 Å from the N5 of the isoalloxazine ring). If in NQO2 the hydride anion is transferred to the closest atom, then reduction will take place at one of the oxygen atoms of the nitro group of CB1954. This would imply that the reaction mechanism is via a nitroso intermediary product, an idea previously alluded to[32] and supported by experimental work that revealed an extremely fast reduction of the CB1954 nitroso derivative by NQO1[33] and of a nitrofurazone nitroso derivative by *E.coli* NTR.[27] Such a mechanism requires two successive two-electron reduction steps and hence, since the enzyme active site can be occupied by only one substrate at any one time, it is presumed that the intermediary product has to dissociate and leave the enzyme for the FAD to be reduced again by an electron donor. In stoichiometrical terms the overall reaction from the nitro to the hydroxylamine derivative is a four-electron reduction as indicated in Figure 1, in contrast to many diagrams in the literature in which the stoichiometry is not referred to. It is worth noting that the reduction of the nitroso derivative of CB1954 can be achieved nonenzymatically by NADH and other weak biological reducing agents.[33]

Our structure provides crucial insights into the molecular interactions governing the recognition of the

prodrug by the enzyme. One unusual feature is the arrangement of the nitro group in front of Trp105, with some interatomic distances down to 3.3 Å (Figure 4b). Given the chemical properties of the indole ring and the nitro group, their close proximity is not expected to be energetically favorable and is presumably compensated by the numerous other favorable interactions between the prodrug and the protein. Interestingly, in *E.coli* NTR, despite its very different active site structure, an aromatic group is also relatively close to the nitro group, with a geometry reminiscent of that reported here in terms of the distances between the N5/nitro group to the aromatic side chain (in NTR distances from N5 to Phe124 atoms are in the 6−7 Å range and from 4-NO_2 to Phe124 in the 3.6−4.0 Å range; in NQO2, N5 to Trp105: 4−5 Å range and 4-NO_2 to Trp105: 3.2−3.8 Å range). The residue Phe124 was extensively mutated in engineering experiments on NTR and several changes were found to improve the activity with CB1954, in particular mutations to Lys and Asn.[34] On the basis of the structure of NQO2 in complex with CB1954 presented here, we hypothesise that mutagenesis of Trp105 may produce better engineered variants as potential candidates for DEPT.

Experimental Section

The procedures for cloning, expression, and purification are available as Supporting Information. Below details of the crystallographic work are provided.

Crystallization and Data Collection. Crystals of NQO2 were grown in sitting-drops using the vapor diffusion method. A volume of 10 μL of protein solution was mixed with an equal volume of reservoir solution (1.66 M ammonium sulfate in 100 mM Na−HEPES pH 7, 12 μM FAD, 1 mM dithiothreitol) and allowed to equilibrate. Crystals grew in about one week; they were then transferred into a solution of artificial mother liquor containing the CB1954 prodrug at a concentration of 2 mM (from a stock solution prepared in DMSO). Crystals were left soaking for 3 days. For the X-ray diffraction experiments, crystals were frozen using a mixture of 50% glycerol, PEG 400, and MPD (all at 20% final concentration) as cryoprotectant in a solution of artificial mother liquor. Data were collected at −180 °C on a Rigaku R-AXIS IV^{2+} detector using Cu Kα radiation from a Rigaku Micromax-007 rotating anode. Reflections were indexed, integrated, and scaled using the programs *Mosflm*[35] and *SCALA*.[36] Details of the data processing statistics are reported in Table 1. Efforts to collect synchrotron higher resolution data were made but were not successful.

Model Refinement and Structural Analysis. Crystals were isomorphous with those previously obtained and described,[25] apart from some small changes in the cell dimensions. Refmac[37] was employed to carry out rigid body refinement using the native (apo) human NQO2 as a starting model (PDB code 1QR2[25]), after all waters were removed from this file. This procedure, using only data to 3.0 Å, decreased the *R*-factor from values above 50% to 34.2% and the R_{free} from above 50% to 34.9%. Visualization of electron density map, rebuilding, and model fitting were carried out on a Linux system using the program COOT.[38] Positional and *B*-factor refinement using Refmac reduced the R-factor down to 23.8% and the R_{free} to 27.0%, which was followed by limited manual rebuilding and addition of water molecules. Further refinement gave an *R*-factor of 20.0% and an R_{free} of 25.3%.

No ligand atoms were included in the model till this point; omit maps were carefully examined and refinement procedures with the CB1954 model in four different orientations were carried out. Analysis of difference maps allowed us to rule out two possible orientations. It is not possible, however, to discriminate between the two other orientations of the ligand: one orientation with the 4-NO_2 group placed toward Trp105, the other with the 2-NO_2 group placed toward Trp105. Refinement procedures, carried out for two models with the ligand in either of the above orientations, gave the same results: 19.3% and an R_{free} of 24.8%.

Refinement carried out with coordinated in which models for both orientation were included, each with the occupancy of the ligand atoms set at 0.5, gave comparable results with the previous refinement procedures (*R*-factor 19.3% and R_{free} 24.7%).

The final model was evaluated with PROCHECK,[39] and structural analyses were carried out using COOT,[7] DeepView[40] (www.expasy.org/spdbv), and CCP4 programs.[41] Figures were drawn using either the software PyMOL (http://pymol.sourceforge.net) or VMD.[42]

Coordinates have been deposited in the Protein Data Bank with accession code 2BZS.

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Note Added in Proof

Upon submission of our atomic coordinates, 2BZS, to the Protein Data Bank, it was noted that two files for the same complex were recently deposited by other groups with accession codes 1ZX1 and 1XI2. The latter was described in a paper that has just been published by Z. Zhang and colleagues.

Supporting Information Available: Procedures for cloning, expression, and purification; table with statistics for the crystallographic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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