

## Original Article

# Molecular dynamics simulation on the low sensitivity of mutants of NEDD-8 activating enzyme for MLN4924 inhibitor as a cancer drug

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Received August 24, 2015; Accepted September 24, 2015; Epub October 15, 2015; Published November 1, 2015

**Abstract:** MLN4924 is an experimental cancer drug known as inhibitor of NEDD8-activating enzyme (NAE). This anti-tumor candidate is a selective small-molecule inhibitor of NAE which is conjugated to cullin protein on Cullin-RING ligases (CRLs). This covalent modification activates cullin complex to recruit an ubiquitin-charged E2 and leads to downstream target protein polyubiquitination and proteasomal degradation. MLN4924, which can form a covalent adduct with NEDD8, and block NAE at the first step in this pathway, has shown anti-tumor activity in many kinds of cancer cell lines and also xenograft models, including lung cancer, colon cancer, melanoma and lymphoma. The anti-tumor activity of MLN4924 results from inactivation of CLRs, which causes DNA re-replication and inhibition of nuclear factor (NF)- $\kappa$ B signaling, thus leading to cancer cell death. A mutation can reduce the enzyme's sensitivity to MLN4924. Verma et al. in 2013 studied on molecular dynamics simulation of a mutant A171T and consequently found out that this mutation reduce MLN4924 interaction with DNA Binding site of enzyme as a result of reduction of enzyme affinity to ATP. One year later, in 2014, Wei Xu et al. carried out a research on inhibitor resistant cell lines and revealed that a couple of mutations so called Y352H and I310N leads to enzyme resistance to MLN4924 inhibitor, interestingly, the cause reported was the increase of enzyme affinity to ATP. As in Wei Xu et al. experiment the molecular dynamics simulation was not considered, present study is conducted to identify enzyme mutation mechanism by molecular dynamics approach using advantages of Gromacs software version 4.5.6.

**Keywords:** MLN4924, NEDD8-activating enzyme (NAE), Y352H, I310N, mutation, drug discovery

## Introduction

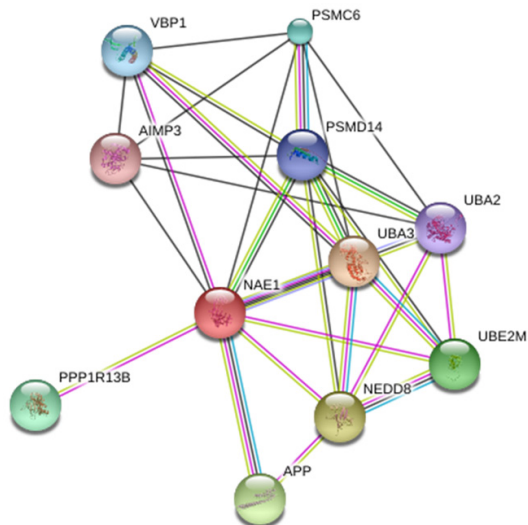
Cancer cells are adopting essentially cell signaling not only for progressing of cell cycle but also for preventing programmed cell death, so-called apoptosis. This signaling requires specific regulatory proteins such as ubiquitin-proteasome system (UPS) that the check points on this process, include a family of E3 ubiquitin ligases called Cullin-RING Ligases (CRLs) [1, 2]. The ligase activity of these complexes requires modification of the cullin protein, a family of hydrophobic proteins providing a scaffold for ubiquitin ligases (E3), situated at their core with a ubiquitin-like protein (ULP) called NEDD8 [1, 2].

NEDD8 is a protein encoded in humans by the NEDD8 gene and in *Saccharomyces cerevisiae*

this protein is known as Rub1 [3]. This ULP, which becomes covalently conjugated to a limited number of cellular proteins in a manner analogous to ubiquitination, an enzymatic post-translational modification (PTM) process in which a ubiquitin protein is attached to a substrate protein. Human NEDD8 shares 60% amino acid sequence identity to ubiquitin. The only known substrates of NEDD8 modification are the cullin subunits of SCF ubiquitin E3 ligases. The NEDDylation of cullins is critical for the recruitment of E2 to the ligase complex, thus facilitating ubiquitin conjugation. NEDD8 modification has therefore been implicated in cell cycle progression and cytoskeletal regulation [3].

The Ubiquitin-Proteasome System (UPS) is responsible for the bulk of protein turnover in

## MD simulation on the NAE mutants



**Figure 1.** Interaction partners of NAE predicted by STRING Database ([www.string-db.org/](http://www.string-db.org/)).

eukaryotic cells and plays a major role in maintaining cellular protein homeostasis. Among UPS substrates are proteins involved in cell cycle regulation, cellular growth and proliferation, Intracellular signaling, DNA repair, membrane receptor regulation, and proapoptotic and antiapoptotic signaling [4-8].

The initial step required for attachment of NEDD8 to a cullin is catalyzed by the E1, NEDD8-activating enzyme (NAE). The NAE interaction with other proteins is illustrated in **Figure 1**. The inhibitor of NAE; MLN4924 has been revealed to block the activity of NAE and prevent the subsequent neddylation of cullins. Preclinical studies have demonstrated antitumor activity in various solid tumors and hematological malignancies, and preliminary clinical data have shown the anticipated pharmacodynamic effects in humans. MLN4924, a small molecule with Molecular Weight of 443.5193 g/mol (**Table 1** and **Figure 2**). Inhibiting NEDD8-activating enzyme (NAE), is thought to be a potential anticancer drug [9] and previously in clinical trials has shown clinical activity in some solid tumor and hematologic malignancies [10] and recently in complications with Lymphoma or Multiple Myeloma [11].

Furthermore, it exhibits potent in vitro cytotoxic activity against a variety of human tumor derived cell lines [12]. Treatment of tumor cells with MLN4924 increases the abundance of known CRL substrates. In the human cell lines

studied, the mechanism of cell death seems to be a consequence of uncontrolled DNA synthesis in the S-phase of the cell cycle leading to DNA damage and induction of apoptosis. While still in the early stages of clinical development, the encouraging preclinical and clinical activity of MLN4924 supports investigation into the mechanisms of sensitivity and resistance to this drug [12]. It was shown that mutations in the nucleotide binding pocket and NEDD8 binding cleft of NAEb affect MLN4924 adduct formation and dissociation from NAEb. The NAEb mutations that have been detected in MLN4924-resistant cells derived in tissue culture or in vivo occur in two areas of the gene, the nucleotide binding pocket at Alanine 171 and at various residues that are within or close to the NEDD8 binding cleft [13]. The inhibition of NAE by MLN4924 occurs through the formation of a NEDD8-MLN4924 covalent adduct resembling NEDD8-AMP, a process that requires NAE catalytic activity [14]. Notably, the NEDD8-MLN4924 adduct is a tight binding inhibitor of NAE, and it has been proposed that the tight binding nature of the inhibitor-protein adduct is crucial for MLN4924 potency [15]. Inhibition of NAE has emerged as a highly promising approach to treat cancer through the adenosine sulfamate analog MLN4924. A mutation can reduce the enzyme's sensitivity for MLN4924. Verma et al. in 2013 studied on molecular dynamics simulation of a mutant A171T and consequently found out that this mutation reduce MLN4924 interaction with DNA Binding site of enzyme as a result of reduction of enzyme affinity to ATP [16]. A year later, in 2014, Wei Xu et al. carried out a research on inhibitor resistant cell lines and revealed that a couple of mutations so called Y352H and I310N leads to enzyme resistance to MLN4924 inhibitor, interestingly, the reported cause was the increase of enzyme affinity to ATP. As in Wei Xu et al. experiment the molecular dynamics (MD) simulation was not considered, this study is conducted to identify mutation mechanism in enzyme using Gromacs software version 4.5.6.

### Methodology

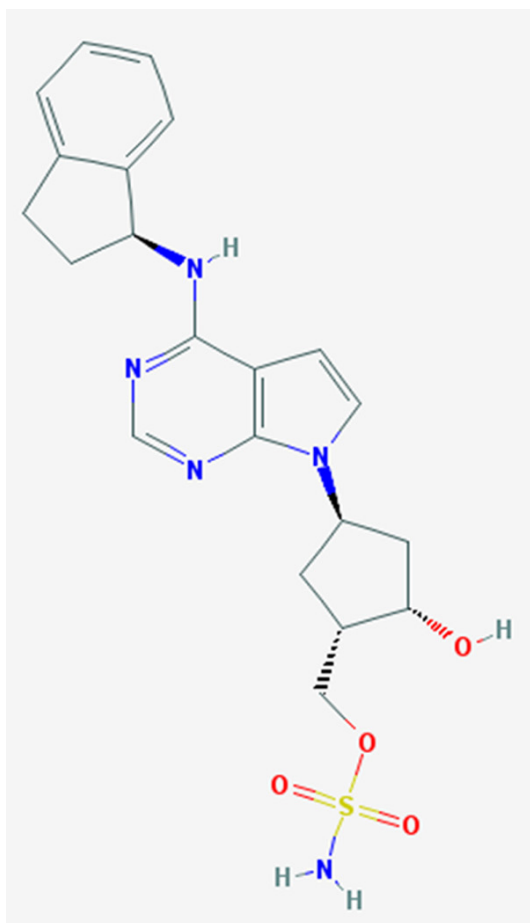
#### *Prediction possible interaction NAE partners*

Initially, in present study possible interaction partners of NAE partners predicted by taking

## MD simulation on the NAE mutants

**Table 1.** Computed Properties of MLN4924, Pfam database

Molecular Weight	443.5193 g/mol
Molecular Formula	C <sub>21</sub> H <sub>25</sub> N <sub>5</sub> O <sub>4</sub> S
XLogP3	1.7
Hydrogen Bond Donor Count	3
Hydrogen Bond Acceptor Count	8
Rotatable Bond Count	6
Exact Mass	443.162725 g/mol
Monoisotopic Mass	443.162725 g/mol
Topological Polar Surface Area	141 Å <sup>2</sup>
Heavy Atom Count	31
Formal Charge	0
Complexity	734
Isotope Atom Count	0
Defined Atom Stereocenter Count	4
Undefined Atom Stereocenter Count	0
Defined Bond Stereocenter Count	0
Undefined Bond Stereocenter Count	0



**Figure 2.** Chemical structure of MLN4924 visualized by Pfam database ([www.http://pfam.xfam.org/](http://pfam.xfam.org/)).

advantages of STRING database ([www.http://string-db.org/](http://string-db.org/)). This interactions include direct (physical) and indirect (functional) associations.

### *Computation properties and Chemical structure of MLN4924*

Also, by Pfam database ([www.http://pfam.xfam.org/](http://pfam.xfam.org/)), we evaluate Molecular Weight, Molecular Formula, XLogP3, Hydrogen Bond Donor Count, Hydrogen Bond Acceptor Count, Rotatable Bond Count and etc. beside Chemical structure of MLN4924.

### *Molecular dynamics simulation and molecular mechanics minimization*

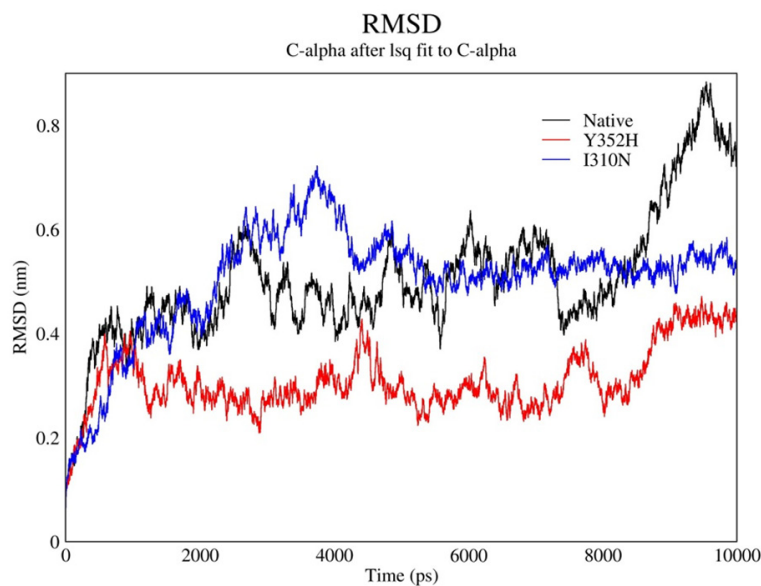
Molecular Dynamics (MD) simulation and molecular mechanics (MM) minimization were performed using Gromacs 4.5.6 package under anamber.ff99SB. The GAFF topologies for MLN4924 inhibitor were generated using the Antechamber software using partial charges calculated with the restrained electrostatic potential method. The GAFF topologies were converted into GROMACS format using the acpype tool. MD simulations were carried out with periodic boundary conditions. Van der Waals forces were treated with a cut-off of 10 Å. The Particle-Mesh Ewald method was used with a 10 Å cut-off. Also, crystallographic structure of NEDD8-activating enzyme (PDB code: 3GZN) was used for theoretical studies. Modeller program 9.13 was used to construct mutant Y352H and I310N.

The frequency to update the neighbor list was 5. The protonation state of Gromacs package was used to calculate the total charge of NEDD8-activating enzyme and mutants. MD simulation was accomplished in four steps. In the first step, the entire system was minimized using the steepest descent followed by conjugate gradients algorithms. In the second step or equilibration step, heavy atoms were restrained using a force constant of 1000 kJ/mol nm and the solvent and ions were allowed to evolve. This was done through minimization and molecular dynamics in the NVT ensemble for 100 ps and in the NPT ensemble for 100 ps. Then in order to obtain equilibrium geometry at 298 K and 1 atm, the temperature of the system was increased and the velocities at each step were reassigned according to the Maxwell-

## MD simulation on the NAE mutants



**Figure 3.** 3D structure of Nedd8-activating Enzyme in complex with Nedd8 and MLN4924 (<http://www.ncbi.nlm.nih.gov/Structure/MMDB/mmdb.shtml>).



**Figure 4.** RMSD chart shows changes during time.

Boltzmann distribution at that temperature and equilibrated for 100 ps. Temperature coupling

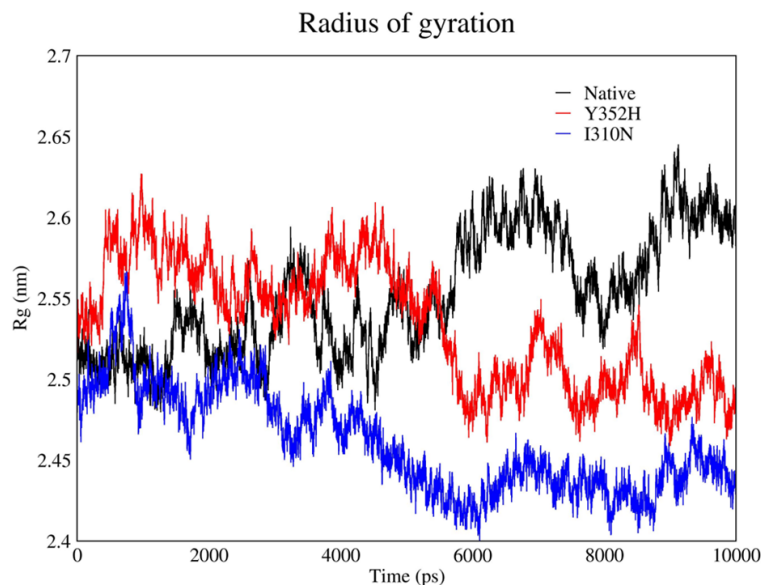
was set to 0.1 ps and pressure coupling to 2 ps. The Berendsen algorithm was used for thermostat and barostat during the equilibration step. All bonds were constrained via the LINCS algorithm. In the final step or production phase, a 20 ns MD simulation was performed under an NPT ensemble. In order to retain temperature and pressure stable in production step, Nosé-Hoover thermostat and Parrinello-Rahman barostat were used with removing position restraints. The temperature was at 298 K with a time step of 2 fs. In addition, the LINCS algorithm was used to constrain the lengths of hydrogen-containing bonds in this step. Na<sup>+</sup> and Cl<sup>-</sup> ions were used to neutralize the system charge.

## Result and discussion

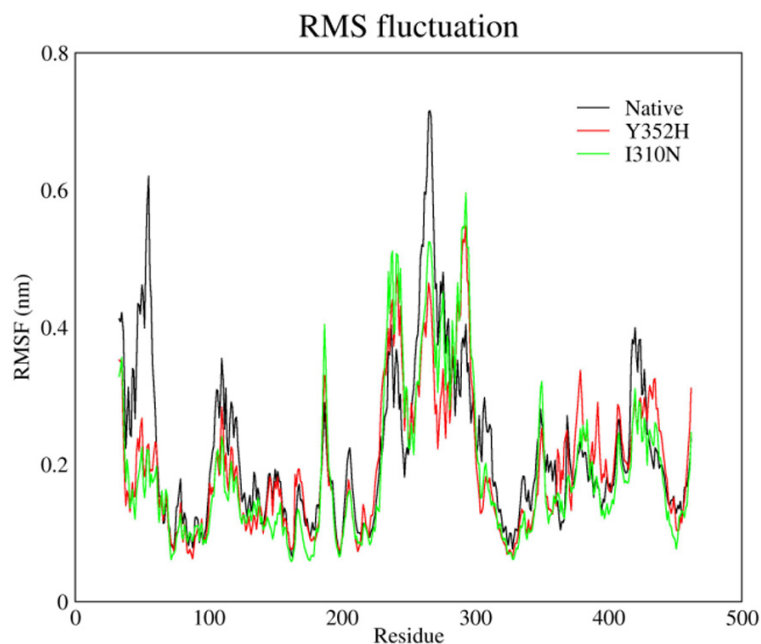
### *Results of predicting interaction partners*

Below figure represent how NAE may interact with other proteins, predicted by String database. In detail, NEDD8 activating enzyme E1 subunit 1; Regulatory subunit of the dimeric UBA3-NAE1 E1 enzyme. E1 activates NEDD8 by first adenylating its C-terminal glycine residue with ATP, thereafter linking this residue to the side chain of the catalytic cysteine, yielding a NEDD8-UBA3 thioester and free AMP. E1 finally transfers NEDD8 to the catalytic cysteine of UBE2M. Necessary for cell cycle progression through the S-M checkpoint. Overexpression of NAE1 causes apoptosis through deregulation of NEDD8 conjugation (534 aa).

## MD simulation on the NAE mutants



**Figure 5.** Radius of gyration obtained in MD analysis.



**Figure 6.** RMS fluctuation plot of Native, Y352H and I310N.

### *Results of computation properties and Chemical structure of MLN4924*

In next step, we computed properties and Chemical structure of MLN4924 and results are arranged in **Table 1** and **Figure 2**. These data are useful to deep understanding physico-chemical properties of compound which will be used in MD.

### *Molecular dynamic simulations*

After basic analysis, we precede molecular dynamic simulation. **Figure 3** shows Nedd8-activating Enzyme in Complex with Nedd8 and Mln4924.

In MD analysis, Root Mean Square Deviation (RMSD) of simulated structure upon time is an appropriated and common criterion to verify Molecular dynamics stimulation stability. Therefore, RMSD changes of main enzyme chain atoms for both natural and 2 mutants of that upon simulation period-which lasted 10 Nanoseconds-are calculated in comparison with experimental structure and the results are shown as following chart (**Figure 4**).

As it can be seen, RMSD after initiatory minimizing energy during first 2 nS has increased and it is the indicator of that the molecule based on surface has to be transferred to the places with lower energy level according to balance distance. After 2 nS this trend went downward whereas the fluctuation slightly decreased so that, they performed simulations are on acceptable conditions due to their stability and there is not any evidence of significant deviation of produced structure during simulation period from previous structures. Thus, to a large extent it can be claimed that the different regions of structures interact with each other and every structures met all the criteria to achieve ultimate conformation in simulation.

Furthermore, any modification in protein motif and shape can be evaluated by Gyration Radius (**Figure 5**). This parameter provides information about total protein volume distribution in spher-

## MD simulation on the NAE mutants

**Table 2.** Summary of natural protein and its connector energies

Energy type	Calculated energy
Van der Waal energy	-207.177 ± 18.524 kJ/mol
Electrostatic energy	-129.819 ± 29.047 kJ/mol
Polar solvation energy	282.588 ± 37.288 kJ/mol
SASA energy	-19.621 ± 1.176 kJ/mol
SAV energy	0.000 ± 0.000 kJ/mol
WCA energy	0.000 ± 0.000 kJ/mol
Binding energy	-74.030 ± 17.434 kJ/mol

**Table 3.** Summary of I310N Mutant and its connector energies

Energy type	Calculated energy
Van der Waal energy	67.683 ± 12.619 kJ/mol
Electrostatic energy	-1522.361 ± 62.344 kJ/mol
Polar solvation energy	260.484 ± 40.990 kJ/mol
SASA energy	-1.675 ± 0.066 kJ/mol
SAV energy	0.000 ± 0.000 kJ/mol
WCA energy	0.000 ± 0.000 kJ/mol
Binding energy	-1195.869 ± 83.891 kJ/mol

**Table 4.** Summary of Y352H Mutant and its connector energies

Energy type	Calculated energy
Van der Waal energy	-220.654 ± 18.622 kJ/mol
Electrostatic energy	-141.197 ± 28.372 kJ/mol
Polar solvation energy	312.992 ± 33.450 kJ/mol
SASA energy	-19.995 ± 0.898 kJ/mol
SAV energy	0.000 ± 0.000 kJ/mol
WCA energy	0.000 ± 0.000 kJ/mol
Binding energy	-68.854 ± 42.452 kJ/mol

ical state. In other words, Gyration Radius demonstrates tensed molecular shape and as it is obviously indicated on following chart, this parameter is approximately constant on final section.

The results suggest that the Gyration Radius of mutants were downwards that is clue of constructed secondary structures while the natural Gyration radius was increased. Moreover, because of conformation changes to create more tensed structure compare to primary structure; the radius constantly showed fluctuation in simulation upon time. The evaluation of similarities between three structures revealed that Gyration Radius of natural sample was

larger than the mutants and it seems that this is a result of limitation of main chain movement due to mutation. Besides, flexibility of interacting molecules specially those chains which are involved, have priority in importance of interaction process and eventually affect protein output efficiency.

In some region of these structures the flexibility change and the change pattern for those of natural make difference to those of mutants that can be considered on the comparing below chart (**Figure 6**).

As it can be taken into account from above chart, both mutations in natural protein led to dramatic decline of flexibility on N-terminal end of this protein whereas, mutant's flexibility slightly increased on two regions nearby mutation region at 230-240 and on other regions the flexibility decreased sharply.

The results suggested that this mutation that occurred in natural protein led to decreasing of structural flexibility as an effect of entire structure and eventually the mutant structure became tenser. The connectors' energy for these states (natural and mutants) is summarized in **Tables 2-4**.

### Conclusions

The potential cancer drug, MLN4924 that inhibits NAE is affected by mutation and this can lead to reduce the enzyme's sensitivity to MLN4924. Molecular dynamics simulation of a mutant A171T indicated that interaction with DNA Binding site of enzyme because of reduction of enzyme affinity to ATP is reduced. Y352H and I310N mutants lead to enzyme resistance to MLN4924 inhibitor and molecular dynamics simulation on this experiment identified enzyme mutation mechanism and related changes in natural protein which flexibility is reduced and the mutant became denser in its structure.

### Acknowledgements

We would like to express our gratitude to Dr. Majid Taghdir, the assistant professor of Tarbiat Modares University, for sharing his pearls of wisdom with us during the course of this research. We are also immensely grateful to Mr. Ammar Mohseni for his meaningful contribution for conducting this research.

**Disclosure of conflict of interest**

None.

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