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# Molecular docking and ADMET studies of the interaction of 4-carboxyl-2,6-dinitrophenylazohydroxynaphthalenes with bovine serum albumin

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**Abstract**

Previous spectrophotometric investigations revealed strong binding affinities between four potential monoazo colourants (code-named AZ-01 to 04) and bovine serum albumin (BSA) which could dictate the tissue distribution and toxicity of the additives. The molecular docking interactions of the dyes with BSA were analyzed using AutoDock vina and PatchDock in order to elucidate the functional groups involved in complex stabilization. Docking conformations confirmed the ligands preferentially inserted into the hydrophobic cavities of BSA site I. Structure-BSA binding relationships revealed the binding of AZ-02 was driven by hydrogen bond donation from its free *p*-hydroxynaphthalene substituent to Ser-479 while the predominantly hydrazone form of its positional isomer, AZ-01, increased its lipophilicity and tendency for hydrophobic interactions. The relatively higher C/H ratio of AZ-03 and -04, which contain additional C-7 substituents, was responsible for their stronger binding and the extensive involvement of their aromatic rings in ligand-site I complex stabilization *via* Pi-Pi T-shaped, Pi-alkyl and alkyl-alkyl interactions. Moreso, AZ-01, -03 and -04 exist predominantly as hydrazone tautomers with an overall positive charge which provided complementary modes for interaction with negatively charged aspartic and glutamic acids. The structure-BSA binding relationships of the molecules, which can be employed in synthesis of safer congeners, have been elucidated.

*Keywords*: Binding interactions, Autodock vina, PatchDock, Azo dye, ADMET predictions



## INTRODUCTION

Serum albumin is the predominant transporter protein in the circulatory system. It plays decisive roles in the distribution, metabolism and potential toxicity of many endogenous and exogenous ligands [1]. Extensive binding to plasma proteins can decrease plasma concentration of an unbound drug while weakly bound drugs usually have short duration of action [2]. It is therefore often necessary to investigate the extent of binding of new chemical entities (including food and drug additives) with serum proteins such as bovine serum albumin or human serum albumin [3]. Of the two proteins, bovine serum albumin (BSA) because of its structural homology with human serum albumin (HSA) and the added advantages of low cost and availability has been the most extensively used in binding investigations [2, 4]. Many of such investigations when studied with UV, fluorescence and IR spectroscopy employ

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perturbations in the response parameter of the ligand in the presence of albumin to characterize the interaction [1, 2, 5, 6]. In addition, the recent elucidation of the crystal structure of BSA makes possible the semi-empirical study of ligand-albumin complexation which can provide useful complimentary insight to experimental findings [7]. In particular, molecular docking studies can decipher predominant and other modes of interaction as well the subdomain of BSA in which binding is energetically favoured. Bovine serum albumin comprises 583 amino acid residues and consists of three homologous helical domains, I, II and III which are hydrophobic cavities that can embed almost all of the amino acid residues [1]. Each of the domain consists of two sub domains (A and B) with subdomains IIA, IIIA and IB known as Sudlow's site I, II and III respectively. The main binding sites of BSA for different types of chemical molecules have been identified as subdomain IIA and IIIA both of which contain tryptophan residues [2].

While intensive multi-faceted approaches are often adopted to guarantee the safety of new chemical entities being developed as medicinal agents [3, 8, 9], less emphasis is usually given to the myriads of chemical additives/excipients that are co-formulated and inevitably co-administered with active pharmaceutical ingredients [10, 4]. As a result, despite safety concerns, synthetic food dyes, mostly of the azo chemical class, have continued to be used to improve the elegance of pharmaceuticals and processed foods. There is therefore a need for the continuous multi-technique investigation of the safety of existing and new chemical food/drug additives as they can be equally harmful due to possibility of bioaccumulation when taken over long periods as it is likely when co-formulated with medicines required for management of chronic diseases [10]. Recently, the interactions between a group of 4-carboxyl-2,6-dinitrophenylazohydroxylnaphthalenes (which are being developed as potential food/drug additives) and bovine serum albumin were investigated using UV spectroscopy [11,12]. While very useful conclusions were made from that study, a deeper understanding of the ligand-protein complexation process and the factors contributory to its stabilization are necessary. This understanding is not only useful in predictive toxicology but can be employed in lead optimization and the synthesis of congeners with improved safety properties. The objective of this study was therefore to employ an *in-silico* docking approach to provide a molecular-level understanding of the azo dyes-BSA interactions with the elucidation of the specific chemical functional groups and amino acid residues involved in their complex stabilization. In addition, the absorption, distribution, metabolism, excretion and toxicity (ADMET) profiles of the four ligands were compared.

## EXPERIMENTAL METHODS

**Ligands and protein used.** The test compounds were four congeneric monoazo dyes, 4-carboxy-2,6-dinitrophenylazohydroxynaphthalenes code named AZ-01, AZ-02, AZ-03 and AZ-04 that were previously synthesized and used for binding interactions experiments with BSA [11,12]. The structure of the compounds were built with Marvin sketch and then saved in *mol* format. The structures of warfarin and ibuprofen, used as standard site I and II markers respectively, were obtained from PubChem. Bovine serum albumin (PDB ID: 3V03) was identified from literature [1,13] and its crystallographic structure was downloaded from the RCSB protein data bank. Albumin PDB ID: 3V03, originally isolated from *Bos taurus*, was selected for this study as its crystal structure is available in very high resolution of 2.7 Å and has thus been widely used in similar docking studies [1,3,14]

**Molecular docking with PyRx.** The library of ligands (four test and two standard compounds) was imported into PyRx, minimized and then converted to dockable format using the Open Babel plugin [15]. The crystal structure of the protein was loaded into Chimera 1.14 software [16]. All complexed ions and non-standard amino acid residues were deleted before the protein was minimized at 200 steepest descent steps, 0.02 steepest descent steps size, 10 conjugate gradient steps, 0.02 conjugate gradient steps size and 10 update intervals. Solvents were thereafter removed before the addition of hydrogen bonds and the assignment of charges using the Gasteiger force field. The pdbqt formats of the prepared ligands and protein were loaded into PyRx for docking. The active sites of bovine serum albumin were identified using Computed Atlas of Surface Topography of Proteins, CASTp 3.0 [17]. For the recognition of the two active sites in BSA, the settings of the grid box used for molecular docking were defined as indicated in Table 1. The same grid box parameters were used for all the ligands. For each of the ligand, the maximum eight exhaustive conformational interactions with each binding sites of the protein was carried out using the AutoDock Vina of PyRx software.

**Molecular docking with PatchDock.** Molecular docking was also done using the PatchDock web server [18,19]. The ligands were minimized using UFF force field in Avogadro and then saved as PDB files. The protein was prepared by deleting chain A, all non-standard amino acids, water molecules and co-complexed ligands. The prepared molecules were uploaded into PatchDock and site-directed docking was carried out by specifying the amino acid residues at subdomains IIA and IIIA of albumin obtained from CASTp 3.0. The clustering RMSD was set at 1.5 for protein-small ligand interactions.

Post docking analysis and visualization of output files were carried out using Chimera 1.14 and Biovia Discovery Studio 2020 visualizer [20].

**ADMET prediction.** The pharmacokinetics and pharmacodynamics parameters of the four monoazo compounds were analysed using the ADMETsar 2 server [21]. For this purpose, the SMILES formats of the ligands were generated using Marvin sketch. For each ligand, the number of violations of the Lipinski's rule of 5 and the metabolic promiscuity based upon the Cytochrome P450 modes were also predicted.

## RESULTS AND DISCUSSION

The structures of the four test ligands are presented in Figure 1. Three of the dyes (AZ-01, -03 and -04) possess a hydroxyl group at *ortho* position to the common azo linkage while the hydroxyl group of AZ-02 is *para* to its azo bond. In addition, there are C-7 substituents of propionic acid and butanone on the naphthalene skeletons of AZ-03 and AZ-04 respectively. It is anticipated that the structural differences of the congeners will influence the type, extent and magnitude of their binding with serum albumin.

Molecular docking results revealed that the ligands preferentially interacted with different amino acid residues in either of the two active sites in BSA *via* a variety of modes of interactions including polar, ionic and hydrophobic forces. When incorporated into polypeptide chain as found in BSA, the amino and carboxylic acid functional groups in amino acids lose their charges [22]. However, five of the 20 common amino acids have side chains that can be charged. Thus, at physiological pH, glutamic and aspartic acids which contain acidic side chains are negatively charged while the trio of lysine, arginine and histidine with basic side chains are positively charged. In addition, all five amino acids contain at least one hydrogen bond donor and/or acceptor atoms in their side chains and are therefore polar, hydrophilic and because of their charges, capable of ionic bonds *via* electrostatic attraction. Asparagine, glutamine,

serine, threonine and tyrosine are the remaining five amino acids with side chains that contain hydrogen donor and/or acceptor atoms though uncharged. These amino acids are therefore polar, hydrophilic (glutamine, asparagine) or neutral (serine, threonine, tyrosine) and frequently engaged in hydrogen bonds. Amino acids without hydrogen bond donor and/or acceptor atoms are non-polar and often interact *via* hydrophobic modes including Van der Waals forces [22].

**Comparative affinity of test ligands with standards.**

**Preferential binding modes of test ligands**

The four ligands and standards used as site markers were subjected to molecular docking analysis against the two active sites in BSA. Warfarin and ibuprofen were selected as BSA site I and II markers respectively based on several spectroscopic displacement studies that have used the two compounds as site probes as well as docking studies that have demonstrated the preferential binding of these compounds to the respective binding sites of BSA [3,14,23]. Using Autodock vina, the best conformations of the test ligands that occupied the same pocket as the standards were selected with their binding affinities reported in Table 2. The four test ligands showed higher binding affinities for site II than ibuprofen while only AZ-03 and 04 showed a higher affinity for site I than warfarin. The results revealed that the binding of the dyes to the site I of BSA was more energetically favourable compared to binding to site II.

Similar results were obtained with PatchDock which is based on a shape complimentary algorithm [18]. In PatchDock, ligand-protein solutions are ranked using scoring functions that include both geometric fit and atomic desolvation energies. As depicted in Figure 2, the four test ligands bound preferentially with site I of albumin and with energies that were greater than those obtained with warfarin, the site marker. In addition, the site I complexes of AZ-03 and AZ-04 were better stabilized than those of the positional isomers, AZ-01 and AZ-02. Expectedly, the approximate interface area of the complexes of AZ-01 and 02, being positional isomers, were very similar.

Thus, ligand-site I complexes were better stabilized and may show prolonged storage periods as the stronger binding affinity is an indication of increased albumin solubilization that generally increases body distribution of xenobiotics and their access to storage site [24]. More importantly though, preferential binding of the ligands to site I might be a pointer to possible clinically relevant additive-drug interactions arising from the competitive displacement of bound drugs from the same binding site. This is particularly remarkable for AZ-03 and AZ-04 which showed higher binding affinities for site I than warfarin in both PyRx and PatchDock simulations. A comparison of the binding conformations of warfarin (a site I marker) and the monoazo ligands within site I of BSA are depicted in Figure 3.

As shown in Figure 3, although the ligands inserted into the hydrophobic cavities of BSA, they were surrounded by amino acid residues of varying polarity, hydrophobicity and overall charges. The intermolecular forces of interaction and the amino acid residues involved in the formation and stabilization of ligand-BSA site I complexes are depicted in Table 3. A comparison of the intermolecular forces employed by warfarin, known to bind preferentially to BSA site I marker were also included.

The docked conformations of AZ-01 and 02 within site I revealed both ligands were surrounded by amino acid residues such as Phe-205, Arg-208, Ala-209, Ala-212, Leu-346, Ala-349, Lys-350, Glu-353, Glu-357, Ser-479 and Leu-480. The nitro groups of AZ-01 were involved in hydrogen bond formation with side chains of Arg-208, Lys-350 and Leu-480 at bond distances of 2.287, 2.150 and 2.228 Å respectively. In contrast, the

carboxylic acid and naphthol substituents in AZ-02 dictated its complex formation as they were involved in hydrogen bond formation with Glu-357, Ser-479 and Leu-480. Also present in the close vicinity of the ligands were positively charged hydrophilic amino acids such as Lys-350 and negatively charged amino acids such as Glu-353, Glu-357 which could interact with the nitro and azo functional groups respectively on the ligands. Furthermore, the amino acid residues surrounding the docked conformations of AZ-03 and AZ-04 in the hydrophobic cavity of Site I included Phe-205, Arg-208, Ala-209, Ala-212, Leu-326, Leu-330, Leu-346, Ala-349, Lys-350, Glu-353, Glu-478, Leu-480, Val-481, Ser-479. Analysis revealed hydrogen bond interactions between the oxygen atom of the phenyl nitro groups of both ligands and the side chains of Arg-208, Lys-350 and Leu-480. Hydrogen bond interactions of AZ-03 occurred with bond distances of 2.453 Å (Arg-208), 2.118 Å (Lys-350) and 4.390 Å (Leu-480) while bond distances of 2.193 Å (Arg-208), 2.107 Å (Lys-350) and 4.540 Å (Leu-480) were observed with AZ-04. Lysine and arginine are very basic with pKa of 10.5 and 12.5 respectively [22]. In particular, the positive charge on the arginine side chain is stabilized by resonance and thus the amino acid is sufficiently basic to abstract proton not only from the propionic acid substituent of AZ-03 (as shown in Figure 3c) but also from the less acidic butanone residue of AZ-04 as shown in Figure 3d. In addition to the hydrogen bond donor-acceptor interactions so described, several hydrophobic amino acids including Ala-209, Ala-212, Leu-330, Leu-346, Ala-349, Lys-350 could be observed in an extensive network of alkyl interactions involving the naphthalene and propionic acid residues of AZ-03. Less extensive alkyl interactions were observed with AZ-04. Similarly, few charged amino acid residues such as Lys-211, Lys-350, Arg-208 and Glu-353 could be observed in the close proximity of both ligands in their binding pockets.

**Structural considerations in binding affinities and complex stabilization.** The binding patterns revealed some useful generalizations that can be attributed to the chemical structures of the test ligands and their overall hydrophilicity-lipophilicity balance. AZ-01 and 02 are more polar than the pair of AZ-03 and AZ-04 both of which contain additional propionic acid and butanone substituents respectively at C-7 position of their naphthalene residues. The higher C/H ratio and the associated increased lipophilicity of the latter two congeners can explain their relatively stronger binding affinities and the extensive involvement of their aromatic rings in ligand-site I complex stabilization *via* Pi-Pi T-shaped interactions with phenyl ring of Phe-205 and a complex network of Pi-alkyl interactions with Arg-208, Ala 209, Ala-212, Leu-346, Ala-349, Lys-350. In addition, alkyl interactions between the propionic acid substituent in AZ-03 and side chains of Ala-212, Leu-350 as well as carbon-hydrogen interactions between the butanone substituent of AZ-04 and side chain of Gly-327 were contributory to binding of the two molecules to Trp-213 active site.

AZ-01 and AZ-02 are isomers differing in only the position of the hydroxyl group relative to the azo linkage as shown in Figure 1. *Para*-phenylazonaphth-2-ols such as AZ-01 can undergo intramolecular hydrogen bonding involving *ortho* hydroxyl and nitrogen of azo linkage. The presence of a strong intramolecular hydrogen bonding and electron withdrawing nitro groups shifts equilibrium towards the keto form resulting in an increase in the ring skeleton of the molecule as shown in Scheme 1 [25].

**Table 1:** Parameters of grid boxes in molecular docking of standard and test ligands

| Parameter | Domains | |
|---|---|---|
| | Site I | Site II |
| Coordinates of centre | 109.57, 25.68, 21.02 | 75.17, 10.80, 6.59 |
| Size | 48.38, 53.59, 53.29 | 40.90, 36.01, 55.85 |

Site I is the subunit domain IIA of BSA that contain tryptophan 213 residue, Site II is the subunit domain IIIA of BSA that contain tryptophan 134 residue

**Table 2:** Binding affinities of standard and test ligands for BSA sites I and II

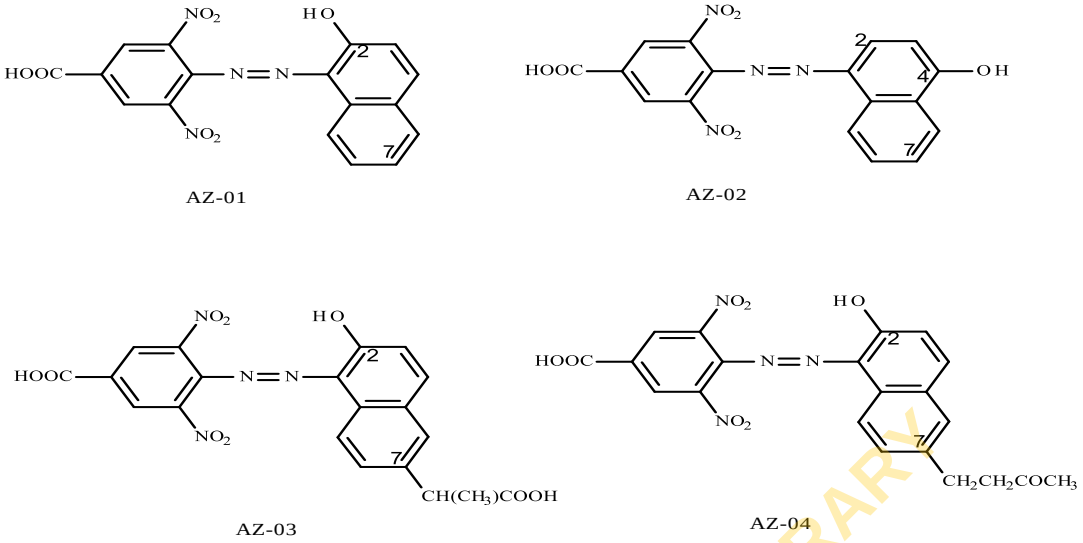
| Ligand | ΔG energy (kcal/mol) | |
|---|---|---|
| | Site I | Site II |
| AZ-01 | -7.4 | -7.3 |
| AZ-02 | -7.6 | -7.4 |
| AZ-03 | -8.7 | -8.4 |
| AZ-04 | -8.5 | -8.1 |
| Ibuprofen | | -7.2 |
| Warfarin | -8.2 | |

**Table 3:** Intermolecular forces and residues involved in Ligand-BSA site I complex stabilisation

| Intermolecular force | Interacting amino acid residues | | | | |
|---|---|---|---|---|---|
| | AZ-01 | AZ-02 | AZ-03 | AZ-04 | Warfarin |
| Hydrogen bond | ARG 208; LYS 350; LEU 480 | GLU 357; SER 479; LEU 480 | ARG 208; LYS 350; LEU 480 | ARG 208; LYS 350; LYS 480 | ARG 208; LEU 346 |
| Van der Waals | | LYS 350 | ALA 209; ASP 323 | GLY 327; PHE 205 | |
| Pi-Pi T shaped | PHE 205 | | PHE 205 | | |
| Pi-sigma | VAL 481 | | | ALA 212 | ALA 212 |
| Pi-alkyl | ALA 209; ALA 212; LEU 346; ALA 349 | LYS 350; VAL 481 | ALA 209; ALA 212; LEU 346; ALA 349; LYS 350 | ALA 209; ALA 212; LEU 346; ALA 349; LYS 350 | ARG 208; ALA 212; LEU 326; LEU 330; ALA 349 |
| Pi-cation | | | | | ARG 208; LYS 350 |
| Alkyl | | | ALA 212; LEU 330 | | |
| Attractive charge | | GLU 353 | | | |
| Unfavourable acceptor-acceptor | | | SER 479 | | |
| Unfavourable positive-positive | ARG 208; LYS 350 | LYS 350 | | | |

**Table 4:** ADMET properties of the test ligands

| ADMET Properties | | AZ-01 | AZ-02 | AZ-03 | AZ-04 |
|---|---|---|---|---|---|
| (a) Lipinski filter | Molecular weight | 382.29 | 382.29 | 454.35 | 452.38 |
| | AlogP | 4.48 | 4.48 | 4.66 | 5.0 |
| | H-Bond acceptor | 8 | 8 | 9 | 9 |
| | H-Bond donor | 2 | 2 | 3 | 2 |
| | Rotatable bonds | 5 | 5 | 7 | 8 |
| | Number of violations | 0 | 0 | 0 | 0 |
| (b) Other admetSAR parameters | Blood brain barrier | + | + | + | + |
| | Carcinogenicity (binary) | - | - | - | - |
| | CYP inhibitory promiscuity | - | - | - | + |
| | Human intestinal absorption | + | + | + | + |
| | Acute oral toxicity mg/kg | 1.97 | 1.51 | 2.43 | 2.62 |
| | Water solubility | -3.45 | -3.52 | -3.43 | -3.87 |




AZ-01: 4[(2-hydroxynaphthalen-1-yl)diazenyl]-3,5-dinitrobenzoic acid
AZ-02: 4[(4-hydroxynaphthalen-1-yl)diazenyl]-3,5-dinitrobenzoic acid
AZ-03: 4[(7(1-carboyethyl)-2-hydroxynaphthalen-1-yl)diazenyl]-3,5-dinitrobenzoic acid
AZ-04: 4[(2-hydroxy-7-(3-oxobutyl)naphthalen-1-yl)diazenyl]-3,5-dinitrobenzoic acid

**Figure 1:** Chemical structures of the test ligands

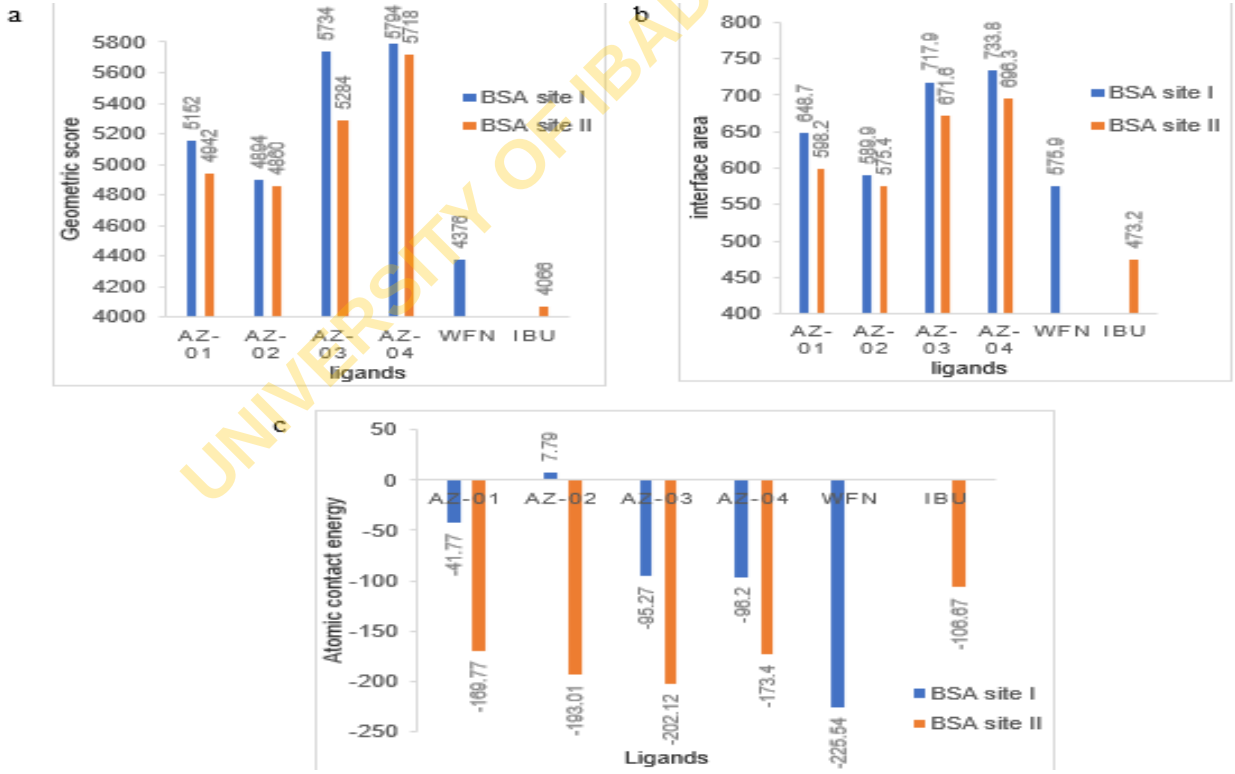
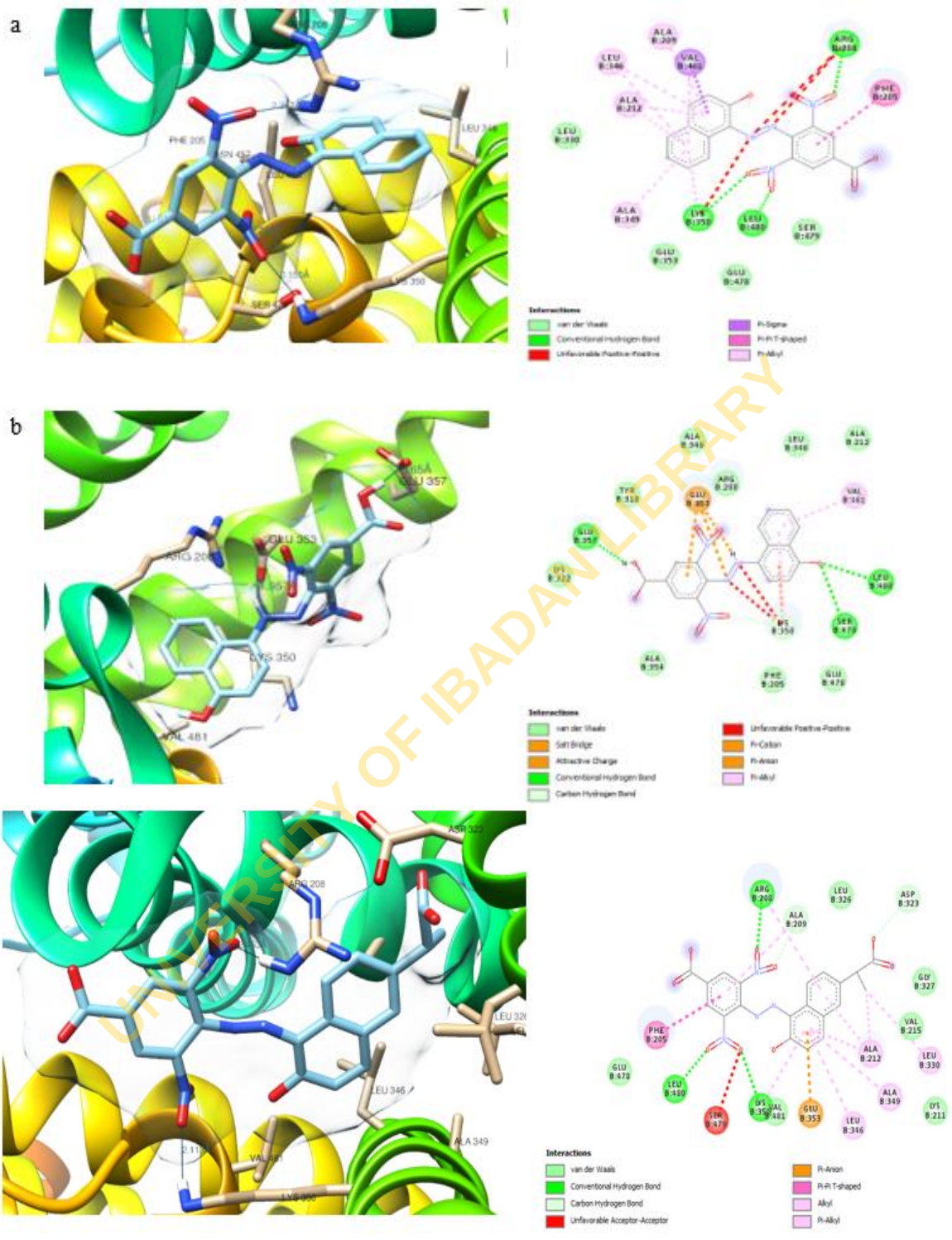



**Figure 2:** Docking analysis of test ligands, warfarin (WFN) and ibuprofen (IBU) using PatchDock



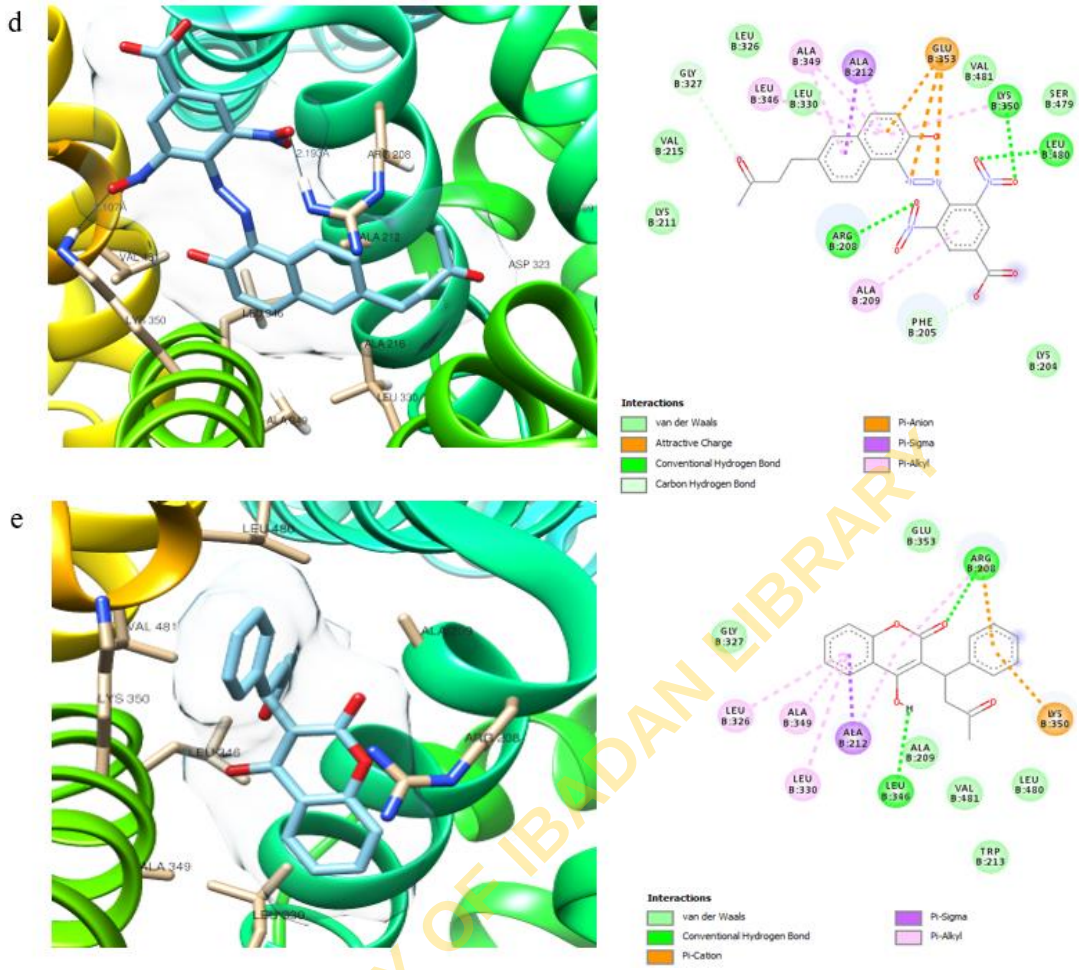
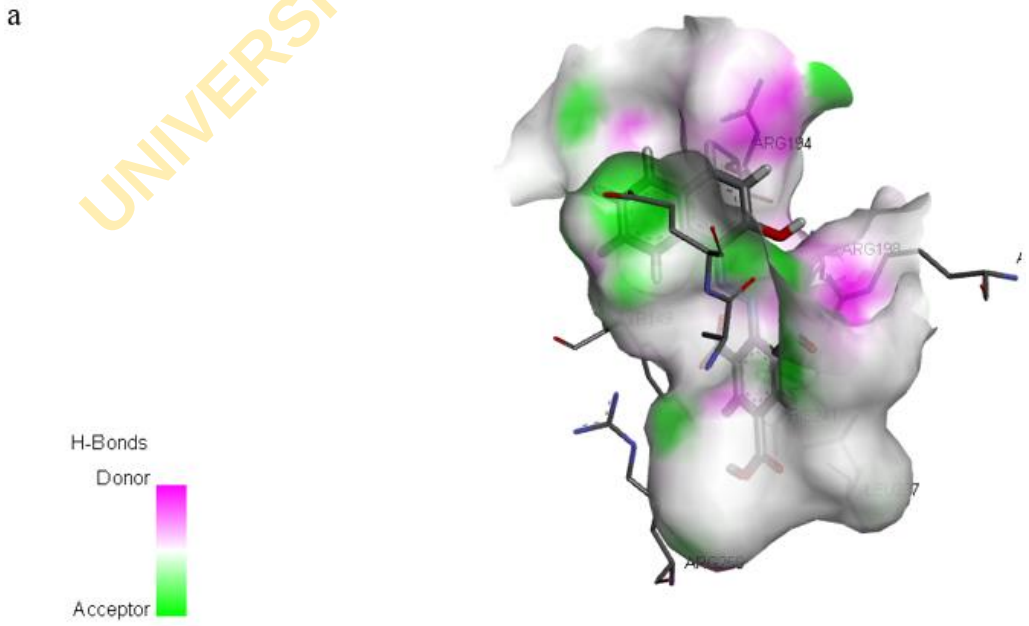



**Figure 3:** 3D (left) and 2D (right) views of molecular interaction of BSA site I with (a) AZ-01, (b) AZ-02, (c) AZ-03, (d) AZ-04 and (e) warfarin



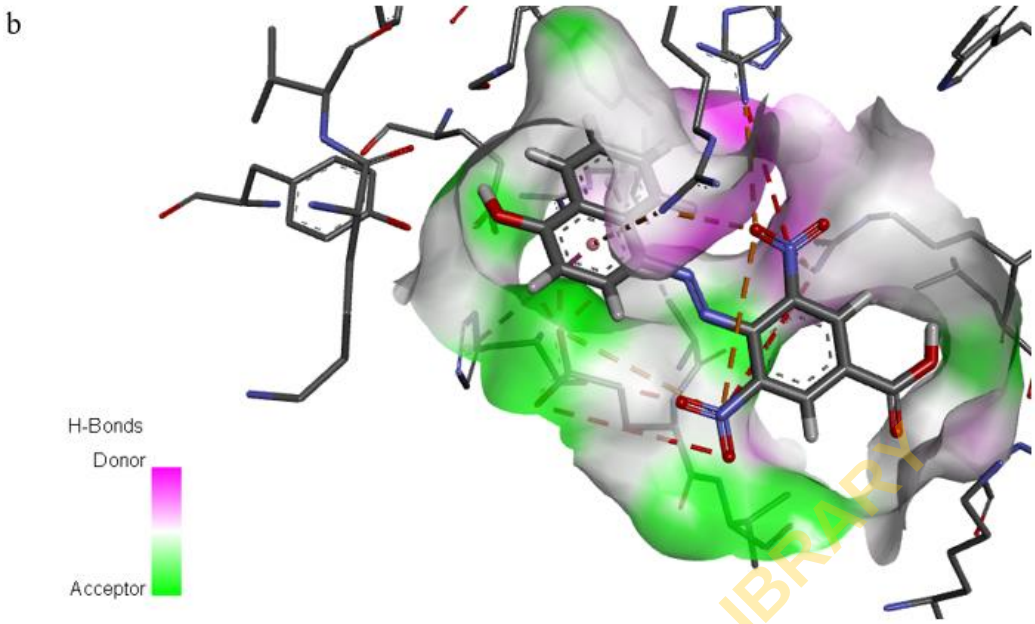
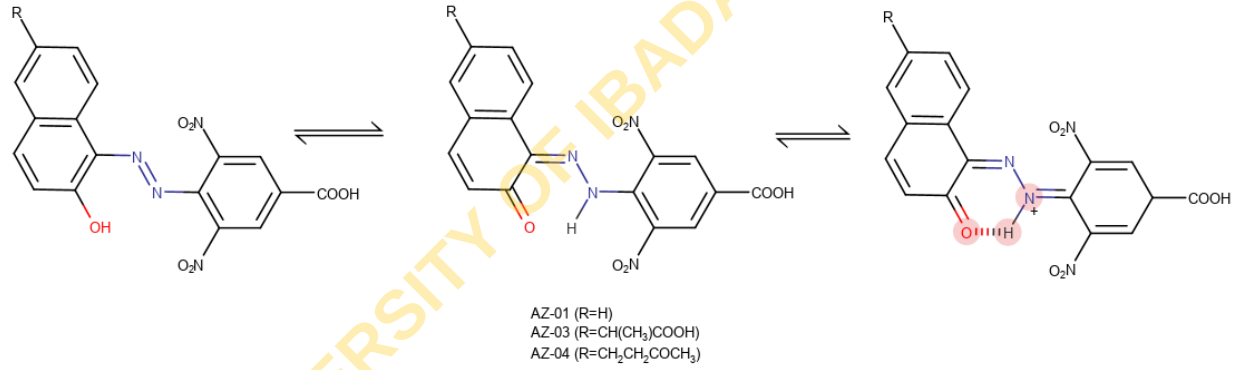



**Figure 4:** Hydrogen bond mapping of receptor surface showing involvement of the common hydroxyl groups of (a) AZ-01 and (b) AZ-02




**Scheme 1:** Azo-hydrazone tautomerism of 4-phenylazonaphth-2-ols

Conversely, 4-phenyl azonaphth-1-ols such as AZ-02 cannot undergo such rearrangement reactions. Thus, the *para* hydroxyl group in AZ-02 has been shown by previous NMR and solvatochromic studies to be free, labile and available for intermolecular interactions while the *ortho* hydroxyl group in AZ-01 is not available for such interactions because of its involvement in an intramolecular hydrazone formation [26]. This structural difference in the two isomers can therefore explain some of the differences in their binding energies and the substituents employed for complex stabilization. The formation of the hydrazone tautomeric form of AZ-01 will lead to an increase in the ring system of the molecule, its relative lipophilicity and an improved ability for hydrophobic interactions. Consequently, AZ-01 which exists predominantly in the more lipophilic hydrazone form, was involved in pi-pi stacked, pi-sigma and eight pi-alkyl interactions compared to only two pi-alkyl interactions that were observed with AZ-02. Additionally, the keto-enol shift of the isomers will dictate the substituents used in complex stabilization. Using PatchDock, the hydrogen bond mapping of the receptor surface revealed

an extensive involvement of the free hydroxyl group of AZ-02 as a hydrogen bond donor in complex stabilization (Figure 4). Similarly, the docked pose of AZ-02 at site I using AutoDock vina revealed a hydrogen bond donation from its free *p*-hydroxynaphthalene substituent to the hydroxymethyl group of Ser-479 (Figure 3b). A second hydrogen bond interaction involving the side chain of Leu-480 could also be observed. Expectedly, no such hydrogen bond involvement could be seen with the *ortho* substituted AZ-01 in either Figure 3a or 4.

As previously mentioned, the test ligands in their binding pockets were surrounded by amino acids of varying charges. This is as a result of different functional groups, present on the ligands, which can readily ionize at physiological pH to give charged ions that provide complimentary modes for interaction with charged amino acids in BSA. For example, AZ-03 and 04, which also contain *ortho* hydroxy groups, are capable of hydrazone formation in a similar manner as AZ-01 and indeed previous NMR investigation of the azo-hydrazone shifts of these molecules have shown that they also exist predominantly in the hydrazone form [12]. This tautomeric shift is associated with the development of an overall positive charge on the three molecules which provides a complimentary mode for their interaction with negatively charged aspartic and glutamic acids that could be observed in the binding pockets of AZ-01, 03 and 04. Similarly, the carboxylic acid group (common to all four ligands) and the propionic acid exclusive to AZ-03 can readily ionize to the carboxylate anion and drive interaction with positively charged lysine side chains.

**Drug-likeness of test ligands.** In order to assess the drug-likeness of the test ligands which is a desired property in their development as potential food and drug additives, the structures of the azo molecules were submitted to the ADMETsar 2 server. The predicted pharmacokinetics and pharmacodynamic parameters of the ligands are depicted in Table 4. The drug-likeness of the congeners were predicted using the Lipinski's rule of five which provides guidelines for drugability of small molecules by the use of five physiochemical properties including molecular weight, lipophilicity, polar surface area, hydrogen bonding and charge. The rule of five predicts a high probability of success for a drug candidate with no more than one violation [27]. As shown in Table 4, all four test ligands have molecular weights less than 500 and their log P did not exceed the limit of 5. Similarly, the number of hydrogen bond donors and acceptors did not exceed 5 and 10 respectively as dictated by the Lipinski's rule. The compounds are non-carcinogenic and can also be well tolerated as their oral acute toxicity were well below 5 mg/kg body weight. In addition, AZ-01, -02 and -03 did not show ligand promiscuity with cytochrome P450 and are therefore likely to cause insignificant, if any, alterations in the pharmacokinetics of co-administered medications that are metabolized by this all-important enzyme system.

**Conclusion.** The molecular docking interactions between four dye molecules of the 4-carboxyl-2,6-dinitrophenylazohydroxynaphthalene series have been investigated using Autodock Vina and PatchDock. The binding of the four dyes to site I was energetically favoured compared to binding to alternate site. The molecules did not bind by covalent bonds but by a variety of weak reversible intermolecular forces involving different amino acid residues in the hydrophobic cavities of bovine serum albumin. The structure-BSA binding relationships of the molecules, which can be employed for synthesis of safer congeners, have also been successfully elucidated.

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