RESEARCH PAPER

COMPUTATIONAL ANALYSIS OF NAT2 VARIANTS: LINKING PROTEIN STABILITY TO ISONIAZID-INDUCED HEPATOTOXICITY

Mohanasundaram Nachimuthu[1], Narayana Saibaba KV[2*]

[1]Department of Biotechnology, GITAM School of Core Engineering, GITAM University, Visakhapatnam, Email: mnachimu@gitam.edu

[2]Department of Biotechnology, GITAM School of Core Engineering, GITAM University, Visakhapatnam. Email: skvn@gitam.edu,

* Corresponding Author mnachimu@gitam.edu, skvn@gitam.edu

Abstract

Isoniazid is antituberculosis drug in prophylaxis treatment of tuberculosis, but it multifaceted with idiosyncratic adverse drug reaction of hepatotoxicity. Genetic diversity in N-acetyltransferase 2 (NAT2), enzyme in aryl amine and hydrazine drug metabolism, is a predisposing factor for drug induced liver injury. Slow acetylator phenotypes are particularly susceptible to drug-induced liver injury. The present studies examined the consequences of NAT2 polymorphisms with protein folding, stability, and substrate binding with acetyl hydrazine, a reactive isoniazid metabolite. And also investigated how these changes may contribute to hepatotoxicity. Using the NAT2 crystal structure (PDB: 2PFR) as a template, we modelled 21 variants. The protein stability (ΔΔG) was predicted with DUET, intrinsic disorder was determined with PONDR, and binding energy for acetyl hydrazine were determined with Swiss Dock. Computational study was performed to assess the relationship between stability deficit and binding affinities changes. Most of the variants showed reduced stability (ΔΔG: -0.45 to -2.527 kcal/mol) and modest decreases in acetyl hydrazine binding energy values (ΔG: -0.01 to -0.3 kcal/mol). The destabilization of enzymes reduces their ability to bind with acetyl hydrazine, impairing detoxification and leading to the accumulating toxic metabolites Our studies deduce that N-acetyltransferase 2 (NAT2) polymorphisms alter the state of isoniazid metabolism integrated the effects of protein folding, stability, and substrate binding. These findings structural integrity supporting the usage of pharmacogenetic profiling to guide therapy and reduce the risk of drug-induced hepatotoxicity in tuberculosis treatment.

Keywords: *NAT2 polymorphisms, Isoniazid metabolism, Hepatotoxicity, Protein stability Acetyl hydrazine binding, Pharmacogenomics, Tuberculosis therapy*

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1.Introduction

Single nucleotide polymorphisms (SNPs) represent the most conventional type of genetic variation in the human genome., with nearly 88 million variants indexed by the 1000 Genomes Project [1]. The disease-associated SNPs can directly influence gene function and phenotype, regulate susceptibility and therapeutical efficacy [2].Drug-metabolizing enzymes are only determining fate of drug efficacy a toxicity, detoxification and elimination [3]. N-acetyltransferase 2 (NAT2) is particularly important, metabolizing of numerous aromatic amine hydrazine xenobiotic drug and is highly polymorphic, developing phenotype rapid, intermediate, and slow acetylator in diversity of population. More than 45 nucleotide variations have been described, and twelve SNPs define alleles that observable change in enzyme activity, binding affinity with ligand and patient response [4–6]. Variation is not only restricted to drug metabolizing enzyme, encoding receptors, transporters, signaling molecules, and antioxidant enzymes also contribute to differences in drug response [7]. Isoniazid is metabolized by N-acetyltransferase2 NAT2 CYP2E1, and GST enzymes, while SNPs in HLA, UGT, NOS, BACH, and MAFK promote antioxidant pathways and rely susceptibility to hepatotoxicity [8].

Isoniazid induced liver injury frequently occur with elevations in serum aminotransferases [9]. Histopathology may develop subtle inflammatory infiltrates to severe necrosis. Crucially isoniazid itself is not exactly toxic but alternatively reactive metabolites of isoniazid such as acetyl hydrazine and hydrazine prompt immune-mediated injury [10]. Conformation structure studies of NAT2 variants show that amino acid substitutions at specific positions can alter substrate selectivity and enzyme stability [11,12]. The variant *NAT2*19* (C190T, R64W) reduces activity and stability, producing a slow acetylator phenotype associated with elevated isoniazid plasma levels and increased hepatotoxic risk [13–15]. Some other variants *NAT2*14* (G191A ,R64Q) and *NAT2*7*(G857A G286E), weaken catalytic efficiency, while *NAT2*5*(T341C ,I114T) modifies substrate affinity without destabilizing the protein [16–18]. Excluding NAT2, CYP2E1 promote to hepatotoxicity by generating reactive metabolites that covalently bind hepatic proteins, tailoring immune responses [19,20]. Dysregulation of bile transporters BSEP and MRP2 has also been implicated in cholestatic injury via the SIRT1/FXR pathway [21].

Clinical report invariably shows that slow acetylators have higher isoniazid and its metabolite plasma AUC concentrations and greater risk of hepatotoxicity compared wild type rapid acetylators [22–24]. This result has supported for pharmacogenetic profiling of NAT2, GSTM1, and CYP2E1 prognosis for isoniazid exposure and hepatotoxicity risk. The secondary metabolite acetyl hydrazine of isoniazid, present in plasma, serves as a biomarker for isoniazid-induced hepatotoxicity. [25]. Recent research results that non-coding SNPs and epigenetic regulation, including DNA methylation, add additional control over N-acetyltransferase 2(NAT2) expression [26–28]. The synonymous SNPs, though silent at the protein level, may affect mRNA folding and translation efficiency, progressively modify phenotype [29]. Combinations of these finding underscored that isoniazid-induced hepatotoxicity develops not only from a single genetic variant, but from a multifaceted of polymorphisms, enzyme kinetics, and epigenetic regulation in this study, the impact of N-acetyltransferase 2 (NAT2) polymorphisms on protein stability and substrate binding with acetyl hydrazine, a reactive metabolite of isoniazid, was examined, and the potential role of these changes in promoting hepatotoxicity was explored

2.Materials and Methods

The effect of NAT2 polymorphism on protein folding, stability, and substrate binding with acetyl hydrazine and how these changes may contribute to hepatotoxicity of drug was investigated in the present study.

2.1.Data Extraction and Data Assessment: Single nucleotide polymorphisms (SNPs) of N-acetyltransferase 2(NAT2) were obtained from published data file (https://nat.mbg.duth.gr/Human_NAT2_alleles) [30]. A total of 73 SNPs were reported, of which 20 were multiple SNPs. The N-acetyltransferase 2(NAT2) gene is highly diverse across populations and its phenotypes are based on its two haplotypes of N-acetyltransferase 2(NAT2) [31]. These polymorphisms are known to modulate substrate binding energy through alterations in native protein folding and stability [32].

2.2.Protein Stability Prediction: The stability of N-acetyltransferase 2(NAT2) variants (ΔΔG) were predicted using the DUET web server (http://structure.bioc.cam.ac.uk/duet). DUET integrates two complementary computational approaches mCSM and SDM into a consensus prediction optimized with Support Vector Machines (SVM).

2.3.Protein Disorder Analysis: Protein sequence disorder was analyzed in Predictor of Natural Disordered Regions (PONDR) (http://www.pondr.com). These tools predict intrinsic disorder based on backbone flexibility and lack of fixed tertiary structure, providing insight into the stability of N-acetyltransferase 2(NAT2) variants [33].

2.4.Molecular Docking: Swiss institute of Bioinformatics developed online web server called Swiss Dock. This software is used to determine the binding affinities on ligand receptor interactions.[34]

3.Results and Discussion

The wild type N-acetyltransferase 2(NAT2) consists of 290 amino acids and conserved a single coding exon. The conserved central region of the sequence, the amino acids display at positions 112–210 determines substrate affinity, whereas residues at position 211–250 contribute to enzymatic function. In addition, conserved cysteine residues at positions at 46, 68, and 223 are essential for the conformational change and intrinsic stability of N-acetyltransferase 2 (NAT2) [35–36]. Highly conserved arginine residues at positions 9 and 64, with positively charged side chains, contribute to protein conformation but are not directly involved in catalytic activity [37].

All NAT2 variant stability ΔΔG (kcal/mol) and their binding energies ΔG (kcal/mol) with ligand acetyl hydrazine are shown in Table 1 and Figure 1. Wild-type N-acetyltransferase 2 *(NAT2*4*) trait is a control having binding energy of –6.32 kcal/mol and a neutral stability (ΔΔG +0.021 kcal/mol) (Table 1). The Intrinsic disorder PONDR score and their stability ΔΔG (kcal/mol) of NAT2 variants are shown in Table 2 and Figure 2. The PONDR intrinsic disorder score (0.2972) predicts a comparatively ordered structural amino acid component associated with the rapid acetylator phenotype (Table 2). This variant serves as the reference for evaluating the effects of amino acid substitutions on enzyme activity.

3.1.Destabilizing Mutations

The effect of stability on binding affinity with N-acetyltransferase 2(NAT2) variants, which display high destabilisation behaviors, is shown in Table 3. Variants such as *NAT2*5A (Ile114Thr, ΔΔG –2.527* kcal/mol*), NAT2*14A (Arg64Gln, ΔΔG –1.333 kcal/mol), and *NAT2*24 (Leu135Val,* ΔΔG –1.719 kcal/mol) show an observable difference of destabilisation. The *NAT2*5A variant, substituted with a nonpolar amino acid, contributes to structural conformation through hydrophobic interactions. Replacement with threonine reduces hydrophobic clustering, thereby weakening protein stability. In the *NAT2*14A variant, arginine at position 64 is highly conserved and essential for both stability and catalytic activity; substitution with glutamine results in a loss of stability and catalytic efficiency. In the *NAT2*24 variant, although both leucine and valine are nonpolar, differences in their size and shape may affect core packing and folding stability. Figure 3 illustrates those destabilising variants with reduced binding affinity. Their binding energies (–6.20 to –6.22 kcal/mol) were weaker than those of the wild-type phenotype rapid acetylator, correlating with

slow acetylator phenotypes. PONDR disorder scores of *NAT2**14 variant and *NAT2**24 (0.2967–0.3007) were slightly increased compared to the wild-type phenotype, suggesting a minor increment in disorder that aggravates structural instability. These findings show that destabilisation, rather than disorder alone, drives reduced enzymatic structural conformation.

3.2.Moderately Destabilizing Mutations

The conformational structure stability on binding affinity with N-acetyltransferase 2(NAT2) variants expressing moderate destabilization is shown in Table 4. Variants including *NAT2*6B (Arg197Gln, ΔΔG –0.627* kcal/mol*), NAT2**19 (*Arg64Trp,* ΔΔG – 0.450 kcal/mol), and *NAT2*27 (Arg197Ter*, ΔΔG – 0.929 kcal/mol), displayed mixed ΔΔG values between (–0.45 to -1 kcal/mol).*NAT2**6B. Substitution of arginine with glutamine might affected steric interactions and folding stability.*NAT2**19. Localization of conserved arginine with tryptophan eliminated hydrogen bonding, inhibiting substrate binding.*NAT2**27: Introduction of a stop codon at position 197shorten the protein and compromised stability.

The structural destabilisation was evident even though the binding energies of these variants are very close to wild-type (–6.30 to –6.31 kcal/mol). The obtained PONDR scores (0.2942– 0.3000) present no substantial deviation, stabilizing that disorder is not the primary determinant of phenotype. Instead, latent destabilisation compromises folding and catalytic activity, explaining their slow acetylator classification. Figure 4 demonstrates that these retain binding affinity but shift toward reduced stability.

3.3.Stabilizing or Neutral Mutants

The effect of stability on binding affinity with N-acetyltransferase 2(NAT2) variants showing stabilizing or neutral is shown in Table 5 Variants with positive ΔΔG values, such as *NAT2*10 (Ser287Pro, ΔΔG +0.255* kcal/mol*), NAT2**13B (*Thr193Me*t, ΔΔG +0.184kcal/mol), and *NAT2*13D (Lys256Glu*, ΔΔG +0.603 kcal/mol), maintained binding energies close to wild-type (–6.29 to –6.31 kcal/mol). The *NAT2**10 substitution of serine with proline incorporates conformational stiffness, stabilizing the protein despite potential disruption of native folding. In *NAT2**13B Methionine, with its Sulphur-containing side chain, provides flexibility and stability in protein structure confirmation. In *NAT2**13D replacement of lysine with glutamic acid alters electrostatic charge and side chain length, contributing to folding stability.

Their PONDR prediction scores (0.2954–0.2987) were comparable to those of the wild-type reference, suggesting a conserved structural order. Despite stabilizing effects, some variants (e.g., *NAT2*10*) persisted slow phenotypes, signifying that foldability and subtle conformational changes may diminish catalytic efficiency even when binding affinity is preserved. Figure 5 shows a significant result for these stabilizing variants, which cluster near wild-type binding energy but diverge in stability.

3.4.Outlier Mutation

*NAT2*22* (Glu203Asp) variant evolved as a notable outlier. Substitution of glutamic acid with aspartic acid modify stability and develop steric hindrance, preventing proper stability. Although its PONDR score (0.2949) was not raised, the variant showed moderate destabilization (ΔΔG – 0.783 kcal/mol) and the weakest binding energy (–6.02 kcal/mol). This considerable deviation implies abnormal substrate affinity and detoxification capacity, independent of disorder predictions. Depict this outlier as a distinct point separated from the main cluster of variants.

Across all variants, PONDR disorder scores remained within a narrow range (~0.29–0.30), indicating that intrinsic disorder is largely conserved. The phenotypic traits are therefore driven primarily by stability shifts (ΔΔG, kcal/mol) and binding energy deviations (ΔG, kcal/mol). Destabilizing mutations consistently correlated with weaker binding and slow acetylator phenotypes, whereas stabilizing mutations preserved binding but occasionally impaired foldability. Integrating disorder predictions with stability and binding data provides a comprehensive framework for understanding how N-acetyltransferase 2 (NAT2) variants alter enzyme function, supporting the conclusion that structural stability rather than intrinsic disorder is the dominant factor influencing acetylator phenotype.

4.Conclusion

This research confirms that protein stability, protein folding, and binding affinity of acetyl hydrazine (AcHz) were affected due to the N-acetyltransferase 2 (NAT2) polymorphisms from *5 to* 27. The variants such as NAT2*6A have shown destabilising effects, reduced detoxification capacity and elevated isoniazid-induced hepatotoxicity risk. Computational analyses using DUET, PONDR, and Swiss Dock show a moderate correlation between ΔΔG destabilisation and weaker AcHz binding, conformational structural changing mechanisms beyond the acetylation process in phenotypes. These understandings validate N-acetyltransferase 2 (NAT2) variants as a key pharmacogenomic profile of TB drug toxicity, supporting genotype-guided dosing to prevent liver injury in high-risk populations.

ETHICS APPROVAL AND CONSENT TO PAR TICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Variant (NAT2*)	Amino Acid Change	ΔΔG (kcal/mol)	Binding Energy ΔG (kcal/mol)	Phenotype Effect	Notes
NAT2*4 (WT)	Reference	0.021	-6.32	Rapid acetylator	Baseline reference
NAT2*5A	Ile114Thr	-2.527	-6.22	Slow acetylator	Loss of hydrophobic clustering
NAT2*6B	Arg197Gln	-0.627	-6.5	Slow acetylator	Steric disruption

NAT2*10	Ser287Pro	0.255	-6.29	Slow acetylator	Stabilizing but reduced foldability
NAT2*13B	Thr193Met	0.184	-6.3	Neutral	Maintains stability
NAT2*13D	Lys256Glu	0.603	-6.31	Neutral	Electrostatic stabilization
NAT2*14A	Arg64Gln	-1.333	-6.19	Slow acetylator	Reduced catalytic efficiency
NAT2*19	Arg64Trp	-0.45	-6.22	Slow acetylator	Loss of hydrogen bonding
NAT2*22	Glu203Asp	-0.783	-6.01	Slow acetylator	Outlier; weakest binding
NAT2*24	Leu135Val	-1.719	-6.2	Slow acetylator	Altered core packing
NAT2*26	Arg197Gln	-1.87	-6.25	Slow acetylator	Steric disruption
NAT2*27	Arg197Ter	-0.929	-6.31	Slow acetylator	Protein truncation
NAT2*28	Arg197Ter (alt)	-0.585	-6.31	Slow acetylator	Protein truncation

Table 1 : NAT2 variant stability ΔΔG (kcal/mol) changes and their corresponding binding energies with acetyl hydrazine.

Variant (NAT2*)	**Amino Acid Change**	**PONDR Disorder Score**	**ΔΔG (kcal/mol)**	**Notes**
NAT2*4 (WT)	Reference	0.2972	0.021	Ordered structure, baseline
NAT2*5A	Ile114Thr	0.2967	-2.527	Slight disorder increase, destabilized
NAT2*6B	Arg197Gln	0.2942	-0.627	Disorder unchanged, moderate destabilization
NAT2*10	Ser287Pro	0.2954	0.255	Disorder stable, conformational rigidity
NAT2*13B	Thr193Met	0.2987	0.184	Disorder stable, neutral stability
NAT2*13D	Lys256Glu	0.2975	0.603	Disorder stable, electrostatic stabilization
NAT2*14A	Arg64Gln	0.3007	-1.333	Disorder slightly elevated, destabilized
NAT2*19	Arg64Trp	0.299	-0.45	Disorder stable, moderate destabilization
NAT2*22	Glu203Asp	0.2949	-0.783	Disorder unchanged, weakest binding
NAT2*24	Leu135Val	0.3	-1.719	Disorder stable, destabilized
NAT2*26	Arg197Gln	0.2942	-1.87	Disorder unchanged, moderate destabilization
NAT2*27	Arg197Ter	0.3	-0.929	Disorder stable, truncation
NAT2*28	Arg197Ter (alt)	0.3	-0.585	Disorder stable, truncation

Table 2: The intrinsic disorder of NAT2 variant PONDR score and corresponding stability

Variant (NAT2*)	**Amino Acid Change**	**ΔΔG (kcal/mol)**	**Binding Energy ΔG (kcal/mol)**	**Phenotype Effect**	**Notes**
NAT2*4 (WT)	Reference	0.021	-6.32	Rapid acetylator	Baseline reference
NAT2*5A	Ile114Thr	-2.527	-6.2	Slow acetylator	Loss of hydrophobic clustering
NAT2*14A	Arg64Gln	-1.333	-6.19	Slow acetylator	Reduced catalytic efficiency

NAT2*24	Leu135Val	-1.719	-6.22	Slow acetylator	Altered core packing

Table 3: The complete destabilization NAT2 variant and its stability.

Variant (NAT2*)	Amino Acid Change	ΔΔG (kcal/mol)	Binding Energy ΔG (kcal/mol)	Phenotype Effect	Notes
NAT2*4 (WT)	Reference	0.021	-6.32	Rapid acetylator	Baseline reference
NAT2*6B	Arg197Gln	-0.627	-6.5	Slow acetylator	Steric disruption
NAT2*19	Arg64Trp	-0.45	-6.22	Slow acetylator	Loss of hydrogen bonding
NAT2*27	Arg197Ter	-0.929	-6.31	Slow acetylator	Protein truncation

Table 4: The Moderate destabilization NAT2 variant and its stability

Variant (NAT2*)	Amino Acid Change	ΔΔG (kcal/mol)	Binding Energy ΔG (kcal/mol)	Phenotype Effect	Notes
NAT2*4 (WT)	Reference	0.021	-6.32	Rapid acetylator	Baseline reference
NAT2*10	Ser287Pro	0.255	-6.29	Slow acetylator	Stabilizing but reduced foldability
NAT2*13B	Thr193Met	0.184	-6.3	Neutral	Maintains stability
NAT2*13D	Lys256Glu	0.603	-6.31	Neutral	Electrostatic stabilization

Table 5: The Stabilizing or Neutral Mutants NAT2 variant and its stability.

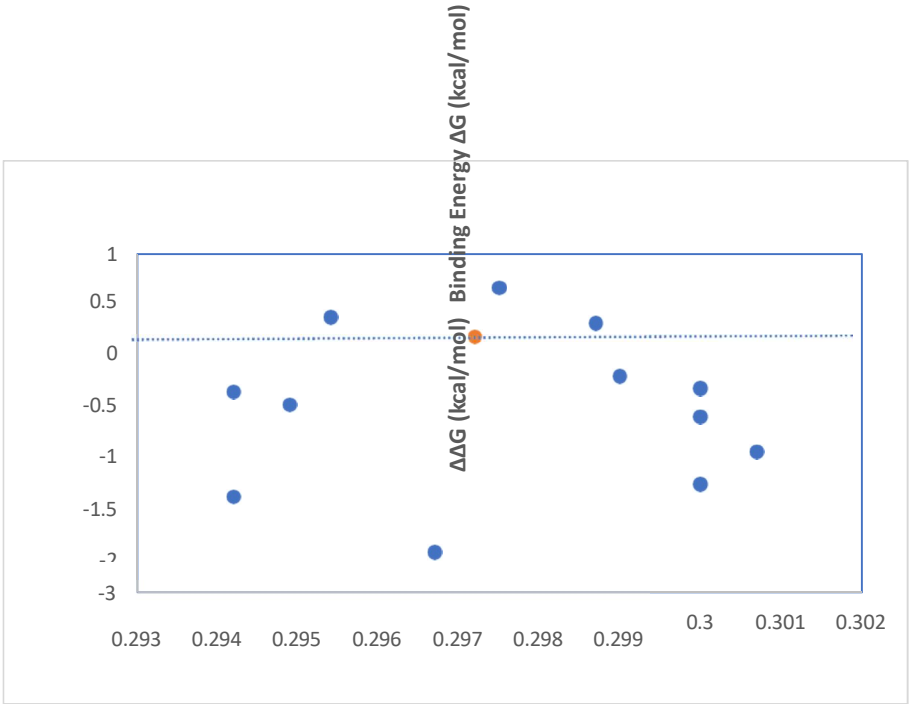

Figure 1: The all NAT2 variant stability ΔΔG (kcal/mol) and binding energy ΔG (kcal/mol) with ligand acetyl hydrazine

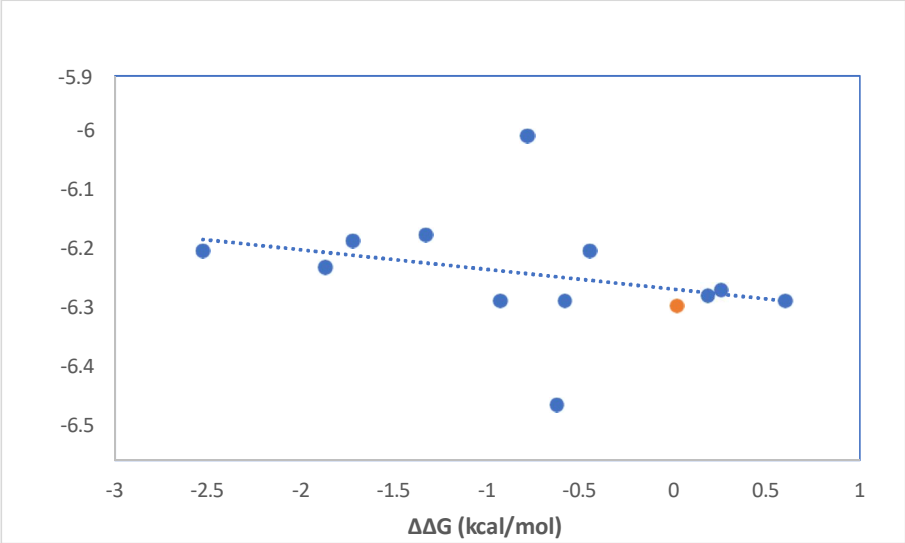

Figure 2: The Intrinsic disorder of PONDR score and stability ΔΔG (kcal/mol) of NAT2 variant

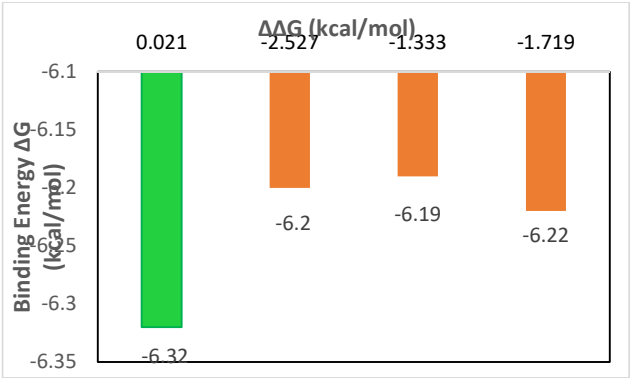

Figure 3: Destabilizing NAT2 variant green colour is wild type NAT2*4 orange colour various destabilizing NAT2 variant

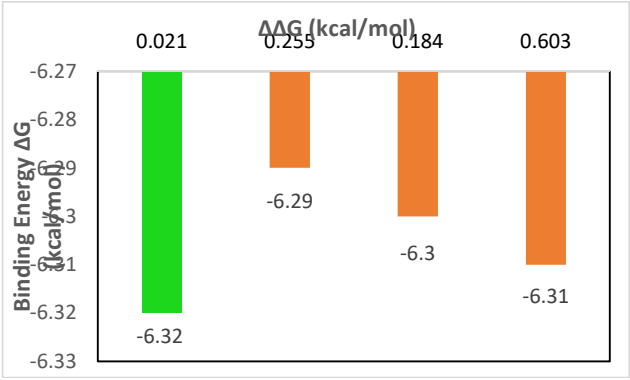

Figure 4: Moderately stabilizing NAT2 variant green colour is wild type NAT2*4 orange colour various destabilizing NAT2 variant

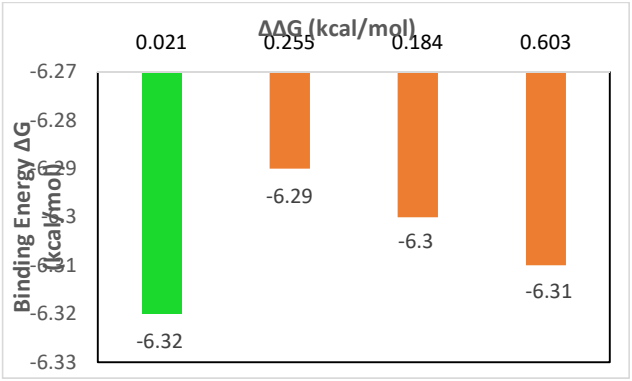

Figure 5: Neutral stabilizing NAT2 variant green colour is wild type NAT2*4 orange colour various destabilizing NAT2 variant