

Natural small molecules as stabilizers and activators of cancer-associated NQO1 polymorphisms

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Abstract

NAD(P)H:quinone oxidoreductase 1 (NQO1) is an antioxidant and detoxifying enzyme involved in the two-electron reduction of a wide variety of quinones. As a non-enzymatic function, it is involved in the stabilization of several tumour suppressors such as p53, p33 and p73 α . NQO1 is overexpressed in several types of tumours, and two common polymorphisms are associated with increased cancer risk, making NQO1 a potential target for new cancer treatments. Here we review the structural and enzymological properties of NQO1, as well as its roles in cancer development and treatment. Particularly, we focus in recent developments on the understanding of the molecular basis leading to loss-of-function in cancer-associated polymorphisms, and propose new approaches to target these molecular defects to develop new pharmacological agents to rescue them. We will focus on pharmacological therapies aimed at correcting the abnormal properties of polymorphic proteins (such as protein stability and dynamics) and modulating intracellular factors leading to loss-of-function (such as accelerated proteasomal degradation).

Keywords: cancer, polymorphism, NAD(P)H:quinone oxidoreductase 1; pharmacological chaperone; protein dynamics; proteasomal degradation.

1. Introduction

1.1 Structure and Functions of NQO1

In 1958 Ernster and Navazio discovered the existence of a highly active oxidoreductase enzyme in the soluble fraction of rat liver homogenates (1). They named it DT diaphorase and subsequently Ernster and colleagues described its properties, in particular its kinetic activity and inhibition (2-6). The enzyme is now better known as NAD(P)H quinone oxidoreductase 1 (NQO1; EC 1.6.5.2). Subsequent biochemical research on NQO1 concentrated on elucidating the enzyme's structural and enzymological properties. It was noted that the enzyme is highly expressed in some cancer cells (7) and, consequently, there have been efforts to identify high affinity, selective inhibitors of NQO1. Paradoxically, reduced cellular NQO1 activity associated with two polymorphic forms of the enzyme is linked to an increased risk of some forms of cancer (8-12). Recent work has shown that these polymorphic forms are less stable than the wild-type protein. Although there has been considerable interest in developing "pharmacological chaperones" and proteostasis modulators for use in some inherited diseases, there has been no work to date on identifying reagents to stabilise cancer-associated variants of NQO1. We postulate that this may be a viable method for the treatment of cancers resulting from these variant forms or as a preventative measure in high-risk groups.

NQO1 is a homodimer with a total molecular mass of approximately 62 kDa and one molecule of FAD bound per monomer (Figure 1A). NQO1 catalyses the oxidation of NADH and NADPH with almost equal activities using various electron acceptors such as quinones, vitamin K₁ and dichloroindophenol (DCPIP) (13). The reaction proceeds via a substituted enzyme (or "ping-pong") mechanism (14) (Scheme 1). It is strongly and competitively inhibited with respect to NAD(P)H by the coumarin-derived, anti-coagulant dicoumarol (14).

The NQO1 homodimer has a two-fold axis of symmetry and interlocking monomers (15-20). Two domains form each monomer, a large catalytic domain (19) with a characteristic α/β flavodoxin fold consisting of a five-stranded parallel β -sheet enclosed between five α -helices and a small C-terminal domain to which binds the adenosine moiety of NADH (Figure 1A) (17).

There are two active sites, each located at the interface between the two subunits (19). FAD binds non-covalently in the active site forming one of its walls with very high affinity (21, 22), and interestingly, some degree of negative cooperativity between sites has been proposed (22). The rest of the boundaries are formed by residues from both monomers (17-19). Specifically, Phe106, Trp105 from one monomer along with Phe178, and the backbone carbonyl of Gly174 from the second monomer, all form the internal boundary of the active site with Tyr126 and Tyr128 from the second monomer forming the roof; the base of the cavity is formed by the isoalloxazine ring of FAD and His161 (Figure 1B). Two water molecules make up the outer boundary. Entrance to the active site is controlled by Gly149, Gly150, and His194 from the first monomer and Pro68 of the second monomer (17).

Each active site has three defined binding spaces, one for the adenine and ribose moieties of NAD(P)H, one for FAD and one for nicotinamide moiety of NAD(P)H or the electron acceptor molecule (Figure 1B-D; Figure 2); the availability of one space for either the electron donor or acceptor is consistent with the substituted enzyme mechanism (17). Comparison of the duroquinone-bound enzyme with the apoenzyme shows that Tyr128 has different conformations; it moves away from the FAD to allow duroquinone to bind and reoccupies its original position partially overlapping the substrate binding site in the apoenzyme while contributing to the closure of the active site's entrance to exclude molecular oxygen and solvent (17). This movement underlines the requirement for control of entry to the active site so that the electron donor and acceptor can enter (17). Dicumarol binds in the active site; it π -stacks parallel to the isoalloxazine ring of the FAD and establishes hydrophobic interactions and hydrogen bonds with residues from both monomers (Figure 1C). Specifically, one coumarin ring hydrogen bonds to the OH group on Tyr128 of one monomer and also to the N_ε of His161 of the second monomer (19).

NQO1 is a versatile protein showing multiple enzymatic and non-enzymatic functions (schematized in Figure 3). NQO1 catalyses the two-electron reduction of quinones (23), quinoneimines (13), nitroaromatics (24-26), hexavalent chromium compounds (27), *p*-benzoquinone and naphthoquinone epoxides (28, 29) and azo dyes (30, 31), even though the main *in vivo* substrates are not well known. Upon NAD(P)H binding to the active site, the nicotinamide moiety is positioned for direct hydride

transfer to FAD (Figure 1D) and in the second step of the catalytic cycle, binding of the quinone is optimal for accepting the hydride from the FADH₂ (32). The two-electron transfer from NAD(P)H occurs to the nitrogen atom in the middle ring of the alloxazine ring system. The subsequent movement of electrons ultimately leads to a negative charge on the O2F oxygen, which is stabilised by acceptance of a proton from Tyr155 (32). This negative charge on the Tyr is stabilised by His161. After the NAD⁺ leaves the active site, the quinone to be reduced binds and the process of electron movement is reversed, the H⁺ on the O2F oxygen is transferred back to Tyr155 causing the His161 to no longer be required for stabilisation, His161 becomes fully protonated and transfers a H⁺ to the hydroquinolate forming the hydroquinone which leaves the active site.

1.2 NQO1 protects cells by catalysing two-electron reductions

By transferring two electrons to quinones, NQO1 prevents the formation of semiquinones which would be produced if one electron was transferred. This protects against the generation of reactive oxygen species (ROS) from redox cycling (33) because the spontaneous reoxidation of semiquinones by molecular oxygen can cause the formation of superoxide free radicals (34). The decrease in concentration of electrophilic quinones also stops them from taking part in reactions leading to sulphhydryl depletion (33). Additionally, NQO1 can directly scavenge superoxide free radicals. (35).

1.3 NQO1 binds to p53, p73α, p33 and Ornithine decarboxylase

NQO1 displays additional non-enzymatic functions by binding to, and stabilising, the tumour suppressor proteins p53 and p73α (36, 37). Additionally, proteasomal degradation of p53 and p73α occurs by two pathways, ubiquitin-dependent and independent. NQO1 regulates the latter by its association with the 20S proteasome and with p53 and p73 (38). These interactions seem to be NADH-dependent even though an enzymatically active NQO1 may not be required (36, 38). NQO1 inhibitors such as dicoumarol, other coumarins, flavones and curcumin, antagonise these interactions (38-43).

2. NQO1 in cancer development and treatment

2.1. NQO1 expression and cancer

NQO1 is present in all tissues with the highest expression levels occurring in epithelial, vascular endothelium and adipocytes and tumour cells, in particular liver tumours (44). Its expression is induced by xenobiotics, antioxidants, oxidants, heavy metals, ultra violet light and ionizing radiations (45). This upregulation occurs via the Keap1/Nrf2/ARE pathway and it is induced in concert with other genes coding for stress response proteins (45).

Inhibition of NQO1 seems counterintuitive as a treatment for cancer since NQO1 provides protection against the production of ROS and stabilises tumour suppressor proteins. However, NQO1 inhibition has been shown to inhibit cell growth in cancer cells, for example, pancreatic cancer cells (46). Dicoumarol mediated inhibition of NQO1 caused an increase in superoxide production in these cells which allowed for the one-electron reduction of quinones by cytochrome p450 reductase and other flavoproteins (46). More recent studies on a variety of coumarin-based inhibitors led to the hypothesis that the inhibition of NQO1 may not be the sole contributing factor in the inhibition of pancreatic cancer cell growth and that it also may be due to mitochondrial uncoupling caused by dicoumarol (47). Inhibition of NQO1 can also be indirectly useful when used in conjunction with other cancer drugs, for example, the drug thymoquinone (TQ) induces apoptosis in breast cancer cells and its mode of action is mediated by reactive oxygen species. NQO1 protects cells against TQ because it scavenges superoxide radicals therefore its inhibition by dicoumarol causes an increase in the concentration of superoxide free radicals and the cells are no longer protected from the action of TQ (48).

2.2. Cancer-associated polymorphisms in NQO1

Two polymorphisms (NQO1*2 and NQO1*3) are found in NQO1 with relatively high frequency. NQO1*2 (rs1800566) is caused by a C to T change at position 609 in the cDNA sequence and results

in a proline to serine substitution at position 187 in the protein sequence (49). p.P187S is quickly degraded via ubiquitin-dependent and independent proteasomal pathways (50, 51). It has about 100-fold lower activity than wild-type NQO1 enzyme and its affinity for FAD is reduced (21, 22, 52). Individuals homozygous for this polymorphism have undetectable NQO1 protein or activity. Heterozygous individuals have reduced NQO1 levels and activity (53). The allele frequency is higher in Asian populations (approximately 50%) compared with Caucasians (approximately 25%) (10). There is a clear association between this polymorphism and cancer susceptibility (54, 55) and also with increased susceptibility to poisoning by benzene and other carcinogenic chemicals (56-58). The types of cancer associated with the NQO1*2 polymorphism include gastrointestinal, oesophageal, liver, urinary, bladder, colorectal, lung, prostate, breast, bone marrow, thyroid and skin (59-69) (70-77).

NQO1*3 (rs 1131341) is caused by a change in the cDNA sequence at position 465, from C to T. In the full-length NQO1 protein sequence, this results in an arginine to tryptophan substitution at amino acid 139 (78). Small differences in enzyme kinetic properties have been reported (22, 79). It also moderately decreases the kinetic stability of the enzyme (as a half-life towards denaturation *in vitro*). Importantly, this polymorphism enhances the alternative splicing of the NQO1 pre-mRNA leading to exon 4 skipping (residues 102-139), which yields a shorter and unstable NQO1 protein with severely compromised catalytic properties (80). Its allele frequency is lower than for NQO1*2 (2% globally) (81). However, it has also been associated with increased risks of some forms of cancer, for example paediatric acute lymphoblastic leukaemia (12, 82).

There is an apparent paradox about the role of NQO1 in cancer. Cancer cells often express high levels of NQO1 and inhibition of NQO1 can lead to cell death. In other words, increased cellular NQO1 activity in this context promotes the cancerous state. This may be because cancer cells are highly metabolically active and thus generate higher quantities of reactive oxygen species which are detoxified by NQO1 and other enzymes. However, normal cells which have reduced levels of NQO1 are more likely to transform to the cancerous state than those expressing wild-type levels. In this case, reduced cellular levels of NQO1 promote the cancerous state. This is likely to be due to the decreased capacity of these cells to deal with reactive oxygen species and the increased likelihood of single electron

reduction of quinone species (since there is insufficient NQO1 activity which results in a larger fraction of the quinone pool being processed by systems other than NQO1). Thus, there is increased free radical load over time, resulting in an increased probability of cellular damage. Reduced NQO1 levels in these cells will also mean less capacity for the stabilisation of p53 meaning that apoptosis is less likely to remove damaged cells which are at risk of transformation.

*2.3. NQO1*2 affects protein function, stability and dynamics*

Recent advances have been made investigating the molecular defects of the NQO1*2 polymorphism. NQO1*2 is able to fold as dimers with similar secondary and tertiary structures to those of NQO1, even though its hydrodynamic behaviour is consistent with a slight expansion of the dimer (21, 22). Its specific activity is severely compromised, due to a 10-100 fold reduction in the FAD binding affinity, thus explaining the very low content in FAD of NQO1*2 as purified (21, 22). Importantly, recombinant NQO1*2 displays much lower thermal and kinetic stability than NQO1, which suggests a shift in the unfolding equilibrium towards non-native states (22, 51). Remarkably, the crystal structure of NQO1*2 has revealed only small and local changes in the structure (21), that therefore cannot explain the devastating effects of NQO1*2 *in vitro* and *in vivo*. To investigate whether NQO1*2 might affect protein dynamics in solution, NMR spectroscopy and proteolysis experiments has been carried out, confirming the presence of partially unfolded states in solution and a particularly high flexibility of its C-terminus (21). Our ongoing research using molecular dynamics simulations and detailed proteolysis studies also support that the reduced FAD binding affinity in NQO1*2 is caused by increased fluctuation at the FAD binding site, while its dimer instability is associated with a highly dynamic monomer:monomer interface (unpublished observations).

3. Cofactors and inhibitors as chaperones for NQO1 polymorphisms

3.1. FAD overcomes some functional, stability and dynamic defects due to polymorphisms

Several lines of evidence support the hypothesis that many of the abnormal properties of NQO1*2 are associated with its poor ability to bind FAD. For instance, detailed mass spectrometry analyses in combination with *in vitro* proteasomal degradation assays, have shown that FAD binding to either apo-NQO1 and apo-NQO1*2 causes a shift in the folding equilibrium towards the native and degradation resistant state (51). These results also support the strong kinetic stabilization observed for NQO1*2 in the presence of exogenous FAD by biophysical techniques (22). Moreover, supplementation with riboflavin to cultured cells expressing NQO1*2 supported a significant stabilization of the polymorphism by FAD, and a concomitant stabilization and recovery of p53 (51). Additionally, enzyme kinetic analyses also support that, upon saturation with FAD, NQO1*2 displays specific activities and kinetic parameters close to those of NQO1, demonstrating that enzyme kinetic defects primarily arise from altered FAD binding (51) (also, our unpublished results). Overall, these studies suggest that supplementation with FAD precursors might open new ways to treat patients bearing the NQO1*2 polymorphism.

However, other results obtained with NQO1*2, in addition to increasing knowledge on the molecular recognition of substrates by the 26S and 20S proteasome leading to their degradation, suggest that other types of ligands are required to stabilize NQO1*2 for treatment of patients. Saturation of NQO1 and NQO1*2 with FAD followed by kinetic analyses of their proteolysis by different proteases as well as by molecular dynamic simulations show that FAD binding does not correct the dynamic alterations caused by the polymorphism in the C-terminus (21) (also, our unpublished observations). This suggests that the dynamics of NQO1 and NQO1*2 are clearly different, which may have deep implications in understanding the degradation of NQO1 and NQO1*2 by the proteasome and in the identification of effective ligands to stabilize NQO1*2 *in vivo*. Degradation of protein substrates by the 20S proteasome depends on the translocation of dynamic regions of the substrate through the proteasome pore to initiate degradation, and these regions are often found at the N- or C-terminus of the substrate (83). In the case of the 26S proteasome, it must recognize a polyubiquitin tag and a disordered initiation site, often in the form of a dynamic tail at the terminus of the substrates (84).

Therefore, the different protein dynamics of NQO1 and NQO1*2 saturated with FAD may not fully correct the instability of NQO1*2 *in vivo*.

3.2. Inhibitors correct dynamic effects at the C-terminus of NQO1*2

As described above, either FAD or ligands that bind to the dynamic C-terminus of NQO1*2 could be used, and even combined, to rescue the function of this polymorphism. A natural binding site that could be used for purpose is the substrate/NAD(P)H binding site. This binding site comprises several residues of the N-terminus and C-terminus of NQO1 (Figure 1C and D), and a ligand binding to the NAD(P)H binding site (such as the inhibitor dicoumarol) induces rigidification of the highly dynamic C-terminus of NQO1*2, thus specifically increasing its steady-state protein levels inside cells (unpublished observations). However, it is still unclear whether this approach might work intracellularly, since the use of an inhibitor such as dicoumarol would abolish the interaction of NQO1*2 with p53 and other oncosuppressors, triggering their degradation. Also, other intracellular targets important for protein homeostasis, such as Hsp90, could be inhibited by dicoumarol (85).

In the search of new potent and specific ligands to pharmacologically rescue NQO1*2, two main strategies emerge. High-throughput screening strategies can be easily adapted for NQO1*2, since simple thermal denaturation and activity assays are available *in vitro*, and these could be used with open (such as those from the Developmental Therapeutics Programs, NCI/NIH; <https://dtp.nci.nih.gov/>) and commercially available compound libraries. Ligands binding to either the N- or the C-terminus of NQO1*2 lead to stabilization of the protein towards thermal denaturation (22) (unpublished observations). However, thermal up-shift and activity assays do not take into account the effect of NQO1*2 on C-terminal dynamics, which may be the key to protecting it towards proteasomal degradation. Therefore, the positive hits obtained from these HTS procedures should be complemented with techniques that also consider protein dynamics, such as *in vitro* partial proteolysis assays and degradation of NQO1*2 by the proteasome in cell-free expression systems. Due to availability of several crystal structures of NQO1, a virtual screening approach can be performed by molecular docking. Indeed, this strategy has been applied to NQO1 to search for potent and more specific

inhibitors than dicoumarol (20, 47, 86-89). These studies have identified a range of symmetrical and asymmetrical quinone-like compounds. Some of these have reduced “off-target” effects such as serum albumin binding and mitochondrial uncoupling when compared to dicoumarol (47, 90)

4. Proteostasis of NQO1*2: proteasomal inhibitors and proteostasis regulators

The protein homeostasis network comprises around a thousand proteins that establishes a protein quality control system, involved in the proper folding, trafficking, disaggregation and degradation of proteins (91, 92). Despite the inherent complexity of understanding the routes that a given protein follows to fold, misfold and eventually be degraded, modulation of protein homeostasis pathways is a promising approach to treat folding diseases. Particularly, different classes of protein homeostasis modulators have been developed and shown to correct multiple folding diseases in cells and animal models. (93). By high-throughput screening procedures, several new proteostasis modulators have been found to operate through HSF-1, FOXO and Nrf-2 pathways, and some of them could be targeted to rescue NQO1*2. HSF-1 forms inactive monomers complexed with Hsp40/Hsp70/Hsp90, and upon release, trimerize becoming active upon phosphorylation. Its activation causes the overexpression of many heat shock and stress proteins, including Hsp70, that negatively feed back (94). As indicated above, Nrf-2 activation leads to overexpression of NQO1.

In the Section 3, we have discussed the potential use of small ligands that affect NQO1*2 stability and dynamics aimed at rescuing its function. Since proteasomal degradation of NQO1*2 seems to be crucial for its loss-of-function, proteasome inhibitors could be used for a similar purpose. Alternatively, protein homeostasis modulators (other than proteasome inhibitors) could be used to boost the efficiency of intracellular folding of NQO1*2.

There are several proteasomal inhibitors approved by FDA (such as Bortezomib) or in clinical trials, so their use in the treatment of several and diverse human disease is promising. Several proteasomal inhibitors has been shown to effectively increase the intracellular stability of NQO1*2, including MG-132, clastolactacystin and Bortezomib (36, 50, 51, 95).

HSF-1 could be pharmacologically targeted to restore NQO1*2 activity. NQO1 is known to interact with Hsp40/Hsp70 along its folding, but not with Hsp90, suggesting that this pathway is required for efficient NQO1 folding. This ternary complex seems to be formed by interaction of the N-terminus of NQO1 with the chaperones. In contrast, NQO1*2 is unable to interact with these chaperones, which may reduce its efficient folding intracellularly (95). Moreover, we might speculate that NQO1 could partially sequester Hsp70 along its folding, increasing the activation state of HSF-1 and its positive effect on the overall intracellular homeostasis.

5. Outlook

There is increasing evidence supporting that pharmacological modulation of NQO1 might be used to treat cancer patients, particularly in those carrying NQO1*2 and NQO1*3 polymorphisms. Remarkably, current and future research on the relationship between dynamic alterations due to NQO1*2 and its enhanced proteasomal degradation will pave the way to search for potent pharmacological agents to rescue its function *in vivo*. We anticipate that multidisciplinary approaches, including HTS techniques, dynamic studies and state-of-the-art assays to measure vectorial proteasomal degradation *in vitro* and in cell model system will be invaluable to identify and optimize such pharmacological agents.

List of Abbreviations

NQO1, NAD(P)H:quinone oxidoreductase 1; NQO1*2, p.P187S polymorphic NQO1; NQO1*3, p.R139W polymorphic NQO1; HTS, high-throughput screening; HSF-1, Heat Shock Factor 1; Nrf-2, NF-E2 related factor 2;

Conflict of Interest

None

Acknowledgments

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Figure 1: Structure of human NQO1. (a) The overall fold of the enzyme showing the two subunits (cyan and green). The FAD cofactors (dotted) are shown in the two active sites. (b) The binding of the FAD cofactor at (light brown) the active site. Note how residues from both subunits (cyan and green) are required for binding. The redox active isoalloxazine ring system (on the right hand end of the molecule as depicted here) is bound into a deep pocket whereas the adenine moiety (left) is more exposed. (c) Dicoumarol (yellow) binds at the active site partially obscuring the isoalloxazine ring of FAD. It thus acts as a competitive inhibitor, blocking NAD(P)H's access to the active site. (d) NADH (purple) binds to the active site such that the nicotinamide ring stacks with the isoalloxazine ring of FAD. Note how the binding of NADH and dicoumarol are mutually exclusive.

Images were produced using PyMol (www.pymol.org) using PDB files 1D4A (17) for (a) and (b) and 2F1O (19) for (c). For (d), the structure of an NQO1 monomer bound to NAD⁺ (kindly supplied by Profs Mario Bianchet and Mario Amzel of Johns Hopkins University Medical School, Baltimore, USA) (15) was aligned to 1D4A using PyMol. The NAD⁺ molecule and the dimeric NQO1 protein (including FAD cofactors) from 1D4A were saved as a pdb file and energy minimised using YASARA (96). The resulting structure files are provided as supplementary information.

Figure 2: Interaction of NQO1 with ligands. The residues interacting with (a) FAD, (b) dicoumarol and (c) NADH. These images were generated using LigPlot+ (97).

Figure 3: Functions of NQO1. Some key functions of NQO1 are summarised. Those associated with its enzymatic roles are grouped on the left and those associated with its interaction with other proteins on the right.

Scheme 1: The mechanism of NQO1. The reaction proceeds through a substituted enzyme mechanism in which NADH first reduces a tightly bound FAD cofactor in the active site of NQO1. NAD⁺ is

released and the enzyme can then accept the second substrate, often a quinone (Q). Two electrons are then transferred from the FADH_2 cofactor generating the reduced form of the quinone (QH_2). This regenerates the initial form of the enzyme, enabling a further round of catalysis.

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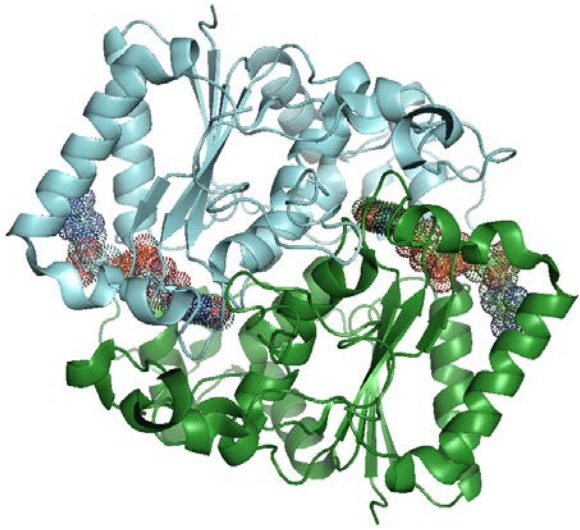
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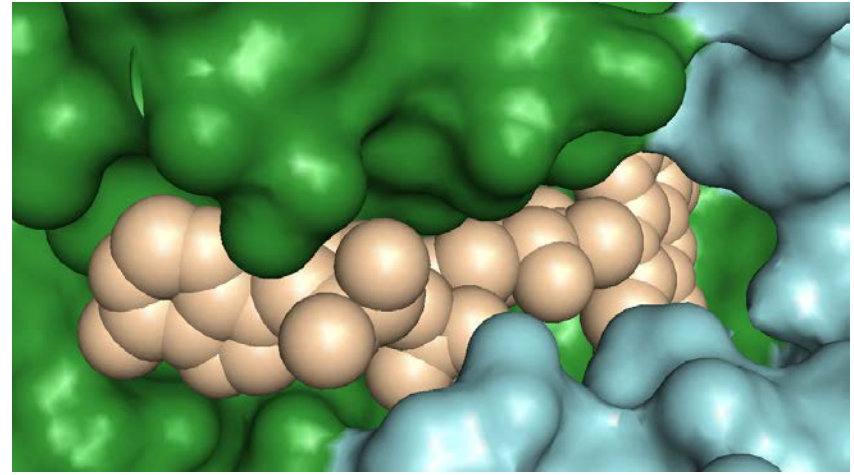
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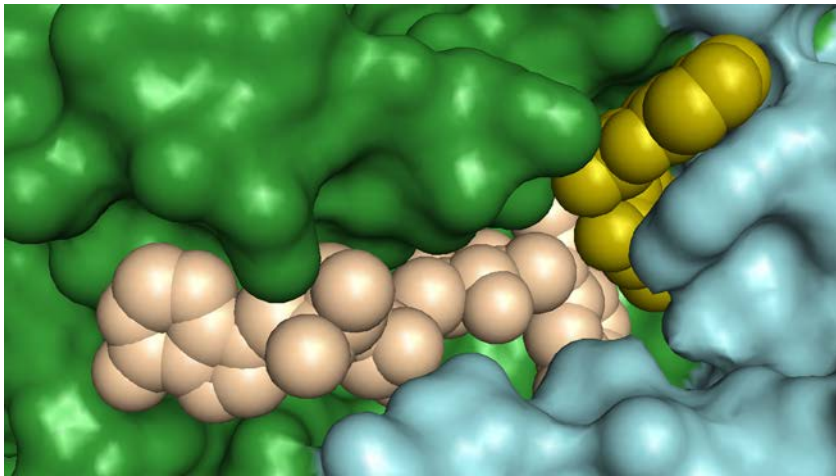
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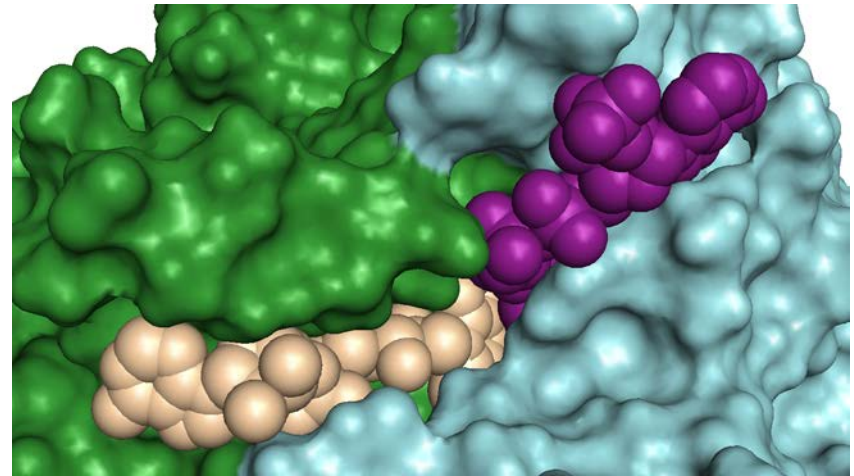
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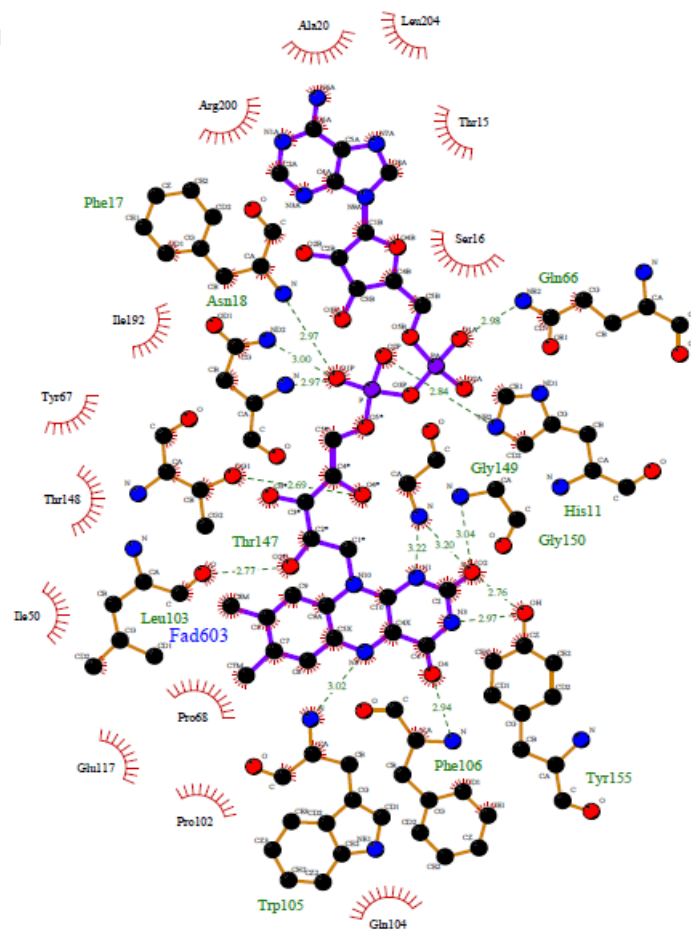
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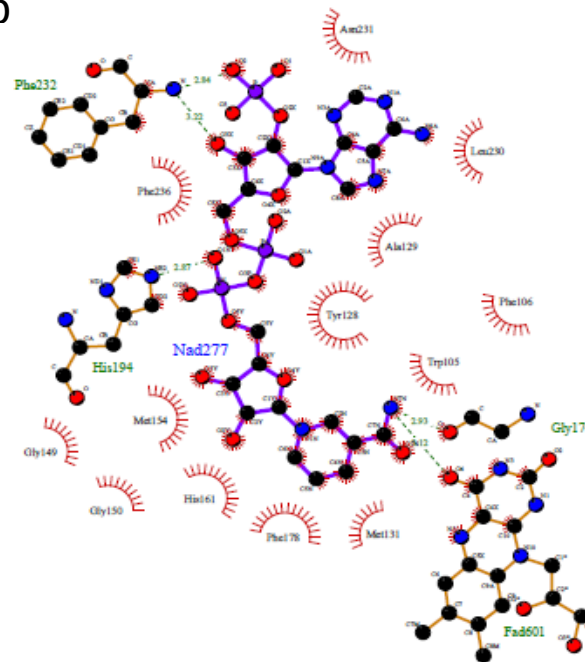
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(a)



(b)



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