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TITLE PAGE

Conformational variations of both PDE5 and inhibitors provide the structural basis for the physiological effects of vardenafil and sildenafil

Huanchen Wang, Mengchun Ye, Howard Robinson, Sharron H. Francis, and Hengming Ke

Department of Biochemistry and Biophysics, The University of North Carolina, Chapel Hill, NC 27599-7260, USA (H.W., M.Y., H.K.); Biology Department, Brookhaven National Laboratory, Upton, NY 11973-5000, USA (H.R.); Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0615, USA (S.F.).

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RUNNING TITLE PAGE

Running title: Crystal structure of PDE5-vardenafil

Corresponding author: Hengming Ke

Department of Biochemistry and Biophysics,

The University of North Carolina,

Chapel Hill, NC 27599-7260

Phone: +1-919-966-2244; Fax: +1-919-966-2852;

Email: hke@med.unc.edu.

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Abbreviations:

PDEs – cyclic nucleotide phosphodiesterases; PDE5 – phosphodiesterase-5.

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ABSTRACT

Vardenafil has higher affinity to phosphodiesterase-5 (PDE5) than sildenafil and lower administered dosage for the treatment of erectile dysfunction. However, the molecular basis for these differences is puzzling because two drugs have similar chemical structures. Reported here is a crystal structure of the fully active and non-mutated PDE5A1 catalytic domain in complex with vardenafil. The structure shows that the conformation of the H-loop in the PDE5A1-vardenafil complex is different from those of any known structures of the unliganded PDE5 and its complexes with the inhibitors. In addition, the molecular configuration of vardenafil differs from that of sildenafil when bound to PDE5. Importantly, the binding of vardenafil causes loss of the divalent metal ions that have been observed in all the previously published PDE structures. The conformational variation of both PDE5 and the inhibitors provides structural insight into the different potencies of the drugs.

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INTRODUCTION

Cyclic nucleotide phosphodiesterases (PDEs) are key enzymes controlling cellular concentrations of the second messengers adenosine and guanosine 3´, 5´-cyclic monophosphates (cAMP and cGMP) (Mehats, et al. 2002; Houslay and Adams, 2003; Goraya and Cooper 2005; Bender and Beavo, 2006; Lugnier 2006; Omori and Kotera, 2007; Conti and Beavo, 2007). The human genome encodes 21 PDE genes that are categorized into 11 families. Alternative mRNA splicing of the PDE genes produces about 100 isoforms of PDE proteins that distribute in various cellular compartments and control myriad physiological processes. PDE molecules contain a variable regulatory domain and a conserved catalytic domain, but show distinct substrate specificity and inhibitor selectivity. Family-selective PDE inhibitors have been widely studied as therapeutic agents for treatment of various human diseases, including cardiotonics, vasodilators, smooth muscle relaxants, antidepressants, antithrombotics, anti-asthmatics, and agents for improving learning and memory (Truss et al., 2001; Rotella, 2002; Schrör, 2002; Lipworth, 2005; Castro et al., 2005; Houslay et al., 2005; Blokland, et al., 2006; Menniti et al, 2006).

The most successful examples of this class of drugs are the PDE5 inhibitors (Fig. 1) sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis) that have been used for treatment of male erectile dysfunction (Rottela et al., 2002). Sildenafil (Revatio) has also been approved for treatment of pulmonary hypertension (Galie et al., 2005). Recently, udenafil (Fig. 1) has been approved by Korean authorities for treatment of male erectile dysfunction (Salem et al., 2006). Although these four PDE5 inhibitors have been successfully approved as the drugs for treatment of human diseases, the enthusiasm for development of novel PDE5 inhibitors continues. PDE5 inhibitors have been shown to have potential for other medical applications, including improvement of memory and treatment of cancer and heart disease (Supuran et al., 2006; Stehlik

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et al., 2006; Blokland, et al., 2006; Salem et al., 2006; Padma-Nathan et al., 2007; Palmer et al., 2007; Zhu et al., 2007; Sander et al., 2007). Recently, much attention has been focused on development of the second generation of PDE5 inhibitors that have the same or different scaffolds from the current drugs, but different pharmacokinetic profiles (Palmer et al., 2007).

Sildenafil, vardenafil, and udenafil have similar chemical formulae (Fig. 1) and possess similar key pharmacophores that provide for their function. These inhibitors also have the same target and interact with many of the same residues at the active site of PDE5, as shown by the crystal structures of the isolated PDE5 catalytic domain in complex with sildenafil and vardenafil (Sung et al., 2003; Huai et al., 2004; Zhang et al., 2004; Card et al., 2004; Wang et al., 2006). The pharmacokinetic and pharmacodynamic analyses, although the head-to-head comparison is still lacking, showed that these PDE5 inhibitors have similar efficacy and tolerance, but they exhibit some functional differences both *in vitro* and *in vivo* (Briganti et al., 2005; Shabsigh et al., 2006; Wright 2006; Supuran et al., 2006; Mehrotra et al., 2007; Doggrell, 2007). For example, vardenafil shows 10-40 fold tighter binding with PDE5 than sildenafil and has an AUC (the area under the curve) of 74.5 μg.hr/L at 20 mg dosage in comparison with 1965 for sildenafil at 100 mg dosage (Shabsigh et al., 2006; Mehrotra et al., 2007).

However, the structural basis for the different potencies of these inhibitors is still puzzling. The early studies on the crystal structures of PDE5 in complex with sildenafil and vardenafil by two groups showed inconsistent results. Vardenafil and sildenafil have the same extended configuration in the crystal structures reported by Sung et al. (2003), in contrast to the folded configuration of both inhibitors in the report by Zhang et al. (2004). Since the PDE5A catalytic domain used in Sung's study is basically inactive and the structure reported by Zhang et

al. contains a chimeric replacement of the PDE5A H-loop with the PDE4D H-loop, the biologically relevant conformation of these drugs has remained a question.

To address this question, the crystal structure of the fully active and non-mutated catalytic domain of PDE5A1 in complex with vardenafil has been determined and compared with the previously published co-crystal structures of the enzyme with sildenafil and vardenafil. The structural comparison shows dramatic differences between the vardenafil and sildenafil complexes in both PDE5 protein conformation and the inhibitor configuration. These differences are likely to contribute to the different properties of these drugs.

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MATERIALS AND METHODS

Protein expression and purification of the PDE5A1 catalytic domain. The cDNA of the catalytic domain of human PDE5A1 was generated by site-directed mutagenesis of the gene of bovine PDE5A, as previously described (Wang et al., 2006). The coding region for amino acids 535-860 of PDE5A1 was amplified by PCR and subcloned into the expression vector pET15b. The resultant plasmid pET-PDE5A1 was transferred into *E. coli* strain BL21 (CodonPlus) for overexpression. The *E. coli* cell carrying pET-PDE5A1 was grown in LB medium at 37^{o}C to absorption A600 = 0.7 and then 0.1 mM isopropyl β-D-thiogalactopyranoside was added for further growth at 15^{o}C overnight. Recombinant PDE5A1 was passed through the Ni-NTA affinity column (Qiagen), subjected to thrombin cleavage to remove the His-tag, and further purified by Q-Sepharose and Sephacryl S300 column chromatography (Amersham Biosciences). A typical purification yielded over 10 mg PDE5A1 with a purity >95% from a 2-liter cell culture.

Protein crystallization and structure determination. The co-crystal of PDE5A1 (535-860) with vardenafil was grown by vapor diffusion. The complex of PDE5A1-vardenafil was prepared by mixing 1 mM vardenafil with 15 mg/mL PDE5A1 at 4^{o}C overnight. The protein drop was set up by mixing 2 μl protein solution with 2 μl well buffer and crystallized against a well buffer of 12% PEG3350, 15% glycerol, 0.1 M sodium acetate pH 4.6 at 25^{o}C. The PDE5A1-vardenafil crystals have the space group $P2_12_12_1$ with cell dimensions of a = 68.9, b = 87.8 and c = 138.5 Å (Table 1). Diffraction data were collected on beamline X29 at Brookhaven National Laboratory and processed by program HKL (Otwinowski and Minor, 1997).

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The structure of the PDE5A1-vardenafil co-crystal was solved by molecular replacement program AMoRe (Navaza and Saludjian, 1997), using the PDE5A1-IBMX structure without the H-loop as the initial model. The atomic model was built by program O (Jones, et al., 1991) against the electron density map that was improved by the density modification package of CCP4. The structure was refined by CNS (Table 1, Brünger et al., 1998).

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RESULTS

Architecture of the PDE5-vardenafil structure

The enzyme of the PDE5A1 catalytic domain used in these studies was fully active and exhibited kinetic properties (k_{cat}, K_m) similar to those for the full length PDE5A1 (Wang et al., 2006). The structure of the PDE5A1 catalytic domain (residues 535-860) in complex with vardenafil consists of 15 α-helices (Fig. 2). Most of the residues in the PDE5A1-vardenafil cocrystal had solid electron density and were traced without ambiguity. Residues 660-672 and 792-806, which are parts of the H- and M-loops, lacked electron density and were disordered. The superimposition of PDE5A1-vardenafil over other previously determined PDE5A1 structures (Huai et al., 2004; Wang et al., 2006) yielded root mean squared deviations (RMSDs) of 0.49, 0.54, 0.51, and 0.47 Å, respectively for Cα atoms of 270 comparable residues (536-657, 686-787, and 813-859) of the unliganded PDE5A1 and its complexes with IBMX, icarisid II, and sildenafil, indicating the overall similarity among the PDE5 structures. However, the PDE5A1-vardenafil structure shows dramatic conformation differences in the H- and M-loops from the known PDE5 structures.

Different conformational changes induced by vardenafil and sildenafil binding

The H-loop of PDE5 was previously shown to have four different conformations depending on the liganded state of the protein: 1) a coil conformation in the unliganded state, 2) two short α-helices (H8 and H9) at residues 664-667 and 672-676 in the IBMX complex, 3) a 3_{10} helix in the sildenafil complex, and 4) two short β-strands in the icarisid II complex (Wang et al., 2006). In addition, these conformation changes of the H-loop upon the inhibitor binding are coupled with the dramatic positional movements, up to 7, 24 and 35 Å in these three complexes, respectively.

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The position and conformation of the H-loop in the PDE5A1-vardenafil structure also differs significantly from those of the known PDE5 structures. First, helix H9 in the PDE5A1-vardenafil co-crystal contains residues Ser675 to Ile680, in comparison with the sequence of 671-675 in the PDE5A1-IBMX complex; the latter composition of the H-loop is similar to the residues of helix H9 in other PDE families (Ke and Wang, 2007). Second, the H-loop in the PDE5A1-vardenafil complex shows a positional shift of as much as 20 Å from that in the unliganded form. Third, the N-terminal residues 680-685 of helix H10 that comprises sequences 680-693 in all the early structures of PDE5 and other PDE families are in a coil conformation in the PDE5A1-vardenafil structure. Finally, residues 792-808 of the M-loop in the PDE5A1-vardenafil structure are disordered. This disorder is similar to features of this region found in the unliganded and IBMX-bound PDE5A1 structures (Wang et al., 2006), but is in contrast to the ordered conformation of the M-loop in the co-crystal structures of PDE5-sildenafil and PDE5-icarisid II. Since the M-loop contains a coiled fragment around Leu804 that contacts the inhibitors and most likely the cGMP substrate (Fig. 2), its disorder in the PDE5A1-vardenafil structure is likely to have some implication for both inhibitor and substrate binding. However, this possibility will require further study.

Vardenafil causes loss of the divalent metal binding

The most surprising feature of the PDE5A1-vardenafil structure is the absence of divalent metals at the active site (Fig. 2). This is in contrast to the absolute conservation of the binding of two divalent metal ions at the active sites of all early reported structures of PDE5 and other PDE families (Wang et al., 2006; Ke and Wang, 2007), even in the presence of the metal-chelating agent EDTA during the protein purification of the PDE4B2B catalytic domain (Xu et al., 2000).

Since the divalent metals and their binding residues are not involved in crystallographic lattice contacts, the loss of the divalent metals in the PDE5-vardenafil complex is unlikely an artifact of the crystal packing. Rather, the loss of the metal ions in the PDE5-vardenafil structure is apparently due to the influence of the conformational changes in the H-loop. A careful examination shows that two of the zinc-binding residues (His617 and Asp764) are well superimposed with those in the other PDE5 structures (Fig. 2D), as shown by small positional differences of 0.15 and 0.19 Å for their Cα atoms between the structures of PDE5A1-vardenafil and PDE5A1-sildenafil. However, two other important metal-binding residues (His653 and Asp654) in the PDE5A1-vardenafil complex show shifts of 0.76 and 0.80 Å from those in the PDE5A1-sildenafil complex although their conformations are retained; the magnitude of these shifts are almost twice the overall RMSD of 0.47 Å for all the atoms in the structures. In addition, the positioning of His684 in the PDE5-vardenafil complex is completely different from that in the PDE5-sildenafil structure and its imidazole ring is now located at the site normally occupied by the second metal ion or magnesium (Fig. 2D). Thus, the positional and conformational changes of Asp654 and His684 apparently act to eliminate binding of both divalent cations. Because Asp654 and His684 are located respectively at the N- and C-termini of the H-loop, the conformational change of the H-loop upon vardenafil binding appears to be the driving force that causes loss of the metals. This suggests that loss of catalytic activity in the presence of vardenafil is due to two factors: 1) direct competition between vardenafil and cGMP for access to the catalytic site, and 2) vardenafil-induced loss of divalent cations from the catalytic site.

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To study whether the inactive PDE5-vardenafil complex can regain the catalytic activity, the PDE5A1 catalytic domain (residues 535-860) was mixed with 1.5 mM vardenafil for 4 hours and then passed through a Sephacryl S300 gel filtration column in a plain running buffer of 20 mM Tris.HCl pH 7.5, 50 mM NaCl, and 1 mM 2-mercaptoethanol without inhibitor vardenafil. The specific activities of the native PDE5, the PDE5-vardenafil complex, and the fraction eluted from the S300 column were measured at 5 repeats by using the previously described method (Wang et al., 2006) and the assay buffer of 20 mM Tris.HCl, pH 7.5, 1.5 mM DTT, 10 mM $MgCl_2$, 0.2 μM cGMP. They were 39.8 ± 0.6 nmol/min/mg for the native PDE5 catalytic domain, 0.09 ± 0.02 for the protein in complex with 1.5 mM vardenafil, and 2.0 ± 0.4 for the protein after passed the S300 column. Addition of 10 and 100 nM zinc to the assay buffer increased the specific activity for the protein eluted from the S300 column by about 2-fold (3.9 ± 1.1 and 4.4 ± 1.0 nmol/min/mg). The second time passing through the S300 column did not further gain the activity. These experiments suggest that the limited catalytic activity can be regained from the inactive PDE5-vardenafil complex by passing the gel filtration column. The small percentage (10%) of activity recovery implies the improper elution conditions or the trap of vardenafil in the closed active site.

Different configurations of vardenafil and sildenafil when bound to PDE5A1.

Vardenafil directly competes with cGMP for access to the catalytic pocket of PDE5A1, as does sildenafil (Figs. 2 and 3). The binding involves three hydrogen bonds formed respectively between 1) O^6 and N1 of imidazotriazinone of vardenafil and Nε2 and Oε1 of Gln817 of PDE5A1, and 2) a sulfonamide oxygen of vardenafil and the backbone nitrogen of Cys677 of PDE5A1. In addition, two water molecules bind to O^6 and N8 of the imidazotriazinone. For

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hydrophobic interactions, the propyl-imidazotriazinone of vardenafil stacks against Phe820 of PDE5A1 and also contacts residues Tyr612, His613, Ile680, Leu765, Ala767, Ile768, Leu782, Phe786, and Gln817. The ethoxy group orients to a hydrophobic pocket and interacts via van der Waals forces with Ala779, Val782, Ile813, and Gln817. The phenyl group forms hydrophobic interactions with Tyr676, Met816, Gln817, and Phe820. The ether oxygen of the ethoxyphenyl group has a distance of 3.18 Å to the amide oxygen of the side chain of Gln817. This distance is an unfavorable interaction because both oxygen atoms have no proton for formation of a hydrogen bond and also because Gln817 is unlikely to switch its side chain orientation due to its pre-existing hydrogen bond with Gln775. The sulfonamide group ($-SO_2N$) of vardenafil contacts Tyr676, Cys677, and Ile680 of the H-loop, in addition to the stack against Phe820. The ethylpiperazine group orients to the surface of the binding pocket and interacts with Met816, Gly819, and Phe820.

Vardenafil binding to PDE5 shares a number of similarities with the binding characteristics of sildenafil, including similar location of their ethoxyphenyl and imidazotriazinone/pyrazolopyrimidinone groups, the same stacking against Phe820, and the hydrogen bonds with Glu817 (Fig. 3). In addition, both inhibitors are buried in the catalytic pocket. The solvent accessible area of the bound vardenafil is only 8%, which compares well with 9.4% of the bound sildenafil (Wang et al., 2006). However, the bound vardenafil shows different molecular configuration and interactions from the bound sildenafil (Fig. 3). The key difference is the orientation of the piperazine portion of two drugs. The ethylpiperazine of vardenafil orients to the surface of the binding pocket and is extended to interact with residues of Tyr676 to Ile680. In comparison, the methylpiperazine of sildenafil folds back to its molecular

entity and interacts with residues Asn662 to Ile665 of the H-loop, but not Tyr676-Ile680, via van der Waals' interactions.

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DISCUSSION

Vardenatil possesses a chemical structure very similar to sildenafil, but is 10-40 fold more potent than sildenafil for PDE5 inhibition and a smaller clinically administered dosage for treatment of erectile dysfunction (Saenz de Tejada et al., 2001; Corbin et al., 2004; Setter et al., 2005; Supuran et al., 2006; Mehrotra et al., 2007). Thus, the structural basis for their different biochemical and physiological properties has been a puzzle. The results of the early studies on the crystal structures of PDE5A isolated catalytic domain in complex with sildenafil and vardenafil showed the similar binding mode of these inhibitors, but did not entirely agree (Sung et al., 2003; Zhang et al., 2004). Vardenafil and sildenafil had the same extended configuration in the structures by Sung et al. (2003), in contrast to the folded configuration of both inhibitors in the report by Zhang et al. (2004) (Fig. 3). The different orientations of the piperazine tails of the inhibitors appear to result from the rotation of the single C-S bond. Although the energy barrier for the single bond rotation is minimal in theory, it is rare that the same inhibitors adopt different conformations when bound to their receptors. Since the PDE5A catalytic domain used in Sung's study (2003) is basically inactive and the Zhang's structure (2004) contains a chimeric replacement of the PDE5A H-loop with the PDE4D H-loop, the biologically relevant configurations of these inhibitors is in question. The configuration of vardenafil in our structure is similar to that in the structure reported by Sung et al. while our sildenafil configuration is similar to that reported by Zhang et al. Since our PDE5 protein is fully active and contains all native PDE5 residues, the configuration difference between vardenafil and sildenafil is likely to be relevant to the biological and physiological properties of these inhibitors.

Our previous studies have already shown that the H-loop of PDE5A is highly flexible and can adopt different conformations and positional locations upon binding of the inhibitors (Wang et

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al., 2006). This study adds significant new structural information pertaining to the interactions between PDE5 and vardenafil. While it is not clear if the individual conformation of the H-loop can be exploited for design of new PDE5 inhibitors, the flexibility of the H-loop is likely to be an important allosteric mechanism that impacts substrate and inhibitor binding. The isolated PDE5A1 catalytic domain showed similar binding affinity with vardenafil and sildenafil and the vardenafil affinity is significantly boosted by the involvement of GAF-B of PDE5A1 regulatory domain (Blount et al., 2006). This implies that the unique features associated with the interaction of each of these inhibitors with the PDE5 catalytic domain are likely to play a major role in determining the influence of the regulatory domain on inhibitor affinity. A full understanding of the molecular effects of these inhibitors will require further structural study within the context of the PDE5 holoenzyme.

It is interesting to note that the PDE4-selective inhibitor rolipram caused relocalization of the full-length PDE4A4 and was suggested to trigger a conformational change on a loop interacting with Mg^{2+} (Terry et al., 2003). Besides, the binding of cAMP to the GAF domain of trypanosome PDEB1 (Laxman et al., 2005) and cGMP to the GAF domain of human PDE5 (Zoraghi et al., 2005) induced the allosteric conformational changes of the enzymes. These observations, together with the structural study done here, suggest that conformational changes promoted by the binding of inhibitors and substrates in the certain PDE families are essential for the regulations such as phosphorylation of PDE4 and cGMP/cAMP binding to GAF domains of PDEs. The H-loop of PDE5 likely serves as a key mediator of the allosteric regulation of enzymatic activity.

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FOOTNOTES:

a) This work was supported by NIH GM59791 (HK) and by NIH DK58277 (SF).

b) The atomic coordinates and structural factors have been deposited into the Protein Data Bank with accession codes of 3B2R.

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FIGURE LEGENDS

Fig. 1. Chemical structures of PDE5 inhibitors. Sildenafil, vardenafil, tadalafil and udenafil are drugs for treatment of erectile dysfunction. IBMX is a non-selective inhibitor of most class I PDE families.

Fig. 2. PDE5 structures. (A) Structural superimposition between the complexes of PDE5-vardenafil and PDE5-sildenafil. The cyan ribbons represent their comparable structures. The H- and M-loops of PDE5-sildenafil are shown in gold. The H-loop in the PDE5-vardenafil structure is shown in green. The dotted lines represent the disordered residues in the H- and M-loops. (B) Interactions between vardenafil and PDE5A1 residues. (C) The electron density for vardenafil. The (Fo-Fc) map was calculated from the structure with omission of vardenafil and is contoured at 3 sigmas. (D) The superimposition of the metal-binding residues between PDE5A1-vardenafil (green) and PDE5A1-sildenafil (gold). The zinc and magnesium ions were drawn from the sildenafil complex, but were absent in the PDE5A1-vardenafil complex.

Fig. 3. Superimposition of PDE5A1-vardenafil (green and cyan ribbons and green bonds) over PDE5A1-sildenafil (golden ribbons and blue bonds). As the consequence of the different positions of both H-and M-loops between the PDE5A1 complexes of vardenafil and sildenafil, Tyr676, Cys677 and Ile680 interact with vardenafil, while Asn662, Ser663, and Leu804 interact with sildenafil. (B) Superposition of vardenafil (green) over sildenafil reported early by our group (Wang et al., 2006). (C). Superposition of sildenafils reported by us (green, Wang et al. 2006), Zhang et al. (2004, gold) and Sung et al., (2003, cyan). (D) Superposition of vardenafils from the same three groups.

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Table 1. Statistics on diffraction data and structure refinement

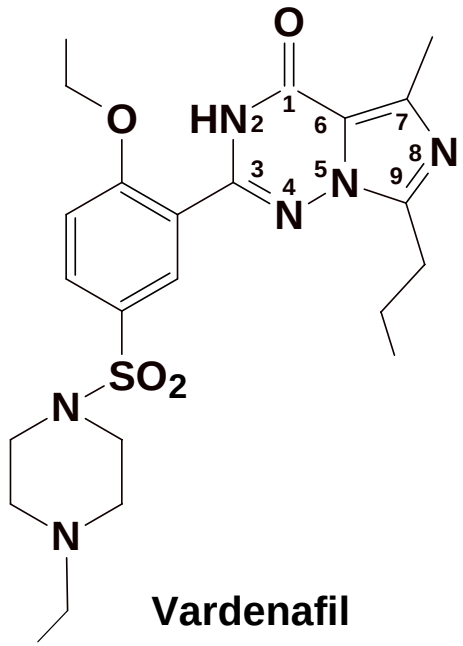
Data collection	
Space group	$P2_12_12_1$
Unit cell (*a, b, c,* Å)	68.9, 87.8, 138.5
Resolution (Å)	2.07
Unique reflections	47,379
Fold of redundancy	12.9
Completeness (%)	91.1 (48.2)*
Average I/σ	12.0 (3.6)*
Rmerge	0.051 (0.28)*
Structure Refinement	
R-factor	0.216
R-free	0.248 (10%)‡
Resolution (Å)	30-2.07
Reflections	45,502
RMS deviation for	
Bond (Å)	0.006
Angle	1.1^o
Average B-factor ($Å^2$)	
Protein	43.4 (4720)§
Vardenafil	35.2 (68) §
Waters	40.3 (183) §

*The numbers in parentheses are for the highest resolution shell.

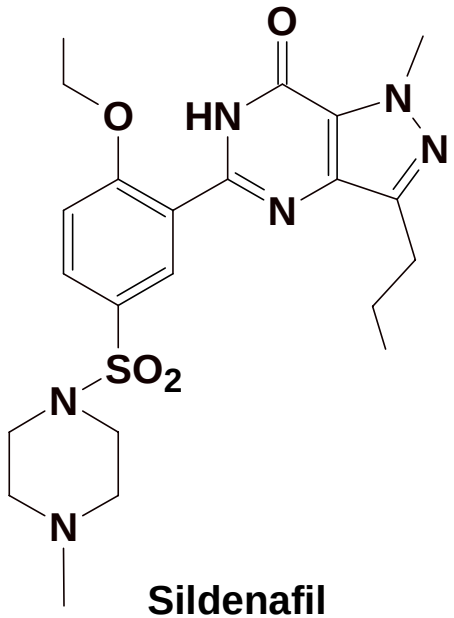
‡The percentage of reflections omitted for calculation of R-free.

§The number of atoms in the crystallographic asymmetric unit.

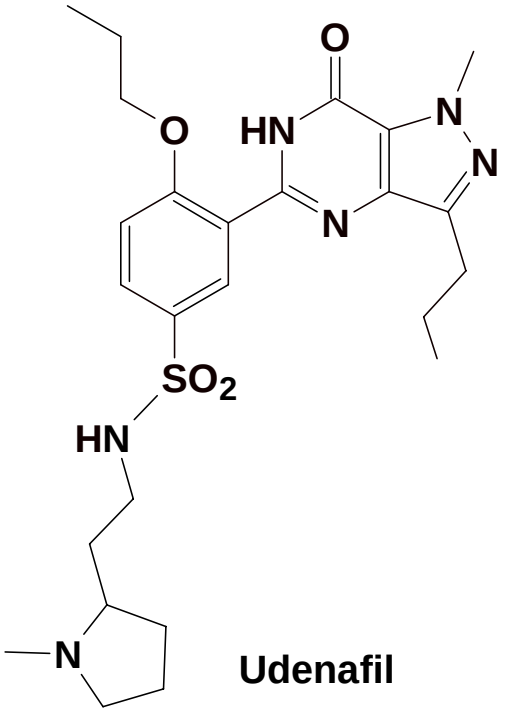
Figure 1



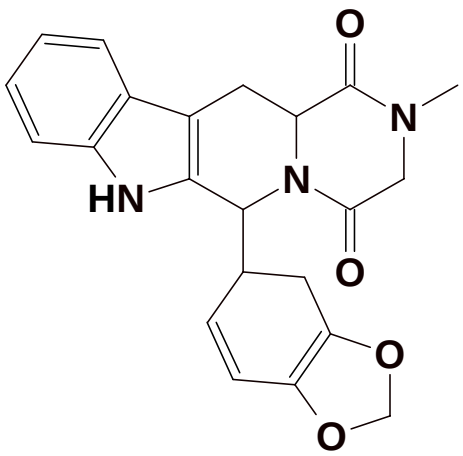
Vardenafil



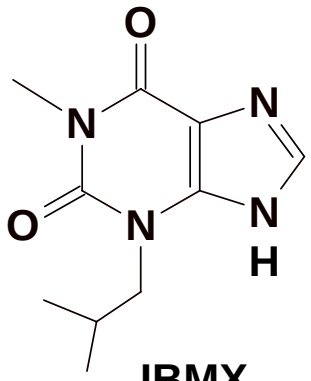
Sildenafil



Udenafil



Tadalafil



IBMX

Figure 2

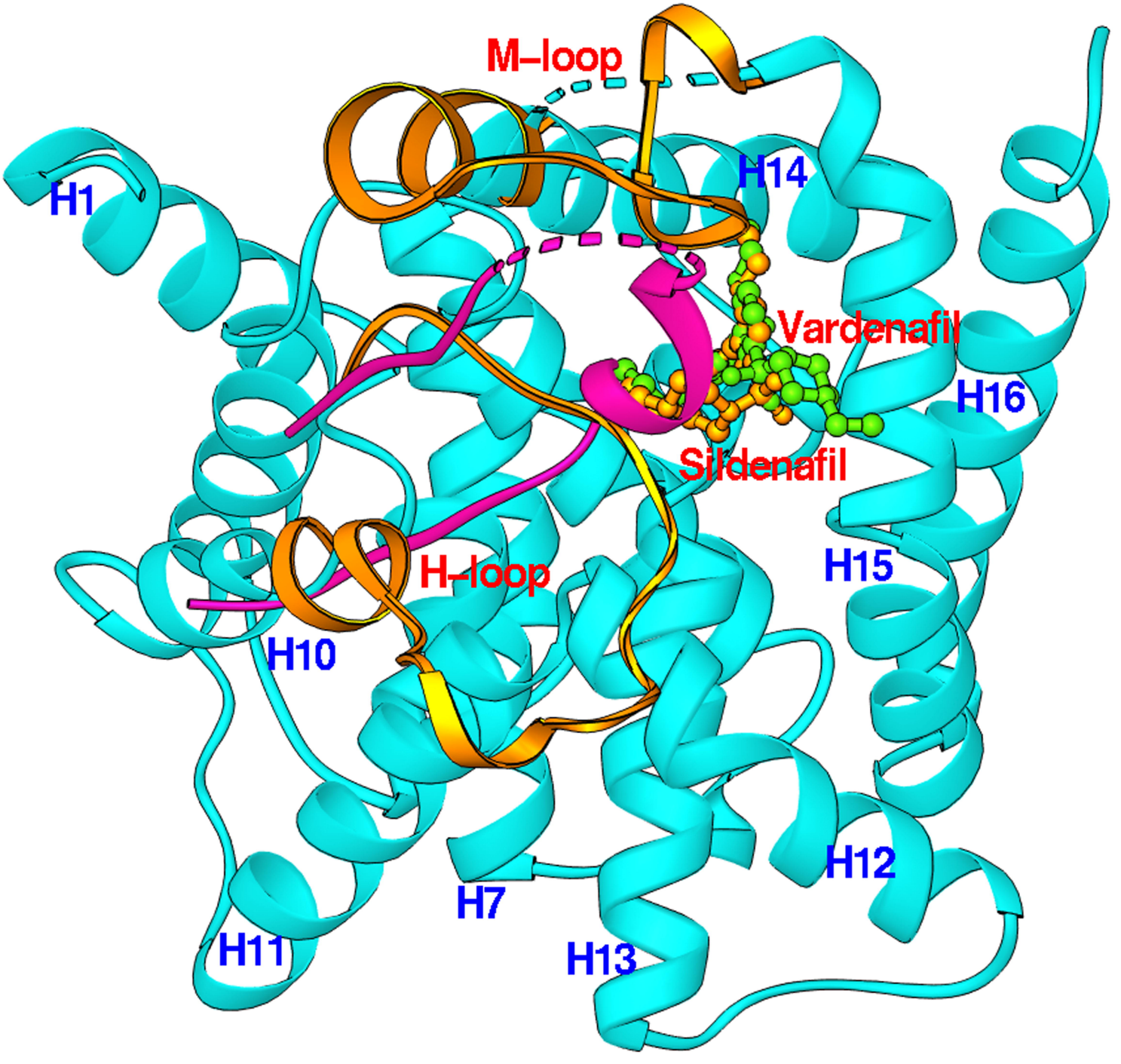
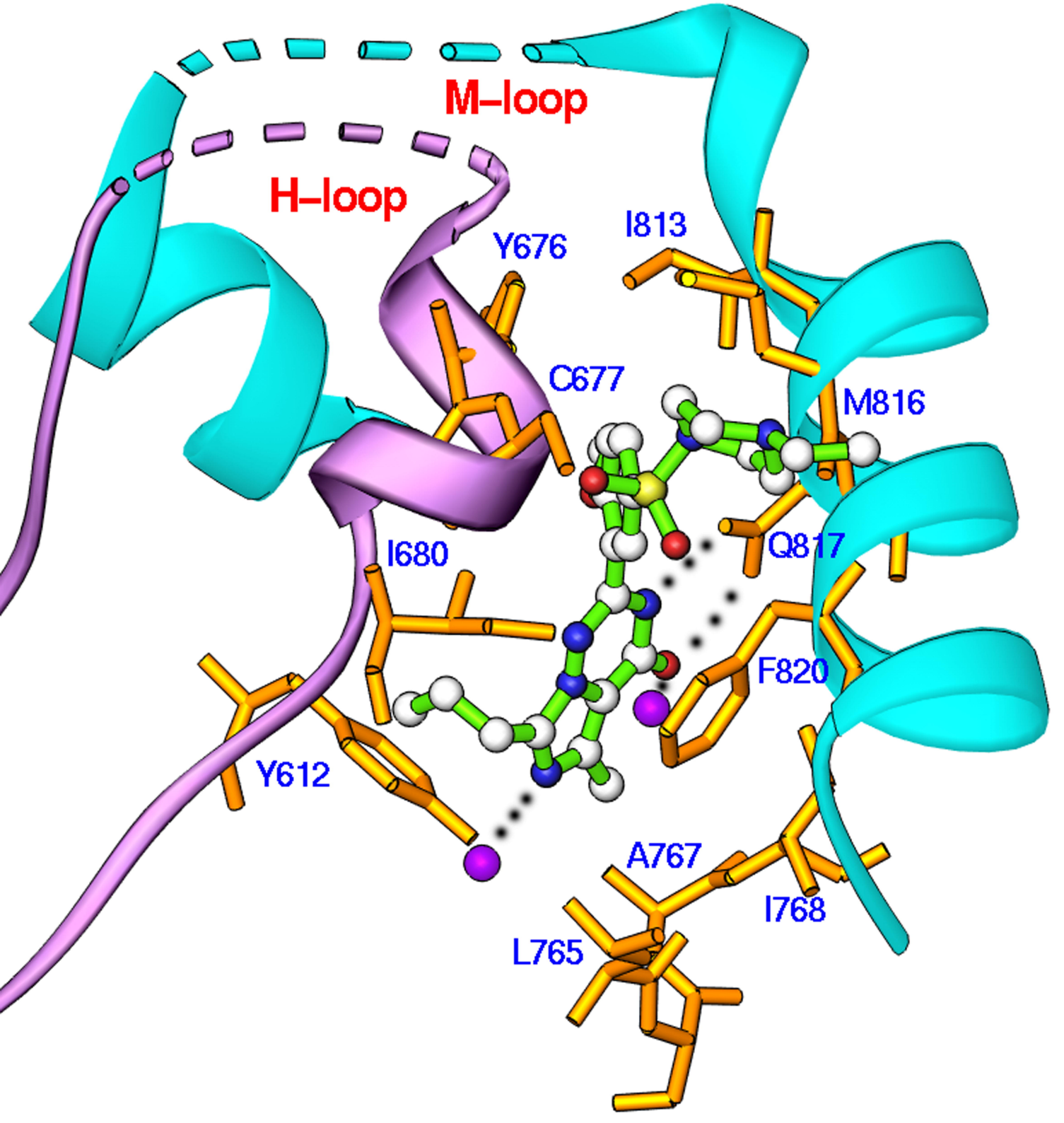

M–loop
H1
H14
Vardenafil
H16
Sildenafil
H–loop
H15
H10
H12
H7
H11
H13

Figure 2


M–loop
H–loop
Y676
I813
C677
M816
Q817
I680
F820
Y612
A767
I768
L765

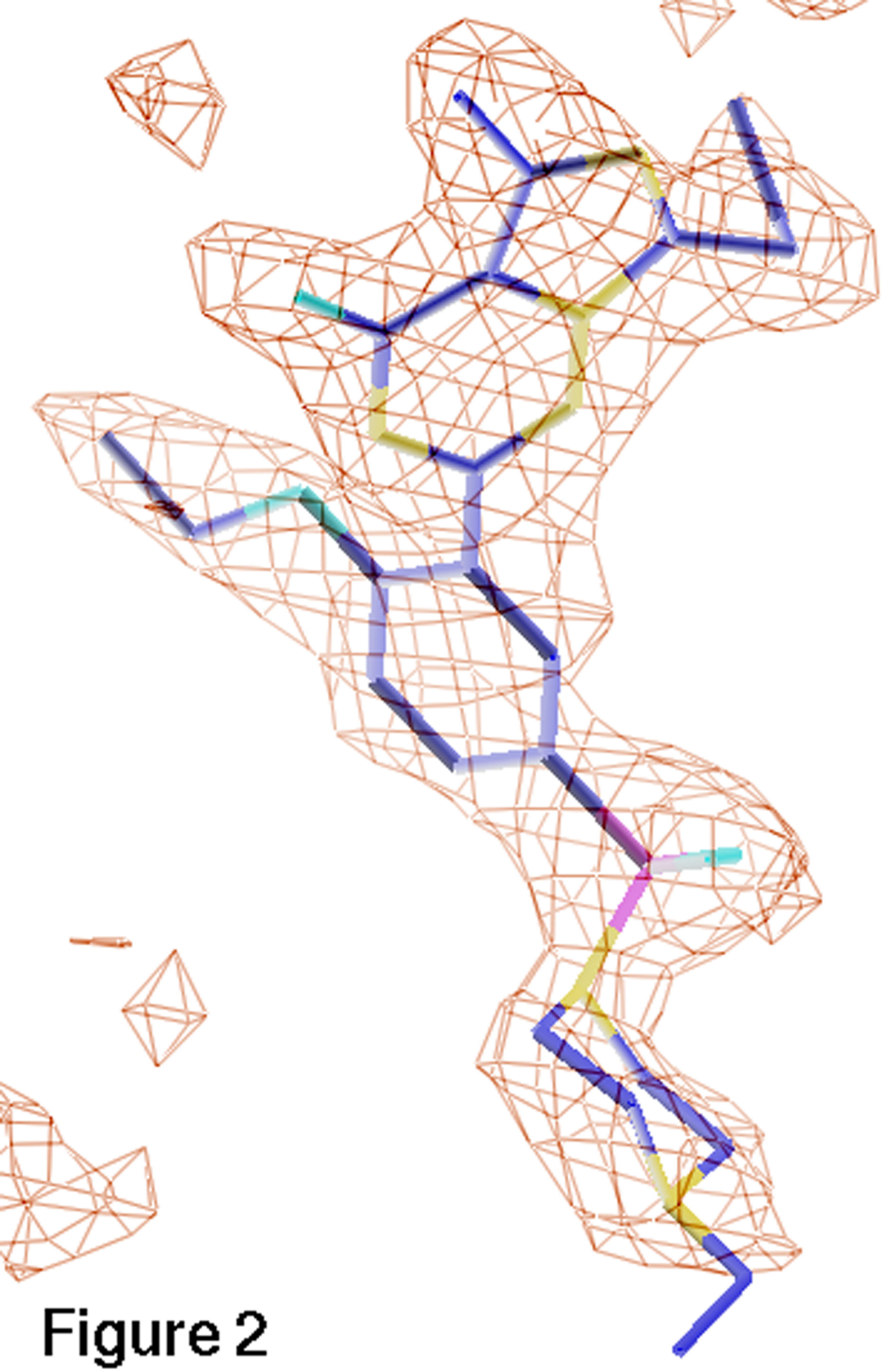


Figure 2

Figure 2

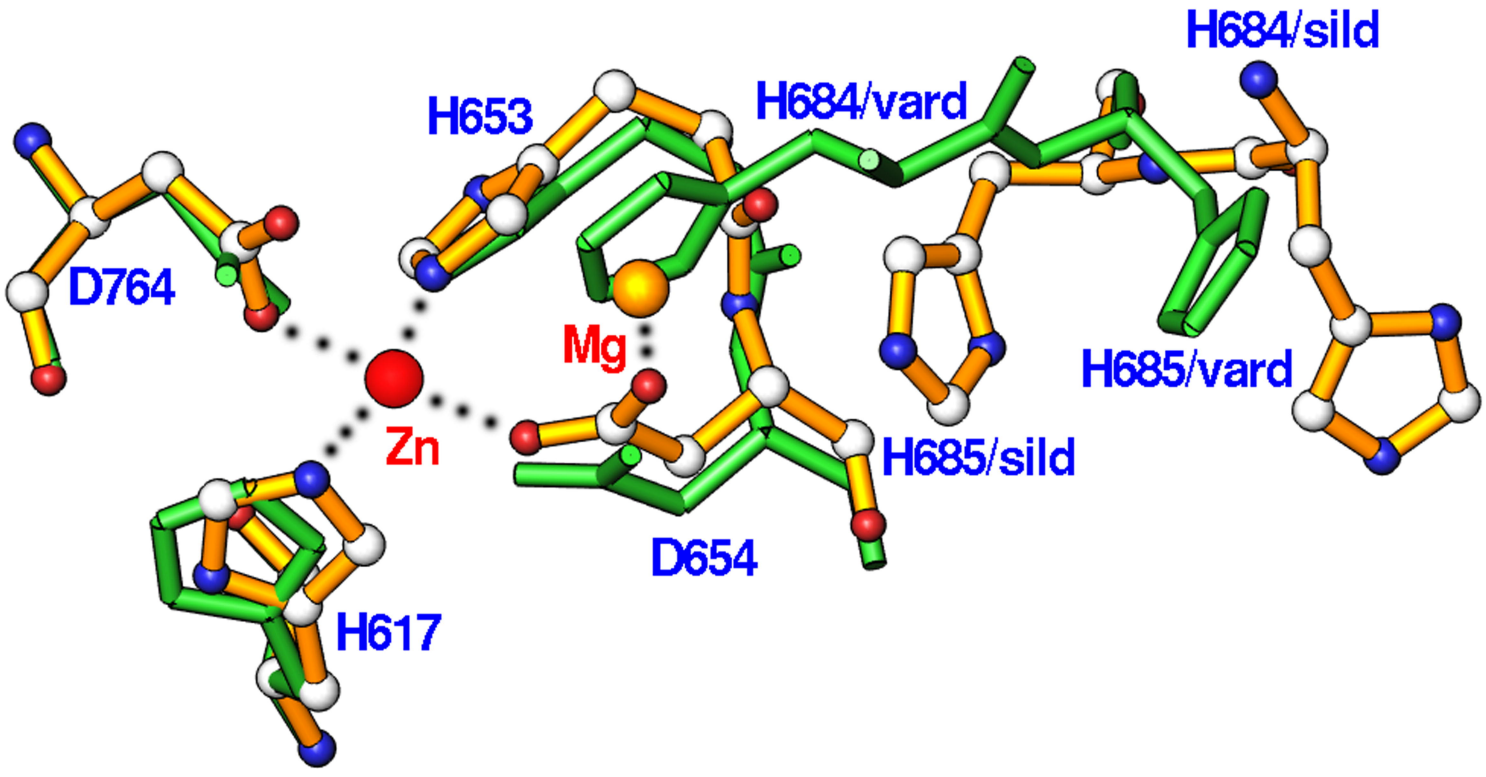

H684/sild
H653
H684/vard
D764
Mg
H685/vard
Zn
H685/sild
D654
H617

Figure 3

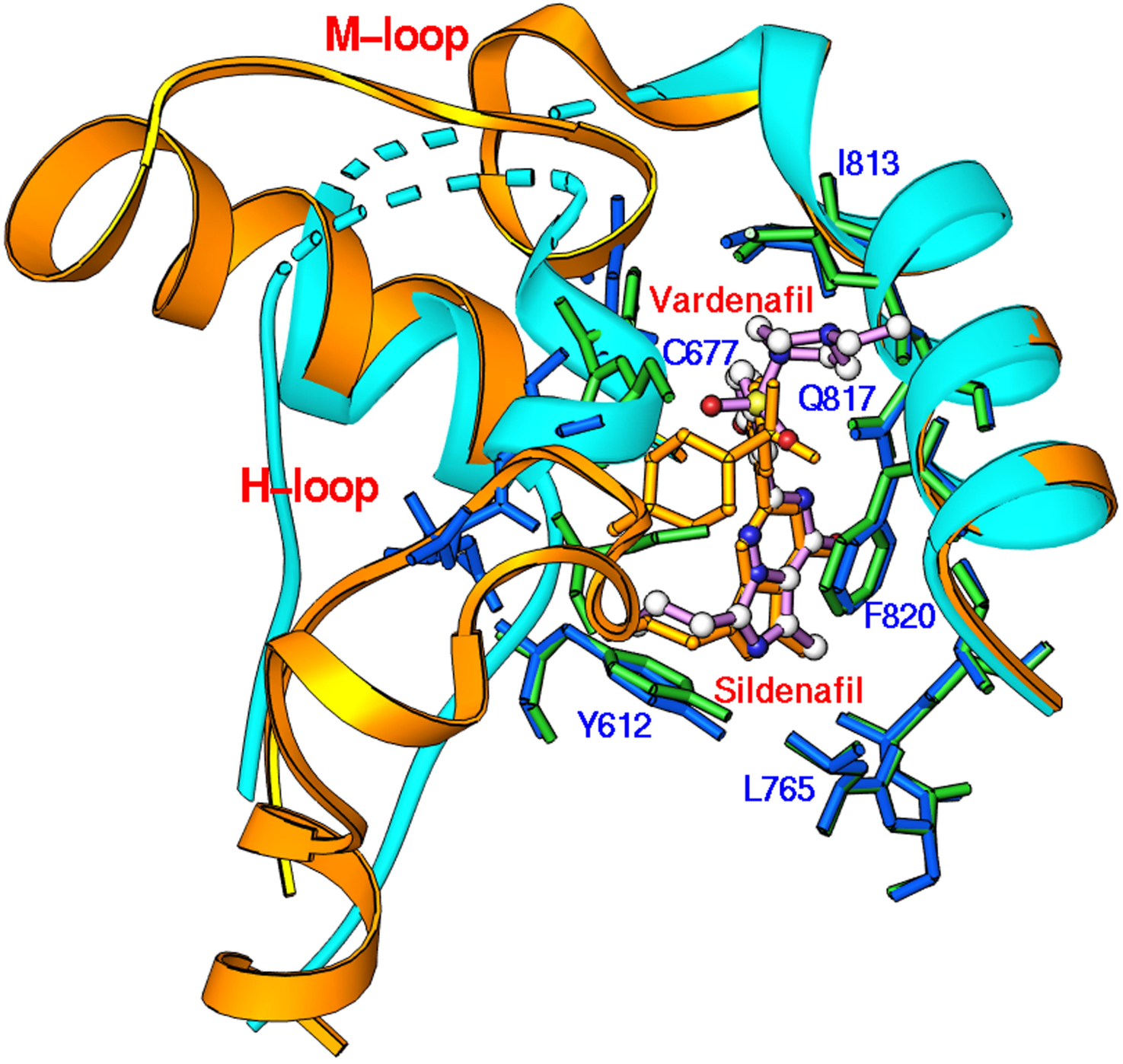

M–loop
I813
Vardenafil
C677
Q817
H–loop
F820
Sildenafil
Y612
L765

Figure 3

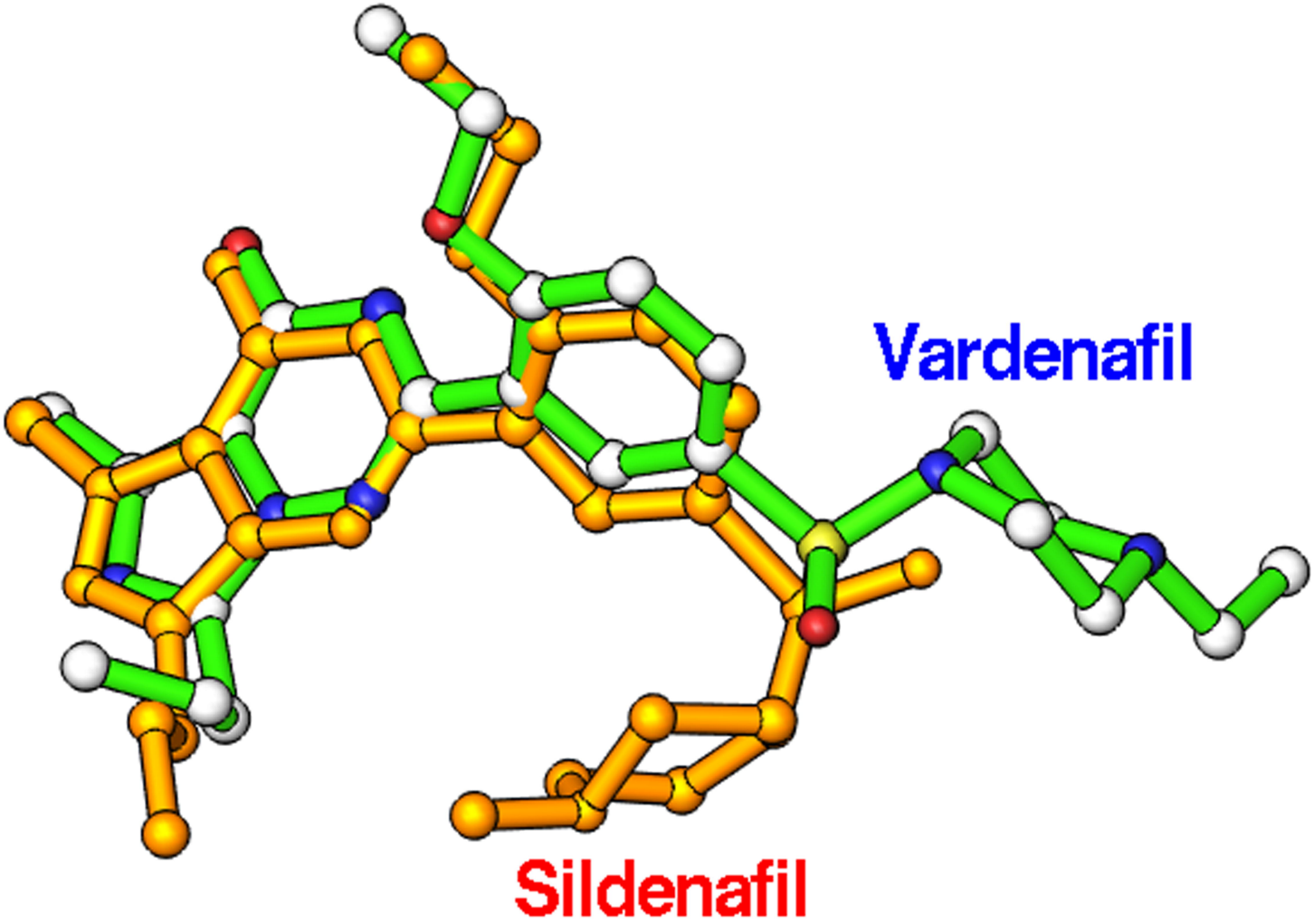


Figure 3

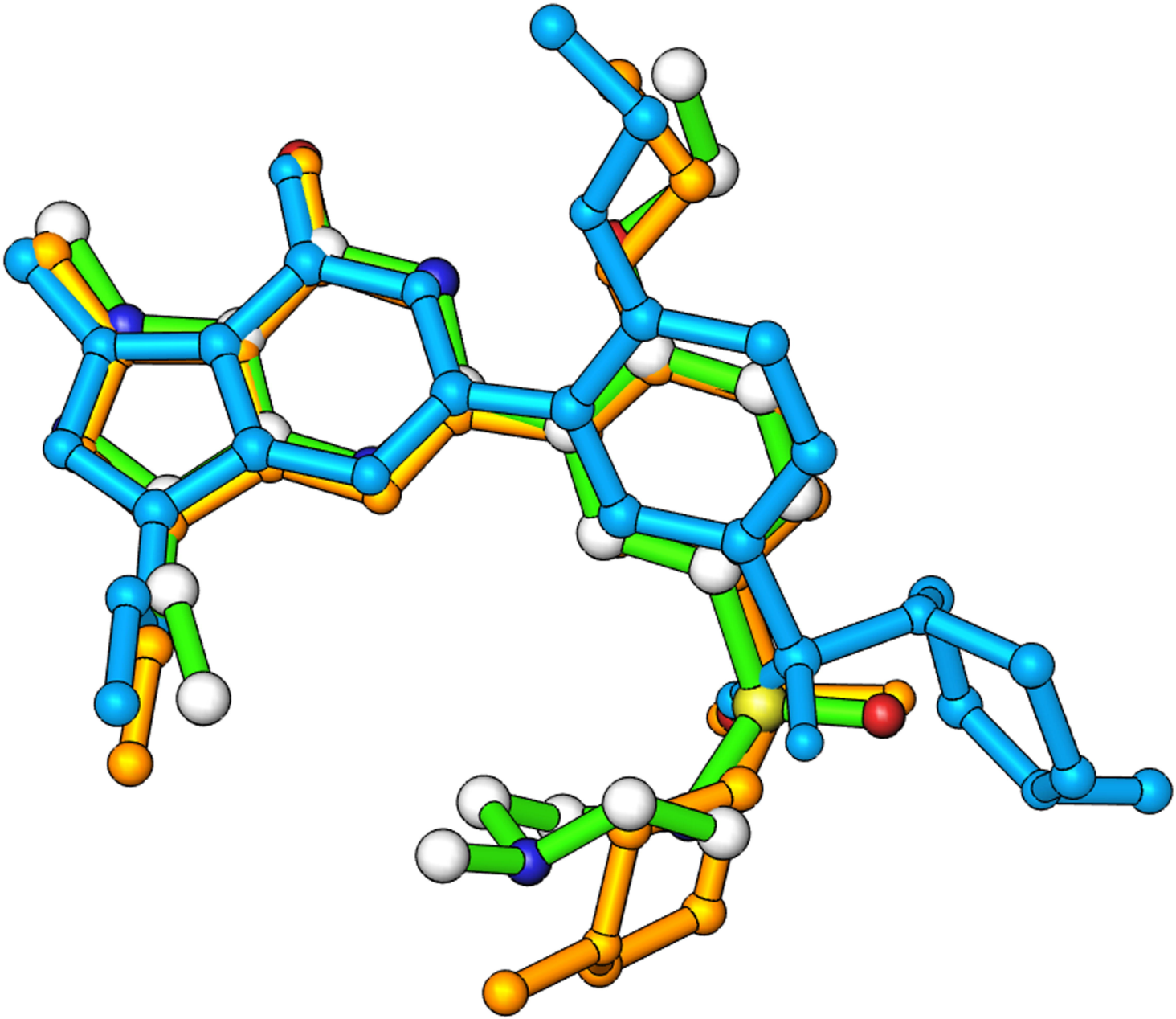


Figure 3